

Combining phenolic grafting and laccase-catalyzed cross-linking: Effects on structures, technofunctional properties and human immunoglobulin E binding capacity of egg white proteins

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ABSTRACT

The efficiency of laccase-catalyzed protein cross-linking can be impacted by substrate protein structure and competing reactions. In this study, chemical grafting of ferulic acid (FA) on protein surface was applied to modulate the cross-linking of two inflexible globular proteins, lysozyme (LZM) and ovalbumin (OVA). The extent of FA-grafting was positively correlated with protein cross-linking extent, and determined the molecular weight profile and structures of the cross-linked product. While laccase-catalyzed reactions (with or without free FA mediator) did not lead to evident cross-linking of the native proteins, oligomeric (up to 16.4%), polymeric (up to 30.6%) FA-LZMs and oligomeric FA-OVA (5.1–31.1%) were obtained upon the enzymatic treatments. The cross-linking on the grafted FA sites occurred mainly through the formation of 8–5'-noncyclic-dehydro-diferulic linkages. The effects of investigated cross-linking approach on the emulsifying, foaming properties and the immunoglobulin E (IgE) binding capacity of LZM and OVA were also evaluated in relation to the structural properties of cross-linked proteins.

1. Introduction

Protein cross-linking has attracted considerable attention as a strategy for modulating structural and mechanical properties of matrix and biopolymer networks as well as for developing biomolecules with targeted functions. Such biomolecules are of great interest in the fields of food, polymer and biomedical science (Permana, Minamihata, Goto, & Kamiya, 2018). In particular, due to its safe and mild process and specificity, the enzymatic cross-linking has a great potential in food processing to modulate the physiochemical properties of proteins (e.g. solubility, thermal stability, surface hydrophobicity) and to enhance their techno-functional properties (e.g. emulsifying, foaming or gelling performance) (Isaschar-Ovdat & Fishman, 2018; Li & Karboune, 2021). Some studies have also shown the ability of protein cross-linking to decrease the protein's allergenicity (Lv et al., 2019; Mihajlovic et al., 2016). Laccase (E.C 1.10.3.2), an oxidoreductase with copper-coordinated redox center, is effective in protein cross-linking. The oxidative cross-linking of protein catalyzed by laccase has shown to

perform mainly through the formation of dityrosine bonds upon the oxidation of tyrosines, which were identified as the prime targets in proteins; the formation of disulfide bonds by the oxidation of cysteines into cystine can also occur (Mattinen et al., 2006; Steffensen et al., 2009). Additionally, laccase-catalyzed oxidation of proteins can result in several amino acid modifications, such as the oxidation of tryptophan into N'-formylkynurenine and further to kynurenine and the formation of carbonyl groups upon the oxidation of methionine, as well as in protein fragmentation (Ercili Cura et al., 2009).

Protein cross-linking-catalyzed by laccase can be hindered by the protein conformation, which can limit the enzyme accessibility to the reactive residues (Mattinen et al., 2006). The use of phenolic acid mediator can promote the protein–protein cross-linking, but it may also favor side reactions, such as the polymerization of phenolic acid mediators and the conjugation between phenolics and proteins (Ma, Forssell, Partanen, Buchert, & Boer, 2011; Steffensen et al., 2009). We hypothesize that the bioconjugation of phenolic moieties on the surface of proteins can create reactive sites for laccase and limit the side reactions,

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thus allowing a better modulation of protein cross-linking. Indeed, the bioconjugation of methoxyphenol groups on the surface of whey protein was achieved via chemical amidation with vanillic acid, and the efficiency of laccase-catalyzed cross-linking of whey protein was greatly improved upon such modification (Ma et al., 2011). Alternatively, the modification of proteins to append a tyrosine-containing polypeptide tag was used to direct the site-specific polymerization of proteins catalyzed by laccase/oxidase, and generate well-structured and highly functional protein polymer assemblies (Permana et al., 2018, 2020). The present study was aimed at the investigation of the modulation of laccase-catalyzed protein cross-linking via altering protein surface phenolic moieties. Two major proteins and known allergens from egg white, lysozyme (LZM) and ovalbumin (OVA), were selected as protein substrates, and were grafted with controllable and quantifiable feruloyl (4-hydroxy-3-methycinnamoyl) moieties, followed by cross-linking treatment catalyzed by *Trametes versicolor* laccase (LacTv). The relationships between the reactivity of ferulic acid (FA)-modified proteins and their enzymatic cross-linking efficiency was discussed with the aim to lay the ground for better modulation of their functionalities.

2. Materials and methods

2.1. Materials

Lysozyme, ovalbumin, ferulic acid, laccase from *T. versicolor*, *n*-hydroxysuccinimide (NHS), 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Folin-Ciocalteu phenol reagent and ethyl acetate were purchased from Sigma Aldrich (St-Louis, MO). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) was from Tokyo chemical Industry Co. Ltd (Tokyo, Japan). Dimethylformamide (DMF) was purchased from Fisher Scientific (Hampton, NH). Serum samples from 6 egg allergic patients were purchased from PlasmaLab (Everett, WA). 3,3',5,5'-tetramethylbenzidine (TMB) is product of Bioshop (Burlington, ON, Canada). Laccase activity was evaluated using ABTS assay as described by Niku-Paavola et al. (1990). One unit activity was defined as the amount of enzyme that oxidize 1 μ mol of ABTS per min.

2.2. Modification of protein substrates

The bioconjugation of FA and protein was performed via a two-step amidation reaction, including NHS-FA ester activation and acylation, according to the modified method of Ma et al., (2011). NHS, EDCI and FA were dissolved in DMF and incubated at 28 °C for 17 h to prepare NHS-FA esters at different concentration levels for each protein. The concentration of FA was maintained in excess at 103 mM, while the concentration of NHS and EDCI was set at 6.1 to 47.3 mM and 6.9 to 54.0 mM, respectively. 10 mg/mL of protein solution were prepared in sodium phosphate buffer 50 mM at pH 7.0. At second step, acylation reaction mixtures at molar ratios of NHS-FA to protein 1.7:1, 3.2:1 and 6.4:1 were prepared by mixing the activated NHS-FA solution at selected concentrations with protein solution and incubated at 28 °C for 24 h. FA-LZMs and FA-OVAs conjugates were recovered upon thorough dialysis to remove free FA and freeze-dried. Protein content of all samples was determined using the Bradford assay (Bradford, 1976).

2.3. Characterization of ferulic acid-modified proteins

2.3.1. Determination of free amine group content

The free amine content in the native and modified proteins was measured via TNBS assay (Habeeb, 1966) with modification. Protein solutions at 0.2 mg/mL were prepared in sodium bicarbonate buffer (pH 8.5, 0.1 M) and added to TNBS solution (0.01%, w/v) at a ratio of 2:1 (v/v); the mixture (0.75 mL) was incubated at 37 °C in the dark. After 2 h incubation, 0.25 mL of SDS solution (10%, w/v) was added to prevent the precipitation of the protein and 0.125 mL of 1 N HCl was added to

stop the reaction. The absorbance of the mixture was measured at 335 nm using a Beckman DU 650 spectrophotometer (Beckman Instruments Inc.; San Ramon, CA). The assay blank without TNBS reagent and control buffer sample without proteins were run in parallel with the samples. Triplicate measurements were performed for each sample. L-leucine solutions at concentrations ranging from 2 to 16 μ g/mL were used for the construction of the calibration curve. The free amine content was expressed as the mole of free amine moiety equivalent per mg of protein.

2.3.2. Measurement of total phenolic content

The total phenolic content of native and modified proteins was determined using Folin-Ciocalteu phenol reagent. 800 μ L of protein solution (0.05–0.2 mg/mL) was mixed with 150 μ L of sodium carbonate (250 g/L) and 50 μ L of phenol. The mixture was incubated at 40 °C for 30 min and absorbance were measured at wavelength 765 nm. Measurements were carried out in triplicates. The calibration curve was constructed using gallic acid solutions at concentrations ranging from 0.001 to 0.1 mM. The total phenolic content was expressed as the mole of phenolic moiety equivalent per mg of protein.

2.3.3. Mass spectrometry analysis

The mass spectrometry analysis of modified proteins was carried out using a Bruker Maxis electrospray ionization-quadrupole-Time-of-Flight-mass spectrometer (ESI-QTOF-MS). Samples were separated on Agilent PLRP-S column (5 μ M, 1000 Å, 2.1 $\times$ 50 mm) using an inline Dionex Ultimate 3000 UHPLC system at 80 °C. The elution was carried out using a gradient from 80% mobile phase A (0.1% formic acid in water)/20% mobile phase B (0.1% formic acid in acetonitrile) to 20% mobile phase A/80% mobile phase B in 12 min. ESI-MS was performed at positive ion mode, and ESI parameters were set as nebulizer pressure at 0.4–1.0 Bar, temperature at 180–200 °C, 4.0–6.0 L/min of dry gas, capillary voltage at 4500 V. The scanning range was *m/z* 200–3500. The mass signal of protein was deconvoluted and analyzed using Bruker Compass Data Analysis software.

