Improving Laccase Catalyzed Cross-Linking of Whey Protein Isolate and Their Application as Emulsifiers

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ABSTRACT: Whey protein isolate (WPI) was chemically modified by vanillic acid in order to enhance its cross-linkability by laccase enzyme. Incorporation of methoxyphenol groups created reactive sites for laccase on the surface of the protein and improved the efficiency of cross-linking. The vanillic acid modified WPI (Van-WPI) was characterized using MALDI-TOF mass spectrometry, and the laccase-catalyzed cross-linking of Van-WPI was studied. Furthermore, the vanillic acid modification was compared with the conventional approach to improve laccase-catalyzed cross-linking by adding free phenolic compounds. A small extent of the vanillic acid modification significantly improved the cross-linkability of the protein and made it possible to avoid color formation in a system that is free of small phenolic compounds. Moreover, the potential application of Van-WPI as emulsifier and the effect of cross-linking on the stability of Van-WPI emulsion were investigated. The post-emulsification cross-linking by laccase was proven to enhance the storage stability of Van-WPI emulsion.

KEYWORDS: Vanillic acid modification, cross-linkability, color formation, emulsion, stability, post-emulsification cross-linking

■ INTRODUCTION

Whey proteins are widely utilized as food emulsifiers. In an oil-in-water emulsion, whey proteins can readily adsorb onto the surface of newly created oil droplets, forming a protective interfacial layer between the dispersed phase and continuous phase and consequently preventing coalescence as both steric and electrostatic repulsive forces between droplets are provided. Protein-stabilized emulsions have been extensively studied, and various approaches have been investigated in order to improve their stability. Conditions such as pH, temperature, protein concentration, pressure, and addition of polysaccharides during the homogenization have been investigated to establish an optimal protocol for the emulsification process.[1−4] Chemical modifications such as succinylation, acetylation, phosphorylation, thiolation, ethylene diamine modification, as well as Maillard reaction have been used to improve the emulsifying activity and/or emulsion stability of protein emulsifiers.[5−9]

Enzymatic tailoring of proteins by, for example, hydrolysis or cross-linking has also been attempted. Hydrolysis of proteins could influence their emulsifying properties. The effect of some proteolytic enzymes such as pepsin, trypsin, chymotrypsin, and plasmin on dairy protein emulsifiers has been investigated and was found to be very dependent on the selection of enzymes, optimization of the degree of hydrolysis (DH), and other conditions of emulsification.[10−12] Another way to enhance emulsifying properties of protein is cross-linking using enzymes such as peroxidase, tyrosinase, transglutaminase, and laccase.[13−16]

Enzymatic cross-linking can significantly influence the stability of protein-stabilized oil-in-water emulsions by changing the structure of emulsifiers at the interface and/or in the continuous phase. The effect can be, however, dependent on whether the enzymatic reaction is carried out before or after emulsification.[17] Cross-linking of protein emulsifiers before emulsification generally retards the adsorption of protein onto the surface of oil droplets and leads to a lower emulsifying activity.[18] In terms of

the emulsion stability, different effects have been reported depending on the enzymes and the extent of cross-linking. Some studies report enhanced emulsion stability (ES) explained by a stronger steric stabilization provided by a thicker layer of protruding branched polymer chain,[18,19] while others observed weakened emulsion stability due to the aggregation of different oil droplets bridged by huge protein polymers.[20,21] In contrast with pre-emulsification cross-linking, post-emulsification cross-linking is believed to be a better approach when aiming at strengthening emulsion stability. Investigations on the stability of emulsions made of α-lactalbumin concentrate after post-emulsification treatment by transglutaminase showed improved ES due to the increase of apparent viscosity in the continuous phase.[17] The same conclusion was drawn from the study of transglutaminase-cross-linked emulsions made of milk proteins.[22] To date, the most commonly used enzyme in the emulsion field is transglutaminase, but some studies on the tyrosinase-catalyzed cross-linking of protein in gels can also be relevant for emulsion makers since similar forces are at stake.[23]