2.4. Laccase-catalyzed oxidative cross-linking of proteins

Laccase-catalyzed cross-linking was carried out using native and FA-modified proteins as substrates. The reaction mixture consisted of 2 mg/mL of protein and 0.024–0.2 U/mg substrate of enzyme solution prepared in sodium phosphate buffer (pH 6.5, 50 mM). Laccase-mediator reactions were also performed with the native proteins using 0.1 to 1 mM of ferulic acid. The cross-linking reactions were carried out for 48 h time course. At selected time intervals, aliquots of reaction mixture were taken and analyzed by electrophoresis. Control reactions without laccase or mediator were run in parallel. After halting the oxidative reactions, the reaction mixtures were dialysed at 4 °C and lyophilized to recover the cross-linked proteins using Gamma 1–16 LSC freeze dried (Christ, Osterode am Harz, Germany).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis was carried out using a mini protein gel apparatus (Bio-Rad, Hercules, CA) following the protocol of Laemmli (1970). 15 μ L of reaction mixture samples (0.2 ~ 0.5 mg of protein/mL) was loaded to 0.75 mm polyacrylamide gels, which consisted of 4% of stacking gel and 12% of resolving gel for OVA samples or 15% for LZM samples. The electrophoresis of the protein was run at 120 V. Protein standards with a broad range of molecular weight (6.5 to 200 kDa, Bio-Rad) were used for molecular weight calibration. After Coomassie staining, the electrophoretic pattern and band relative proportion was analyzed densitometrically using Red Image system and Alphaview software (version 3.3.1, Cell Biosciences, Santa Clara, CA). The bioconversion was estimated from the decrease in the proportion of

monomer upon the enzymatic treatment. The cross-linking extent was defined as the increase in the proportion of high molecular weight fractions, corresponding to cross-linked products.

2.6. Characterization of ferulic linkages of cross-linked proteins

The recovery of phenolic compounds (free grafted ferulic acid and ferulic linkages) from the proteins was performed using an optimized alkaline hydrolysis procedure, in which the protein hydrolysate (12.5 mg/mL) was suspended in 4.2 M sodium hydroxide and incubated at 68 °C for 18 h in darkness. The alkaline hydrolysates were acidified using 10 M hydrochloric acid to adjust the pH to 2.0–2.5. Phenolic compounds were then extracted three times with ethyl acetate at a ratio of 1:1.5 (v/v); the extracts were evaporated until dryness using a Thermo Scientific AES2010 SpeedVac® System (Waltham, MA) and re-dissolved in methanol/water mixture (50:50 v/v). The characterization and quantification were performed by mass spectrometry using Agilent 1290 Infinity II LC system coupled to the 6545 QTOF -MS (Agilent Technologies, Santa Clara, CA). 1 µL of sample was injected and separated on an Agilent poroshell120 EC-C18 analytical column (Agilent Technologies), using a gradient method ramped from 98% of mobile phase A (5 mM ammonium acetate in water)/2% of mobile phase B (methanol/acetonitrile of 1:1, v/v) to 20% of mobile phase A/80% of mobile phase B in 8.5 min, at flow rate of 0.3 mL/min. The MS was operated in both positive (ESI +) and negative ion mode (ESI-). The conditions were set as follows: the drying gas temperature was 200 °C at a flow rate of 10 mL/min, sheath gas nitrogen temperature was 300 °C at a flow rate of 12 mL/min, the pressure of nebulizer was 40 psi, the capillary voltage was 3000 V, the nozzle voltage was 0 V, the fragmentor voltage was 125 V and the skimmer voltage was 65 V. For targeted MS/MS fragmentation, collision energy was 20 V. The identification of oligomerized ferulic acid cross-links was based on the accurate mass and the isotopic patterns, as well as the diagnostic fragment ions if available. The consumption of grafted FA sites on proteins was estimated by the difference between the extracted free grafted FA from the control sample and that from the corresponding reaction sample.

2.7. Assessment of techno-functional properties

The emulsifying property of native and cross-linked LZM samples and the foaming property of the OVA samples were evaluated.

2.7.1. Emulsifying property

Emulsifying property was evaluated according to the method described by Li and Karboune (2021). The emulsion was obtained by homogenizing a mixture of the protein solution (0.5% w/v in 10 mM sodium phosphate buffer, pH 7) and the sunflower oil at a ratio of 3:1 (v/v) using a Fisherbrand™ 850 Homogenizer (Fisher Scientific, Pittsburgh, PA) at 22000 rpm for 90 secs. The initial turbidity of the emulsions and turbidity after 15 min was measured at $\lambda = 500$ nm using Beckman DU650 spectrophotometer, represented by A_0 , A_{15} . The samples were diluted 200 times in 0.1% w/v SDS prior to the turbidity measurement. Emulsifying activity (EA) index and emulsion stability (ES) index were calculated as shown by equation (2.1) and (2.2), respectively.

$$EA_{index} \left(\frac{m^2}{g} \right) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \varphi \times 10^4} \quad (2.1)$$

$$ES_{index}(min) = \frac{A_0}{A_0 - A_{15}} \quad (2.2)$$

where, DF was the dilution factor of the emulsion (200); c was the concentration of the aqueous solution (0.5 w/v); φ was the oil volume fraction (0.25). In addition, micro-images of the non-diluted emulsion samples were taken within 15 min upon the preparation of the emulsion,

using Zeiss Axio Imager Z1 (Zeiss, Jena, Germany) in differential interference contrast (DIC) mode with a 20× magnification objective.

2.7.2. Foaming property

Foaming property were analyzed using a sparging method as described by Waniska and Kinsella (1979) with modifications. 15 mL of protein solution (0.5% w/v in 10 mM sodium phosphate buffer, pH 7.0) was injected via the septum-stoppered inlet into a foam chamber (70 mL), which is a water jacketed glass condenser. Nitrogen was sparged through the solution until the foam chamber was filled, simultaneously liquid was continuously injected to the chamber in order to maintain equal liquid volume as the starting point. The required time for reaching this volume of foam was recorded as T_f . After 5 min, the volume of the liquid drained from the foam was recorded as V_d . Parameters related to foaming properties, which were the percentage of gas entrapped in the foam (G_i), the percentage of foam expansion (FE) and the percentage of liquid retained in the foam after 5 min (R_5), were calculated as the following equations (Eq. 2.3–2.5):

$$G_i = \frac{55 - V_i}{FR \times T_f} \times 100 \quad (2.3)$$

$$FE = \frac{70 - V_0}{V_i} \times 100 \quad (2.4)$$

$$R_5 = \frac{V_i - V_d}{V_i} \times 100 \quad (2.5)$$

where, FR was the nitrogen flow rate (mL/min); V_0 was the initial volume of liquid (15 mL); V_i was the volume of liquid injected during the foaming process.

2.8. Assessment of immunoglobulin E (IgE) immunoreactivity

Inhibition enzyme linked immunosorbent assay (ELISA) was used to evaluate the changes in specific IgE binding capacity of LZM and OVA upon cross-linking (Jiménez-Saiz, Belloque, Molina, & López-Fandiño, 2011). A pool of human sera from individuals with documented egg allergy (acquired from PlasmaLab International, Everett, WA, USA) and containing high level of specific IgE antibodies to LZM and OVA was used for the IgE binding inhibition experiments. Sera containing specific IgE antibody for house dust mite and low total IgE were used as negative controls and non-atopic controls, respectively. Proteins extracts at 10-fold serial dilution (0.001-to 1000 µg protein/mL) as inhibitors were pre-incubated with pooled sera or the control sera overnight at 4 °C for OVA samples, and for 2 h at 25 °C for LZM samples. 96-well microplates (Costar™, Corning, NY, USA) were first coated overnight with 25 µg native OVA or LZM in 10 mM phosphate buffer 7.4 with 137 mM NaCl (PBS) at 4 °C and washed three times with 300 µL of PBS containing 0.1% Tween-20 (PBS-T). The plate was blocked with 5% w/v bovine serum albumin (BSA) in PBS-T for 2 h at 25 °C and washed with PBS-T. The coated plates were added with 50 µL of the inhibitor mixtures and incubated for 2 h at 25 °C. After washing, 50 µL of peroxidase-labeled mouse anti-human IgE (1:1000 dilution with 1% BSA in PBS-T) was loaded as secondary anti-body and incubated for 1 h at 25 °C. The plate was washed again and developed by reacting with 50 µL TMB for 15 min at 25 °C. The reaction was halted by adding 50 µL 1 N H₂SO₄ and the absorbance at wavelength 450 nm was measured (A). Pooled sera with no inhibitors added was used as positive control (100% reactivity) (absorbance recorded as A_0). The percentage inhibition of IgE binding of cross-linked protein were calculated from the absorbance ($\frac{A}{A_0}$)%. IC₅₀ was defined as the concentration of the protein samples, where 50% of inhibition of IgE binding was achieved, which was determined via curve-fitting using Gen5 software (Bio-Tek, Winooski, VT, USA). The percentage of IgE binding were determined by the ratio of the IC₅₀ of the native protein to the cross-linked protein (eq.2.6)

$$\% \text{ of IgE binding} = \frac{IC50(\text{native})}{IC50(\text{cross-linked})} \times 100 \quad (2.6)$$

2.9. Statistical analysis

All experiments were performed in at least duplicates. Statistical analysis was performed by XLSTAT software (Addinsoft, New York, NY, USA) and SPSS Statistics 26 (IBM, Armonk, NY, USA). A two-way ANOVA followed by mean comparison was performed via the Turkey test. p -values ≤ 0.05 were considered as statistically significant.