Laccases (EC 1.10.3.2) are blue copper oxidases that can catalyze the oxidation of various aromatic substrates; in particular, different methoxyphenol compounds in, for example, lignin to corresponding phenoxyl radicals. Atmospheric oxygen is used as the electon acceptor by laccase in the oxidation reaction and a byproduct H_2O is formed.[24] The primary radical reaction products can further react nonenzymatically, resulting in a cross-linked product. Laccases have been reported to polymerize various components, such as lignins and other aromatic compounds.[25,26] Although laccases have been reported to be able to oxidize also tyrosine and tyrosine containing peptides,[27,28] the oxidation of
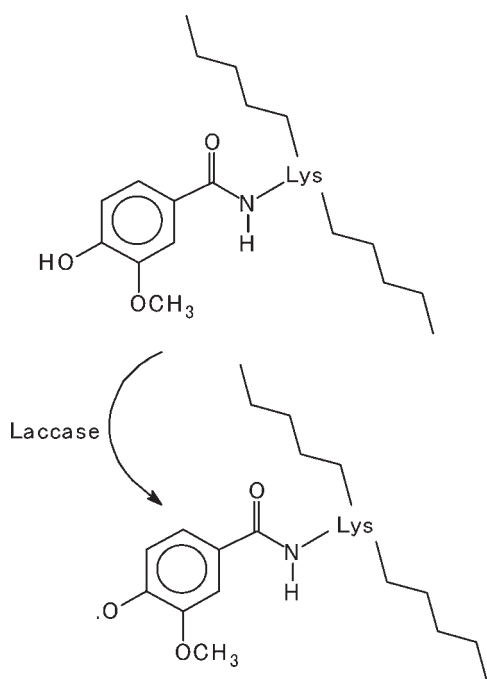
Figure 1. Vanillic acid modified lysine site chain and subsequent activation of the modified protein by lacasse.

proteins has been limited.[22,29] Hence the phenolic groups in protein do not seem to be a very good substrate for laccase-catalyzed reactions. Laccase-catalyzed protein cross-linking has been enhanced by addition of small phenolic compounds as electron transfer mediators which facilitate the formation of homo-cross-links and/or act as bridging agents in heteropolymer structures.[30,31] However, the extent of cross-linking seems to significantly depend on the conformation of the protein substrate. Protein with flexible conformation, casein, for example, can be extensively polymerized by laccase either with high dosage of the enzyme or in the presence of ferulic acid as mediator/bridging agent.[31] On the contrary, the cross-linking of β-lacto-globulin or BSA which has a compact globular structure is only occurring to a very limited extent. Also in this case, the reaction was enhanced by addition of free phenolic acids and high dosage of the enzyme.[29,32] The oxidation and cross-linking in the presence of these commonly used phenolic mediators/bridging agents such as ferulic acid, chlorogenic acid, vanillic acid and caffeic acid cause simultaneous color formation, which is sometimes undesirable for food and other applications.

Introducing methoxyphenol side chains into a protein backbone would be an alternative way to improve the reactivity of protein toward laccase instead of using free phenolic compounds in the reaction. Inspired by lignin, the natural substrate of laccase and abundant with methoxyphenol units, we aimed at creating a "protein−lignin hybrid". This was achieved by chemically incorporating vanillic acid (4-hydroxy-3-methoxybenzoic acid) which is a lignin model compound and commonly used flavoring agent in the food industry with whey protein isolates (WPI) (Figure 1). Making this type of hybrid would give interesting possibilities; one would have different reactive sites in the protein backbone for different biocatalysts to act on. Furthermore, it would give the possibility to form composite biomaterials in which different biopolymers such as, for example, protein and lignin of very different nature are present.

The aim of this work was to covalently functionalize WPI with vanillic acid, whereafter the reactivity of the modified protein toward laccase was studied. Finally, the effect of laccase-catalyzed cross-linking of vanillic acid modified WPI as emulsifier in an oil-in-water emulsion system was studied.

■ MATERIALS AND METHODS

Materials. Whey protein isolate, which was free of lactose (lactose content below 0.5%) and contained a minimum dry protein content of 91%, was obtained from Lacprodan, Arla Foods Ingredients, Viby J, Denmark. Vanillic acid was purchased from Sigma-Aldrich (purity ≥ 97.0%), and EDC was purchased from Thermo Scientific (purity ≥ 97.0%), Rockford, IL, USA. Sulfo-NHS was purchased from Pierce (purity ≥ 98.5%). Flaxseed oil was purchased from Elixi Oil Oy (Somero, Finland), where the fatty acid content was the following: 4% 16:0, 3% 18:0, 12% 18:1, 15% 18:2, and 66% 18:3. Laccase was produced by *T. hirsuta* and purified by anion exchange chromatography and hydrophobic interaction chromatography.[33] Laccase activity toward ABTS was determined to be 7012 nkat/mL (A protein concentration of 3.9 mg/mL).