3. Result and discussions

3.1. Surface modification of proteins and their chemical-structural characterization

The grafting of protein substrates with phenolic moieties was investigated in order to add controlled cross-linking sites and modulate the protein cross-linking efficiency in laccase-catalyzed reactions. Amidation at selected molar ratios of NHS-FA to protein (1.7:1, 3.2:1, 6.4:1) was performed in order to achieve different contents of grafted FA moiety on the protein surface. As the amine side chain of lysine residues and the primary amine of *N*-terminus are the main targeted sites for the amidation via NHS-ester, it can be assumed that the modification extent is dependent on the position and accessibility of lysine residues (Hermanson, 2013). A decrease in the free primary amine content of LZM and OVA was observed upon the amidation reaction. 55.7, 75.6, 92.6% of free primary amine groups on LZM were acylated at NHS-FA/protein molar ratio of 1.7:1, 3.2:1, 6.4:1, respectively; while 17.1, 21.4, 21.8% were acylated on OVA (Fig. 1A). The acylation percentage of LZM was positively correlated with the molar ratio. Similar correlation has been reported for the modification of gelatin by NHS-esters of different saturated fatty acids and for the simultaneous cross-linking of fish collagen with the NHS-adipic acid derivative (Aewsiri, Benjakul, Visessanguan, Wierenga, & Gruppen, 2011; Shen, Tian, Liu, & Li, 2015). Notably, in the case of OVA, a limited increase in the proportion of acylated amines was observed when the molar ratio of NHS-FA/OVA

was increased to 6.4:1. This can be attributed to the limited accessibility of amine groups of some lysine residues of OVA and/or to the changes in the solvation of lysine residues in the acylated product of OVA at higher molar ratio of 6.4:1.

With the increase in the acylation percentage, an increase in the total phenolic content was observed (Fig. 1B). This could be due to either the incorporation of FA to protein surface or protein conformational change which cause the exposure of selected amino acid residues (tryptophan, tyrosine, cysteine) from the protein core. As compared to native LZM, the phenolic content of modified LZM increased by 182.2, 202.3 and 264.2 nmol/mg (2.1-, 2.2- and 2.6-times as the native) upon amidation using NHS-FA to protein ratio of 1.7:1, 3.2:1 and 6.4:1, respectively. Depending on the total phenolic content, the modified LZMs characterized by low, moderate and high modification extent and annotated as L-LZM, M-LZM and H-LZM. Moderate increase (by 52.5 nmol/mg of protein, 1.2-times as the native) in the phenolic content of FA-OVA was obtained upon the modification at NHS-FA/OVA ratio of 6.4:1. However, equally low increase (by 16.0 nmol/mg of protein) in the phenolic content was achieved by using NHS-FA/protein ratios of 1.7:1 or 3.2:1. The modified OVAs were, therefore, characterized by moderate (M-OVA) and low (L1-OVA)/L2-OVA) modification extent. Although the acylated amine percentage in M-OVA was very close to that of L1-, L2-OVA, its increase in total phenolic content was 2.3-fold higher. This may be due to the conformational changes of M-OVA that may have happened upon the incorporation of FA moieties leading to the under-estimation of the acylation percentage. The mass spectrum of M-OVA shows an obviously broader peak with the center of mass signal shifting towards higher molecular weight, as compared to the native and L1/L2-OVA (Fig. S1). This provides the evidence that higher numbers of FA sites were grafted on M-OVA as compared to L-OVAs.

The profiles of FA-modified LZMs were further characterized by mass spectrometry (Fig. 1C). Native lysozyme was detected at mass 14,304.70 Da. The incorporation of one FA molecule on protein via amide bond formation will increase the mass of protein by around 176 Da. Upon surface modification with FA, protein peaks with the masses equal to $14,304.70 + 176n$ Da ($1 \leq n \leq 4$) were present in the mass spectra. By increasing NHS-FA/protein ratio from 1.7:1 to 6.4:1, the mass distribution shifted towards higher molecular weight. The

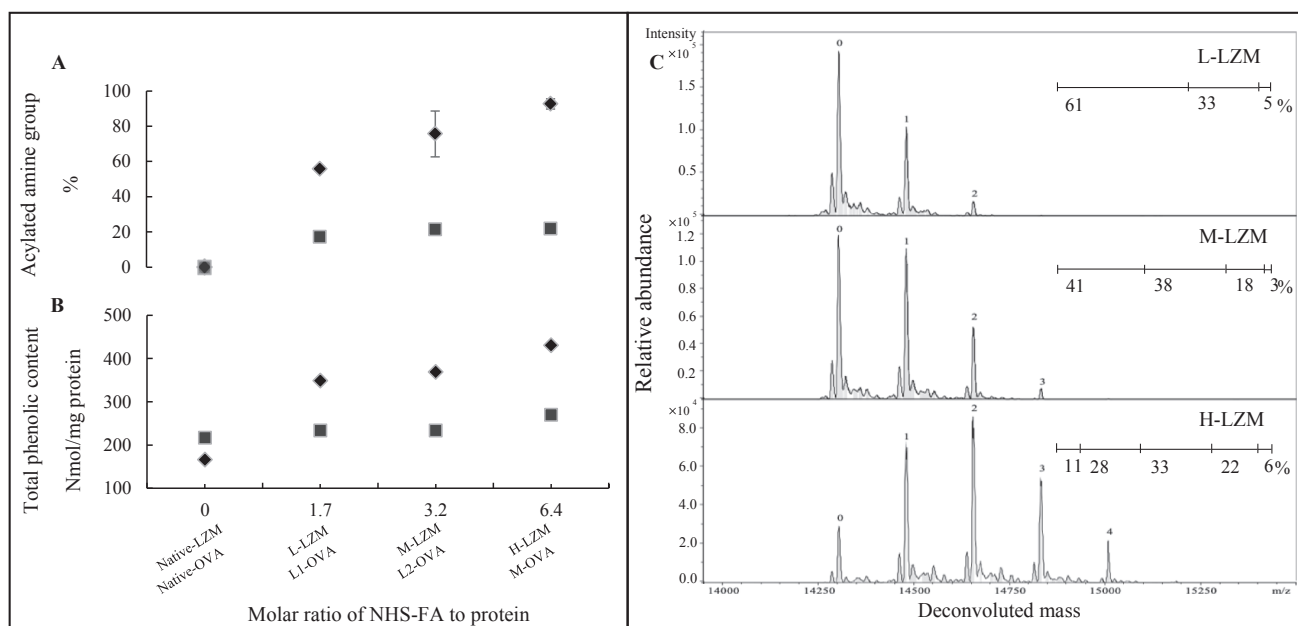


Fig. 1. The chemical properties of modified protein substrates from amidation at different ratios of reactant. Acylation percentage (A); and total phenolic content (B) of lysozyme (LZM, $\blacklozenge$) and ovalbumin (OVA, $\blacksquare$). NHS-FA: *n*-hydroxysuccinimide-ferulic acid ester. C. Deconvoluted mass spectra of modified lysozyme (L-LZM, M-LZM and H-LZM) upon ferulic acid grafting treatment. The mass peaks were marked with numbers, representing the number of ferulic acid adduct that were attached on the protein. The calibration scale refers to the relative proportion of each mass peak.

distribution of protein peaks shifted from unmodified LZM to LZM + FA and LZM + 2FA for L-LZM, and to an additional LZM + 3FA for M-LZM. LZM + 4FA peak was further detected in the mass spectrum of H-LZM. Since the grafted FA is much smaller than the protein, uniform response factors were assumed for native and modified proteins. From the relative abundance distribution of protein peaks, the percentage of FA-modified LZM was estimated at 39, 59 and 89% for L-, M- and H- LZM, respectively. These results are in the agreement with the trend of both the acylated amine percentage and the increased phenolic content (Fig. 1A and B).