Modification of WPI by Vanillic Acid. The modification was performed using a two-step reaction protocol including NHS-ester activation followed by the amine reaction. The NHS-activated vanillic acid was obtained by dissolving vanillic acid, EDC, and sulfo-NHS in DMF at concentrations of 20, 2, and 2.5 mg/mL, respectively, and incubating at room temperature overnight. Then a 10 mg/mL whey protein isolate solution was prepared by dissolving the protein in 50 mM NaP$_i$ buffer pH 7 and 5:1 (volume ratio) mixed with the activated vanillic acid solution. The mixture was stirred at room temperature for 24 h. The product was thoroughly dialyzed against sterilized water and recovered by freeze-drying using a lyophilizer (Steris Lyovac GT 2, Germany). In order to completely remove the residual chemical reagents, the freeze-dried protein was dissolved in sterilized water and purified using an Econo-Pac 10DG desalting column (BIO-RAD). The eluate was collected and freeze-dried again. For the large-scale production of the vanillic acid modified WPI that used as emulsifier, only dialysis was applied.

Analysis of the Extent of Modification. Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was performed to study the extent of the modification. Sinapinic acid was used as matrix and dissolved to saturation in a 1:1 mixture of 0.1% TFA and acetonitrile. The samples of unmodified WPI and vanillic acid modified WPI (Van-WPI) were dissolved in distilled water and 1:1 mixed with matrix. One microliter of the mixture was spotted on the target plate and dried in air for 10 min. The analysis was conducted using a mass spectrometer (Bruker AutoflexII, Germany).

Laccase-Catalyzed Cross-Linking. The reactivity of laccase toward the unmodified WPI and Van-WPI was studied by measuring oxygen consumption during the reaction. The enzymatic reactions were monitored with a single-channel oxygen meter (Precision Sensing GmbH, Regensburg, Germany) in a sealed vial (1.84 mL). Unmodified WPI and Van-WPI were dissolved in 5 mM NaP$_i$ buffer pH 6.0 at a concentration of 3 mg/mL. The vial was filled with 1.82 mL of protein solution and capped. Laccase (18 μL) at a dosage of 10 nkat/mg of the modified or unmodified WPI was injected to start the reaction. Consumption of oxygen was monitored as a function of time. Samples containing the unmodified WPI and different concentrations (0.02, 0.2, and 2 mM) of free vanillic acid were also tested with laccase at a dosage of 2.5 nkat/mg of the protein as a control.

The cross-linking of WPI and Van-WPI with 2.5 and 10 nkat/mg laccase dosage as a function of reaction time (0−24 h) was further studied using a 12% SDS gel (BIO-RAD). The cross-linking reaction was stopped by adding 5 μL of SDS-PAGE loading buffer (Tris-HCl with β-mercaptoethanol and SDS) and 5 μL of water into 5 μL of sample and heating at 98 °C for 10 min. Each well in the SDS-PAGE gel was loaded with 15 μL of sample.

Color Formation. Color formation during the laccase reaction was studied by adding laccase at a dosage of 2.5 nkat/mg into 3 mg/mL WPI, 3 mg/mL WPI with 2 mM free vanillic acid, and 3 mg/mL Van-WPI in 5 mM NaP$_i$ buffer at pH 6.0. The absorbance at wavelength 430 nm was followed using a HITACHI U-2000 spectrophotometer. Reading of the NaP$_i$ buffer was set as a blank.