3.2. Reactivity and cross-linking of native and modified proteins

The cross-linking of native and FA-modified proteins by LacTv was carried out. Prior to enzymatic treatments, FA-modified LZMs/OVAs displayed similar electrophoretic patterns to their native forms (Data not shown), indicating no cross-linking occurred during FA-modification of proteins. The bioconversion of proteins by laccase through oxidation can lead to cross-linking, fragmentation and other types of oxidative modifications. The time course for the bioconversion of protein monomer (Fig. 2) and the protein cross-linking product profile (Fig. 3) were evaluated by SDS-PAGE analysis.

Little evidence of bioconversion of native LZM by laccase was revealed within the investigated reaction time 0–24 h (Data not shown). Upon the addition of free FA as mediator, the bioconversion of LZM was achieved at a relatively low abundance (7.1–11.1%) as shown by reaction time course (Fig. 3A, Fig. S2B). For L-, M- and H-LZM, 24.7 to 29.5% of their monomeric proteins were quickly converted within the first 3 h of reaction, where the highest bioconversion rate was observed with H-LZM, followed by M-LZM and then L-LZM (Fig. 2A). These reveal that all FA-modified LZM exhibited higher reactivity towards laccase action than native LZM. Beyond 3 h, the bioconversion rate slowed down gradually and reached 29.2 to 52.3% at 24 h reaction, with the highest bioconversion obtained with M-LZM. According to the molecular weight profile of the reaction mixtures from FA-modified LZM, the main cross-linked products were dimer, short-chain oligomers ($3 < \text{unit of monomer} \leq 5$) and long-chain oligomer ($5 < \text{unit of monomer} < 10$) within the first 3 h of reactions (Fig. 3A, B and C, Fig. S2A). While during 3 to 24 h, the polymer (unit of monomer ≥ 10) proportion increased with the concomitant decrease in the dimer and oligomer proportions. L- LZM tended to form mainly dimer and oligomers, whereas polymers were formed in M- LZM and H- LZM reaction systems with the highest proportion of polymers (30.6%) achieved in M- LZM upon 24 h of reaction. Although H- LZM had the higher extent of grafted FA sites than M- LZM, its cross-linking at 24 h of reaction was lower. This result can be ascribed to the substrate steric hindrance due to multiple FA sites grafted on H-FA-LZM, affecting its binding in the active site of laccase. When the cross-linking of native LZM was mediated by free FA instead of through the grafted FA sites, the formation of dimer and oligomers were less pronounced, and no polymer formation was observed (Fig. 3A', B' and

C').

No clear evidence of bioconversion or cross-linking was detected on the native OVA when treated with LacTv alone (data not shown) or in the presence free FA mediator (Fig. S2D). In contrast, the treatment of L1, L2-OVAs with LacTv led to a bioconversion of 6.3 and 5.1% at 10 h, respectively, whereas a bioconversion of 31.1% was achieved with the use of M-OVA as substrate (Fig. 2B). Beyond 10 h reaction time, decreased bioconversion as indicated by an increase in the proportion of M-OVA monomer fraction was obtained, revealing the possibility of oxidative fragmentation or precipitation of cross-linked OVA proteins at longer reaction time. As the highest bioconversion was achieved with the use of M-OVA, it can be inferred that the high incorporation of FA into OVA in the M-OVA created additional reactive sites for LacTv oxidative cross-linking action. The long chain oligomers were obtained as the main cross-linked products in the reaction with M-OVA, whereas the cross-linking of L-OVAs resulted in the formation of short-chain oligomers (Fig. 3D, E and F, Fig. S2C).

The overall results confirmed that the enhancement of bioconversion and the modulation of end-product profile of laccase-catalyzed cross-linking can be achieved efficiently by tailoring the extent of FA sites of the proteins. Other cross-linking approaches to obtain oligomeric LZM and OVA has been reported previously (Giosafatto et al., 2012; Liu et al., 2018; Ma et al., 2015; Yang, Lesnierowski, & Xu, 2019). These studies showed that irreversible partial denaturation process or the use of mediator was essential to overcome the steric hindrance originated from the protein structure for the enzymatic cross-linking. The proposed approach in the present study has the advantages of limiting side reactions, using mild reaction condition and leading to a high degree of cross-linking. As per the author's knowledge, the synthesis of polymeric LZM cross-linked products via enzymatic approaches has not been reported previously.

Selected cross-linked proteins with well-defined profiles (* in Fig. 3, Fig. S2A and C) were subsequently used for the structural and functional characterization. The selected cross-linked LZM are L-LZM (3), M- LZM (6) and H- LZM (12) characterized by a cross-linking extent of 18.8, 26.4 and 31.1%, and relative proportions of dimer/oligomer/polymer of 26.1/16.4/0%, 31.3/14.7/7.4% and 26.9/8.4/17.6%, respectively. The selected cross-linked OVA samples, L1- OVA (10) and M- OVA (10), have shown a cross-linking extent of 5.9% and 31.1%, with a relative proportion of short/long chain oligomer of 11.3/0% and 24.0/12.3%, respectively.

3.3. Characterization of ferulic type cross-links

The laccase-catalyzed cross-linking of proteins can happen via multiple functional groups, including tyrosine, cysteine residues and primary amine groups (Mattinen et al., 2006; Steffensen et al., 2009; Wang, Lv, Zhang, Yang, & Gao, 2019). Considering the reactivity of grafted FA sites on modified proteins and their accessibility, the cross-linking most likely occurred on FA sites prior to the amino acid residues. The putative

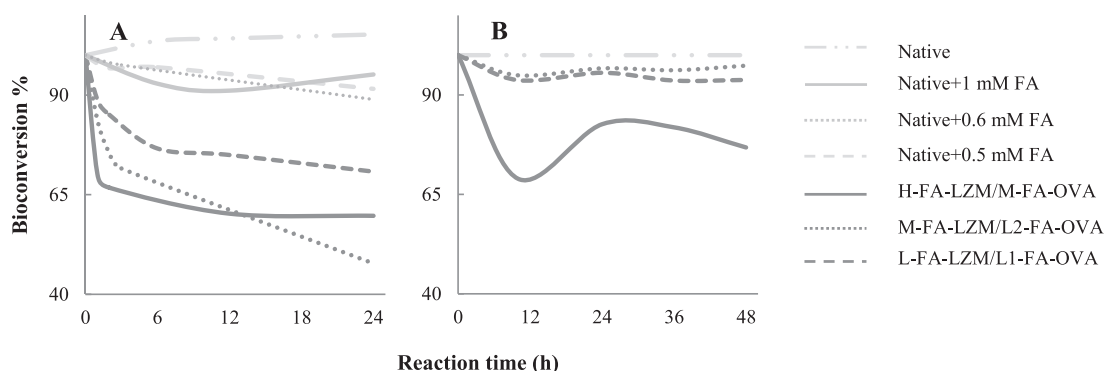


Fig. 2. Time course for the bioconversion of lysozyme (LZM) (A) and ovalbumin (OVA) (B) substrate monomer over time. Abbreviation: FA, ferulic acid.

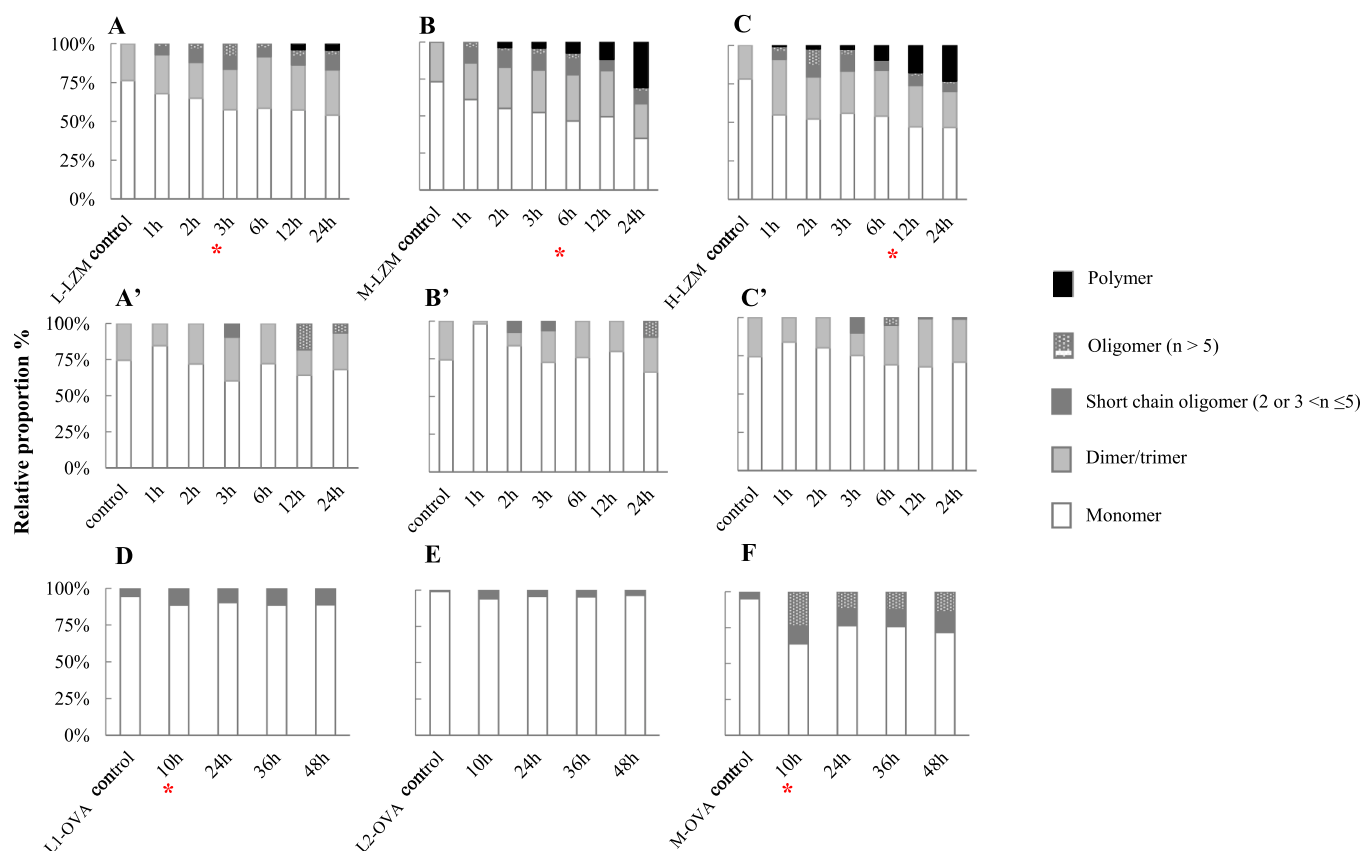


Fig. 3. Molecular weight profile of cross-linked products over time in *T. versicolor* laccase treatment of native/modified lysozyme (LZM) and modified ovalbumin (OVA). FA-modified proteins upon laccase treatment: L- LZM (A), M-LZM (B), H- LZM (C), L1- OVA (D), L2- OVA (E) and M- LZM (F); Native lysozyme upon enzymatic treatment with the addition of free ferulic acid at 0.5 mM (A'), 0.6 mM (B') and 1 mM (C'); these concentrations are equivalent to the extent of grafted FA sites on L, M, and H-LZM, respectively. n, represents the units of monomer.

identification of ferulic type cross-links present in the protein alkaline hydrolysate was based on the matching of mass accuracy and the isotope fidelity with the theoretical values (Fig. 4). From the hydrolysate of cross-linked FA-LZMs, three different types of ferulic oligomer compounds were identified in negative ion mode: dehydro-diferulic acid ($C_{20}H_{18}O_8$) with m/z at 385.0927, dehydro-decarboxylated diferulic acid ($C_{19}H_{18}O_6$) with m/z at 341.1028, and dehydro-triferulic acid with m/z at 577.1344 ($C_{30}H_{26}O_{12}$) (Bunzel, Ralph, Bruning, & Steinhart, 2006; Ward, Hadar, Bilkis, Konstantinovskiy, & Dosoretz, 2001) (Table 1). From the hydrolysate of cross-linked FA-OVA, only dehydro-diferulic acid ($C_{20}H_{18}O_8$) was detected (Table 1). As well, multiple peaks at different retention times were found for $C_{20}H_{18}O_8$ and $C_{19}H_{18}O_6$ with matching score > 96 (Fig. 4A and B). This finding was consistent with the reported data showing that dehydro-diferulates can be formed with various isomer structures (Bunzel et al., 2006; Vismeh et al., 2013; Ward et al., 2001). Among the compound ions, the major one (m/z 385, retention time around 4.0 min) was further confirmed to be 8–5' noncyclic dehydro-diferulic acid, as indicated by the distinguishing ion detected at m/z 309.0753 yielded by positive MS/MS fragmentation (Vismeh et al., 2013) (Fig. 4D insert & Table 1). The product of FA in laccase-catalyzed oxidative cross-linking has been previously identified to be majorly 8–5' dehydrobenzofuran-diferulic acid, with relatively low extent of other forms including 8–8', 8–5' (noncyclic) dehydro-diferulic acid (Aljawish et al., 2014; Carunchio et al., 2001; Ward et al., 2001). In the present work, 8–5' (noncyclic) dehydro-diferulic acid (DiFA(1)) was shown to be the dominant linkage for both cross-linked FA-LZM and FA-OVA samples, which coexisted with other isomers of diferulate cross-links (DiFA(2)).

For all investigated cross-linked FA-modified proteins, >88% consumption of grafted FA sites were achieved, confirming that FA sites

were efficiently used for cross-linking (Table 1). For FA-LZM samples, the content of grafted FA sites that was involved in cross-linking was comparable to the total cross-linked sites estimated based on the corresponding molecular weight distribution. This suggest that the cross-linking of FA-LZMs happened mainly on the grafted FA sites. For FA-OVA samples, grafted FA sites corresponded only to 20–40% of the total cross-linked sites, revealing that other types of cross-links may have been involved in the cross-linked FA-OVAs. Indeed, the FA-grafting process on OVA may have enhanced the exposure of its oxidizable amino acids to laccase action. The composition of the identified FA-type cross-links in each cross-linked protein samples was also characterized (Table 1). The relative percentage composition of FA type cross-links was estimated by the corresponding signal intensity, assuming that the ionization efficiency of these ferulic compounds were similar. The total quantity of FA cross-links increased with the increase of the FA-grafting and cross-linking extent. M- LZM (6) and H- LZM (12) did not show great difference in the content of DiFA (1) type cross-link (~27.00 nmol/mg); however, H- LZM (12) contained more DiFA (2) (19.05 nmol/mg) and tri-FA (0.43 nmol/mg) than those of M-LZM (6) (14.48 and 0.32 nmol/mg, respectively). As for cross-linked FA-OVAs, a total of 1.22 and 8.25 nmol/mg DiFA (1) cross-links was obtained for L1-OVA (10) and M-OVA (10), respectively. Since most of ferulic type cross-links are diferulic, the oligomers/polymers should be mainly in chain shapes (single chains and/or branched chains) connected by diferulate cross-links. Extra branches in the product from H- LZM are expected as it consisted of 28% of LZM that are attached with 3–4 FA sites. Taking the consideration of types and proportion of the FA cross-links, postulated structures for cross-linked FA-LZM/OVA via FA-type cross-links can be elucidated as scheme 1 (in supplementary materials). The molecular shape of cross-linked product directed by the grafted FA-sites could be

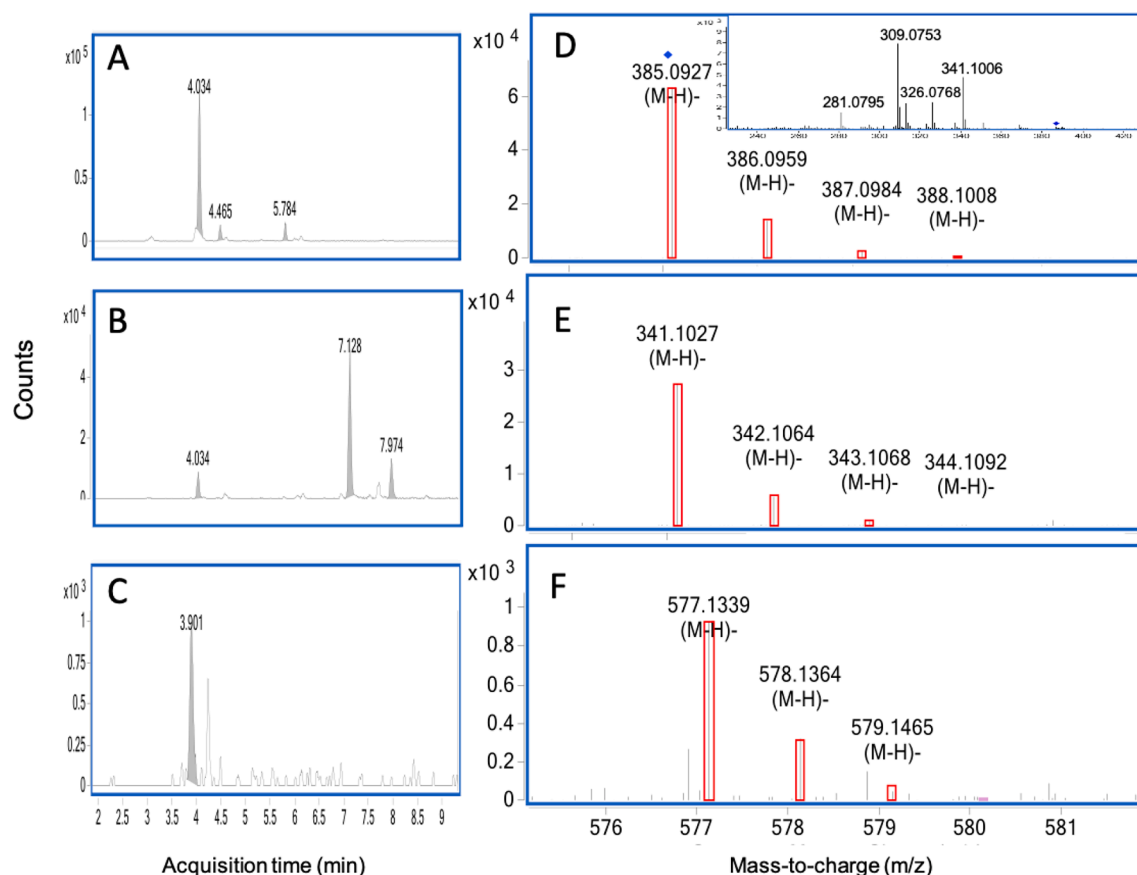


Fig. 4. Extracted ion chromatograms for detected ferulic acid cross-links from alkaline hydrolysates. A. dehydro-diferulic acid ($C_{20}H_{18}O_8$); B. dehydro-decarboxylated diferulic acid ($C_{19}H_{18}O_6$) and C. dehydro-triferulic acid ($C_{30}H_{26}O_{12}$) and the corresponding mass spectra illustrating the isotope distribution for dehydro-diferulic acid ($C_{20}H_{18}O_8$) at retention time 4.0 min (D), dehydro-decarboxylated diferulic acid ($C_{19}H_{18}O_6$) at retention time 7.1 min (E) and dehydro-triferulic acid ($C_{30}H_{26}O_{12}$) at retention time 3.9 min (F) (red rectangles indicate the theoretical isotope pattern for corresponding chemical formula). Insert: Target MS/MS fragmentation mass spectra for $C_{20}H_{18}O_8$ at retention time 4.0 min: in positive ion mode, parent ion with m/z at 387.1080. Collision energy was 20 V. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Identification and quantification of ferulic type linkages from cross-linked proteins.

	[M-H] ⁻ <i>m/z</i> ⁱ	L-LZM (3) nmol/mg	M-LZM (6)	H-LZM (12)	L-OVA (10)	H-OVA (10)
DiFA (1) ⁱⁱ	385.09	24.75 ± 0.09 ^a	27.92 ± 0.71 ^a	27.24 ± 2.32 ^a	1.22 ± 0.00 ^b	8.25 ± 0.00 ^b
DiFA (2) ⁱⁱ	341.10	11.83 ± 0.07 ^b	14.48 ± 0.50 ^{a,b}	19.05 ± 2.09 ^a	N/A	N/A
TriFA (3) ⁱⁱⁱ	577.13	0.20 ± 0.00 ^b	0.32 ± 0.04 ^{a,b}	0.43 ± 0.06 ^a	N/A	N/A
Total quantity of cross-links		36.78 ± 0.02 ^c	42.72 ± 0.25 ^b	46.72 ± 0.17 ^a	1.22 ± 0.00 ^c	8.25 ± 0.00 ^d
Cross-linking extent (%) ^{iv}		95.00 ± 0.00	94.48 ± 0.01	89.75 ± 0.01	100.00 ± 0.00	88.30 ± 0.00

Abbreviations: FA, ferulic acid; LZM, lysozyme; OVA, ovalbumin. Statistical difference was indicated by different letters within the same row.

ⁱ Observed mass to charge ratio of the identified compounds, which is within 1.5 ppm of the theoretical values.

ⁱⁱ Di-ferulate type linkages; DiFA (1) was identified as 8-5' noncyclic dehydrodiferulic acid whereas DiFA (2) represents of other type of isomers of diferulate linkages.

ⁱⁱⁱ Tri-ferulate linkage.

^{iv} Cross-linking extent was estimated by the percentage of grafted ferulic acid site that were consumed upon laccase-treatment when referred to the control samples.

distinguished from those of natural proteins, which were shown to be in the shape of aggregate cluster connected through shared oligo-tyrosine linkages (Dhayan, Sforza, Wierenga, & Gruppen, 2015).

3.4. Evaluation of the cross-linked protein as potential functional ingredients

The emulsification property of native and cross-linked LZM was evaluated. The native LZM had limited emulsification performance, in particular the emulsifying stability (Fig. 5A). This can be explained by its high surface hydrophilicity or inflexible structure (Acton, Kropp, & Dick, 1990). L- LZM (3), with low cross-linking extent of 18.8%, exhibited similar emulsification performance to that of native LZM. With increased cross-linking extent (26.4 and 31.4%) in M-LZM (6) and H-LZM (12), an enhancement in the emulsifying property was observed. Among the LZM samples, cross-linked M-LZM (6) exhibited the highest emulsifying ability, two times higher than that of the native. The micro-image of emulsion made with M-LZM (6) was also characterized by smaller oil droplets with more homogenous size than the other emulsions (Fig. 5B). The presence of LZM dimer and oligomers in M-LZM (6) may have favored its adsorption at oil–water interface. H-LZM (12) led to the highest emulsifying stability among all LZM samples. High proportion of polymeric LZM present in H-LZM (12) have most likely promoted the formation of protective layer around the oil droplets, limiting the creaming and coalescence (Ma et al., 2011). The cross-linking of FA-modified LZM may have also enhanced the surface hydrophobicity of LZM, thus its adsorption and film stability at oil–water interface (Kato,

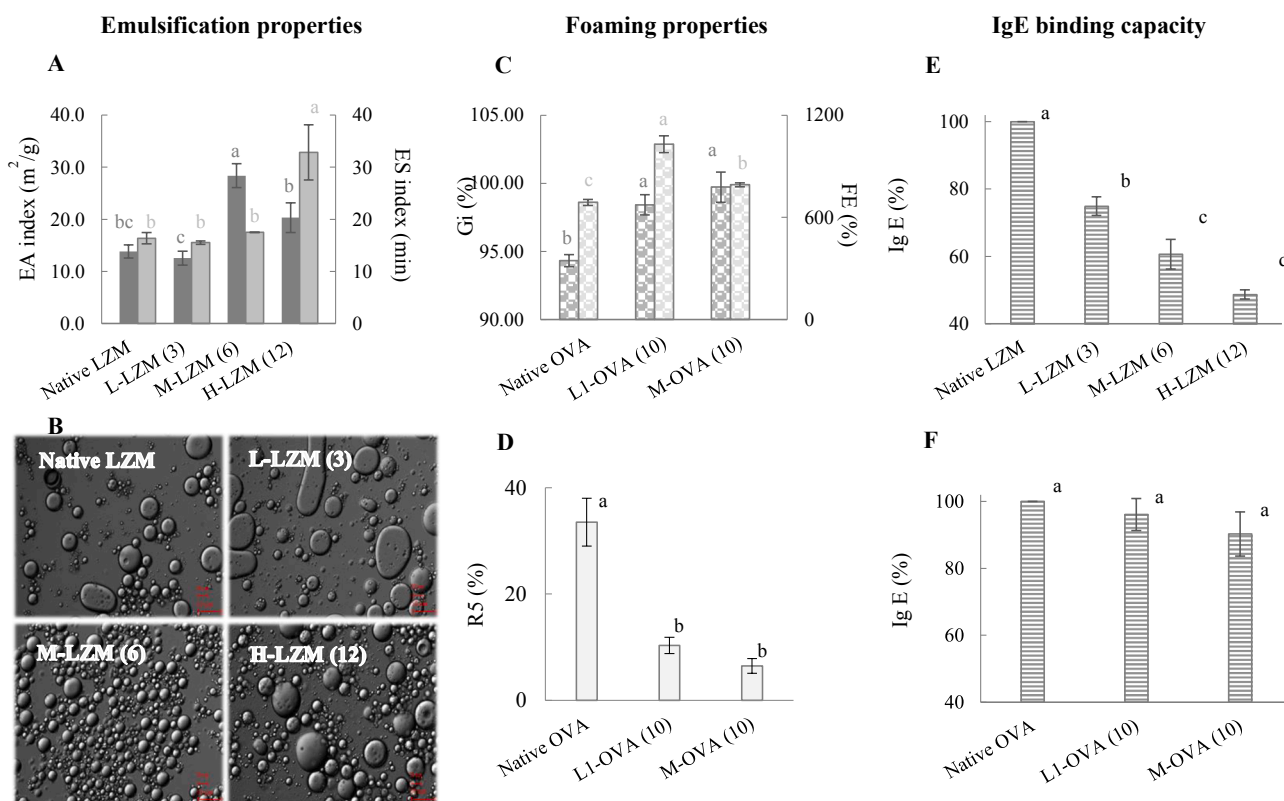


Fig. 5. Effect of cross-linking on techno-functional property and immunoglobulin E binding capacity. A. emulsifying activity index (dark grey) and emulsion stability (light grey); B. micro-images of the emulsion; C. foaming capacity was indicated by percentage of gas entrapped (Gi) (dark grey) and foam expansion FE(%) (light grey); D. foam stability as indicated by percentage of liquid drainage in 5 min; E & F, IgE binding capacity of native/cross-linked lysozyme (LZM, E) and ovalbumin (OVA, F), respectively. Statistical differences were indicated by different letters above the histogram within the same parameter.

Tsutsui, Matsudomi, Kobayashi, & Nakai, 2014; Yang et al., 2019). Improved stabilization effect of LZM as an emulsifier was previously achieved by enhancing the molecular size via the conjugation of LZM with various oligo/polysaccharides (Hamdani, Wani, Bhat, & Siddiqi, 2018; Seo, Karboune, L'Hocine, & Yaylayan, 2013).

The foaming capacity of native and cross-linked OVA was evaluated by measuring the percentage of gas entrapped (Gi) and foam expansion (FE); while the foaming stability was assessed by estimating the liquid drainage after 5 min (R₅) (Fig. 5C&D). Higher foaming capacity was observed with the cross-linked OVA (98.4, 99.7%) than the native one (94.3%) as indicated by the Gi (Fig. 5C). As well, both cross-linked OVA exhibited higher foam expansion than the native one, especially for L1-OVA (10) with relatively low cross-linking extent (6.1%), the FE value of which was 1.5-fold as that of the native (Fig. 5C). On the other hand, both cross-linked FA-OVAs showed impaired the foam stability as indicated by R₅ value (Fig. 5D); a greater decrease in the foam stability was obtained in the presence of M-OVA (10) with relatively high cross-linking extent (31.1%). Overall, the cross-linked OVAs exhibited a higher foaming capacity, while producing less stable foam than native OVA. The enhanced foaming capacity of cross-linked OVAs is most likely associated with low cross-linked extent and with relatively low molecular weight of cross-linked products (short-chain oligomer, 2 < monomer units ≤ 5). It has been previously shown that a proper hydrophobicity-hydrophilicity balance achieved upon oxidative cross-linking treatment can favor the foaming performance of OVA, while the loss in this balance due to prolonged treatments could affect the foaming performance (Li et al., 2019). In contrast with our observation, the increase in the molecular weight of OVA upon heat treatment was associated with enhanced foam stability (Sheng et al., 2019). The presence of FA moieties in the cross-linked OVAs may have either disrupted the intra- or inter- molecular hydrophobic interaction of OVA

that are responsible for film viscoelasticity and stability and/or weaken the electrostatic repulsion between bubbles resulting in faster drainage (Aewsiri et al., 2009).

Cross-linking can alter the integrity of IgE-binding epitopes on protein thus possibly affecting the allergenic potential of the protein ingredients. A decrease in IgE binding capacity was observed for all three cross-linked FA-LZMs, indicating the potential of cross-linking approach for reducing the allergenicity of LZM (Fig. 5E). In addition, the decrease in IgE binding capacity was positively correlated with the FA-grafting/cross-linking extent. Among the cross-linked LZMs, H-LZM (12), containing more branched cross-linked product and polymers, showed the highest reduction (~51%) in IgE binding capacity. Other studies have reported a decrease in the IgE binding capacity of LZM upon glycation and carboxymethylation (Mine & Zhang, 2002; Seo et al., 2013); while urea-induced denaturation of LZM led to a significant increase in allergenicity, as multiple epitope regions of LZM was revealed upon disulfide bonds reduction (Jiménez-Saiz et al., 2014; Mine & Zhang, 2002). No significant difference was shown in IgE binding capacity of the native and cross-linked OVA (Fig. 5F). It can be concluded that the cross-linking did not effectively alter the IgE binding capacity of OVA as compared to LZM. It has been reported that the sequential epitopes of OVA were majorly responsible for the binding with human anti-OVA IgE, while anti-LZM IgE interacted with both conformational and sequential epitopes (Mine & Zhang, 2002). Ma et al. (2015) attributed the insignificant changes in IgE binding capacity of OVA upon transglutaminase-catalyzed cross-linking to low cross-linking extent (Ma et al., 2015). On the other hand, the cross-linking of OVA using poly-phenol oxidase-caffeic acid reaction system was reported to significantly reduce the specific IgE binding capacity and other immunostimulatory responses as a result of extensive cross-linking and conformational changes (Liu et al., 2018; Tong et al., 2018). In literature, the effect of

protein cross-linking by laccase-phenolic mediator reaction system on allergenicity was protein-dependent. Laccase-catalyzed cross-linking lowered IgE binding capacities of fish allergen protein and peanut protein by 34.8% and 50.4%, respectively, while it promoted the allergic sensitization of β -lactoglobulin (Lv et al., 2019; Mihajlovic et al., 2016; Stojadinovic, Pieters, Smit, & Velickovic, 2014).

4. Conclusion

The cross-linking of egg white LZM and OVA were efficiently achieved by *T. versicolor* laccase upon the grafting of FA sites on their surface, especially for LZM, in which polymerization happened. The extent of grafted-FA sites was positively correlated with substrate reactivity/cross-linking efficiency. The cross-linking of the proteins on their grafted FA sites occurred via the formation of di- and/or tri-ferulate, with 8–5' noncyclic dehydro-diferulate being the main ferulic type linkage, resulting in chain-shape molecular assemblies. The cross-linked FA-LZM exhibited enhanced emulsifying property, while the cross-linking of FA-OVA, particularly at low extent and with low molecular weight product, was shown to improve the foaming capacity. Furthermore, the FA-grafting-laccase-catalyzed cross-linking modification approach resulted in a decreased protein IgE binding capacity, which was found to be associated with the modification/cross-linking extent. Our results suggested that FA-grafting-laccase-catalyzed cross-linking approach is a promising one for enhancing the ability of proteins with poor functionalities or non-functional ones as well as for the production of hypoallergenic protein ingredients. Further studies are needed to confirm this potential using additional bio-functional tests, cell or animal allergic models, skin prick test (SPT) or human oral challenges.

CRediT authorship contribution statement

Mingqin Li: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Salwa Karboune:** Conceptualization, Supervision, Methodology, Validation, Writing - review & editing, Project administration, Funding acquisition. **Lan Liu:** Investigation, Methodology, Formal analysis, Writing - review & editing. **Kelly Light:** Investigation, Methodology. **Lamia L'Hocine:** Methodology, Writing - review & editing. **Allaoua Achouri:** Investigation, Methodology, Writing - review & editing. **Melanie Pitre:** Investigation, Methodology. **Cesar Mateo:** Methodology, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.129587>.

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Combining phenolic grafting and laccase-catalyzed cross-linking: effects on structures, technofunctional properties and human immunoglobulin E binding capacity of egg white proteins

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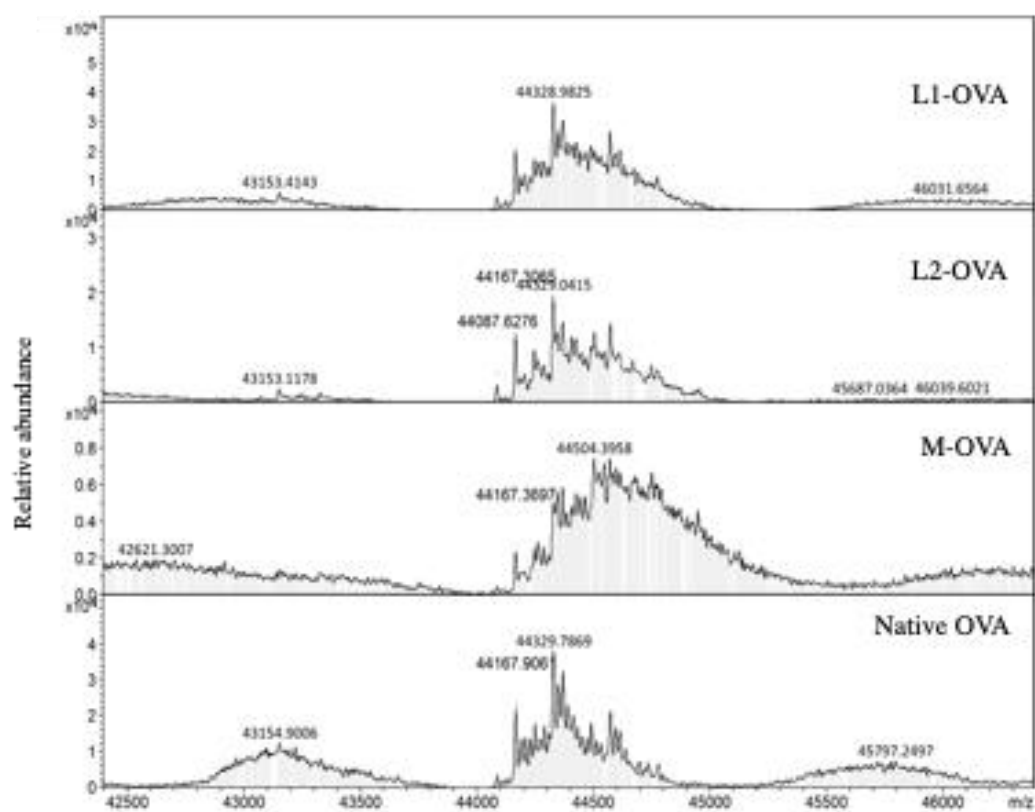


Fig. S1. Deconvoluted mass spectra of native and modified ovalbumin (OVA) at different modification degrees (L1, L2, M).

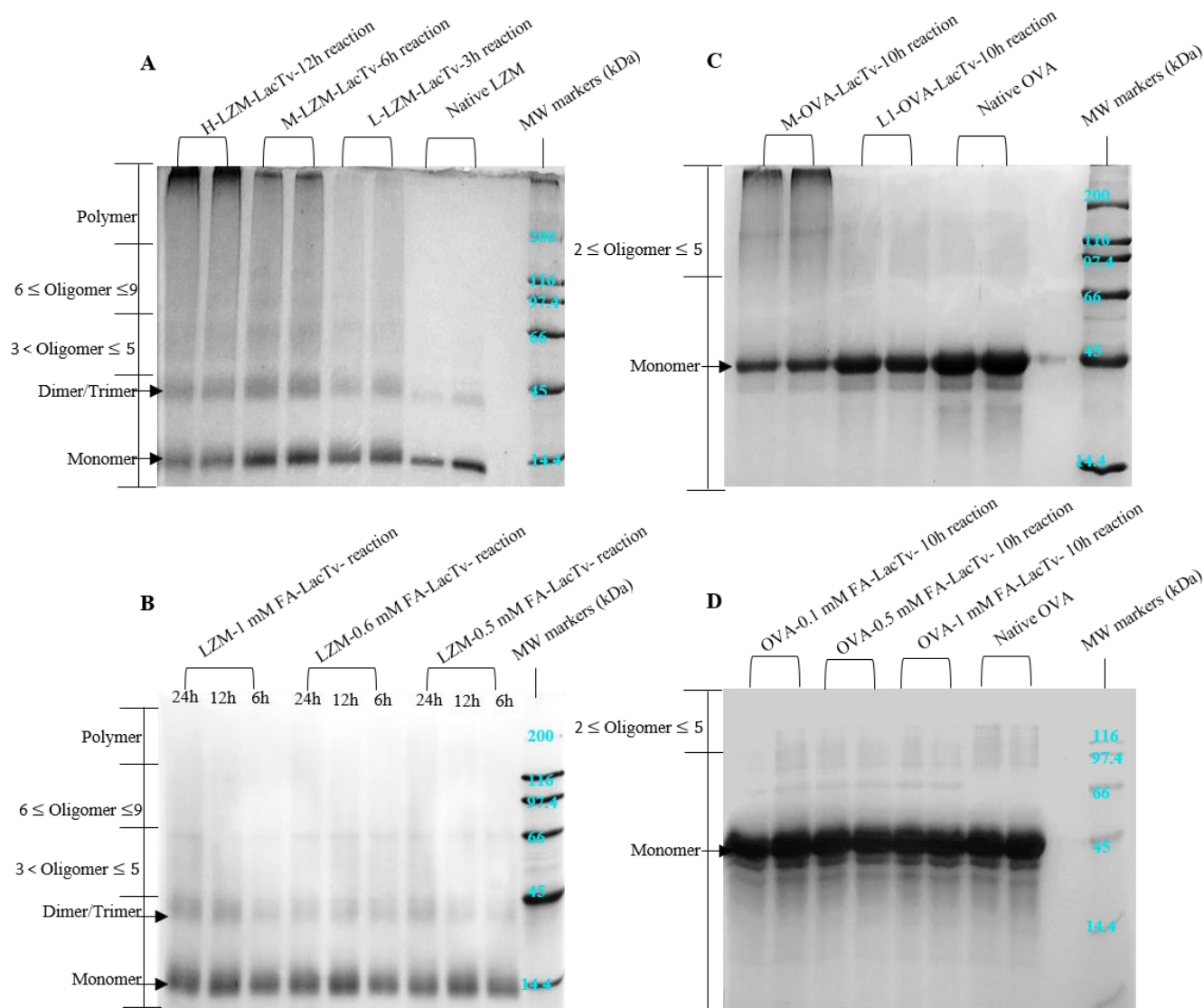
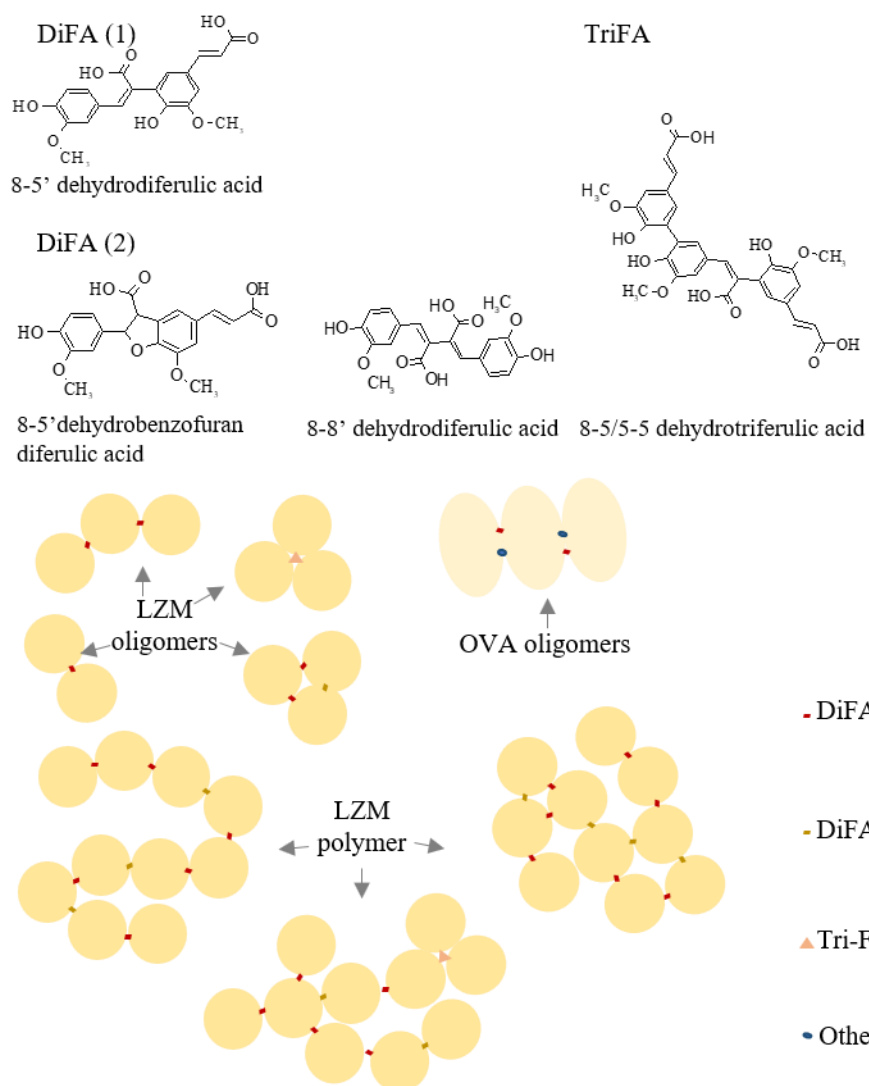


Fig. S2. SDS-PAGE analysis of protein cross-linking reaction mixture catalyzed by laccase from *T. versicolor*. **A.** selected reaction mixture from grafted LZMs and native LZM, all samples were shown in duplicate. **B.** reaction mixture of native LZM with or without free FA mediator (0.5-1 mM) of 6-24h. **C.** selected reaction mixture from grafted OVAs and native OVA, all samples were shown in duplicate. **D.** reaction mixture of native OVA with or without free FA mediator (0.1-1 mM) of 10h, all samples were shown in duplicate. Abbreviation: LZM, lysozyme; OVA, ovalbumin; FA, ferulic acid; LacTv, laccase from *T. versicolor*; MW, molecular weight.



Scheme 1. Potential cross-links involving grafted ferulic acid (FA) sites and associated oligomeric or polymeric product of lysozyme (LZM), ovalbumin (OVA). It is assumed that DiFA (1) linkages are mainly formed at the initial stage of cross-linking, whereas the polymerization and the branching of protein-chains occurred preferably via DiFA (2) or triferulate (tri- FA) type linkages.