Preparation of O/W Emulsion and Post-Emulsification Cross-Linking. Freeze-dried WPI and Van-WPI were solubilized in 100 mL of 5 mM NaP$_i$ buffer pH 6.0 at a protein concentration of 0.3%. A two-step homogenization was applied to a mixture of 90% w/w protein solutions with 10% w/w flaxseed oil. A pre-emulsion was prepared using a stirring-type homogenizer (Heidolph Diax 900, Germany) under constant conditions: 2 × 2 min at 26 000 rpm at room temperature. A pressure homogenizer (Microfluidics M-110Y, Newton, MA, USA) was used to perform the main emulsification at 20 °C and 40 psig (500 bar). The pre-emulsion (100 mL) was circulated in the homogenizer for 10 min, during which it passes through the chamber 30 times. After the emulsification, 20 uL of laccase was added to a 20 mL aliquot of the emulsion made at pH 6 (2.5 nkat/mg) and incubated at room temperature for 24 h. Sodium azide (0.02 wt %) was added to the emulsions to stop the laccase reaction and prevent microbial growth. Emulsions without laccase were also prepared as a control. For emulsifying activity study, the emulsions were also prepared at different pH values by adjusting the pH of protein solutions to pH 5, 6, and 7 using 1 M HCl and 1 M NaOH.

Analysis of Emulsion Properties. Particle size distribution of emulsions with and without laccase was measured by laser diffraction (Beckman Coulter LS230, Brea, CA). The measurement was conducted in an optical model with fluid refractive index 1.33 and, sample refractive index 1.46. The pH of Milli Q water was adjusted to the same value as emulsion samples using 0.1 M HCl and 0.1 M NaOH and used as the measuring media. As an indicative parameter of the emulsifying activity of the proteins, the volume-weighted geometric mean particle diameter was determined from the particle size distribution of two batches of fresh emulsions. Two measurements were taken for each batch. For the storage stability study, samples were consistently taken from the top part of each emulsion at 0, 24, and 72 h. Three repeats were performed to confirm the trend of the change of particle size during storage.

The emulsions were also visualized using confocal laser scanning microscopy (CLSM) equipment consisting of a Bio-Rad Radiance Plus confocal scanning system (Bio-Rad, Hemel Hempstead, Hertfordshire, U.K.) attached to a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan). For imaging, lipids were stained by adding 10 μL of 0.05% (w/v) Nile red in acetone into 1 mL of emulsion. Stained emulsions were examined in 1 mm deep wells on a microscope slide using a 488 nm argon laser line for excitation and a band-pass emission filter at 575−625 nm. Images were assembled of the optical sections taken using a 20× objective (Nikon Plan Apo, numerical aperture 0.75) to the depth of 2 μm with 0.5 μm z step. The stained oil droplets appeared bright. Multiple fields were viewed, and representative images were selected.

■ RESULTS

Modification of WPI by Vanillic Acid. An NHS-ester activation of vanillic acid followed by a reaction with the lysine amine group was used to modify reactive lysine side chains in WPI. Incorporation of the methoxyphenol groups into the protein through formation of an amide bond leads theoretically to a 150.15 Da mass increase of each reacted lysine residue in WPI. Analysis of the precise molecular mass of unmodified and modified WPI by MALDI-TOF MS was used to confirm the occurrence of the reaction described above and to measure the extent of the modification (Figure 2). Two protein peaks were detected in the mass spectrum of unmodified WPI corresponding to α-lactalbumin (M_w 14118 Da) and β-lactoglobulin (M_w 18481 Da).
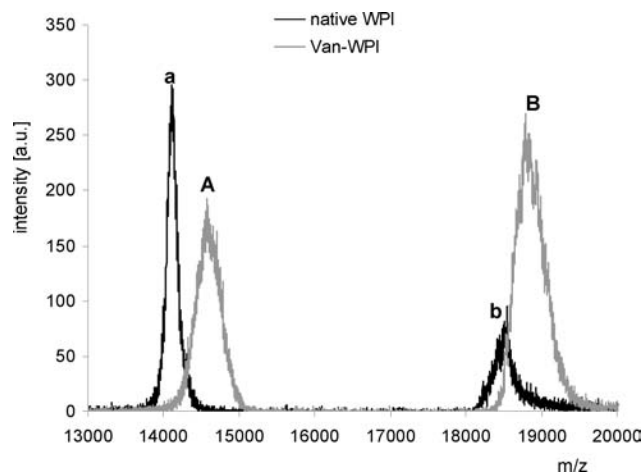

Figure 2. MALDI-TOF mass spectra of the modified WPI. (a) Unmodified α-lactalbumin with molecular mass 14 118 Da; (b) unmodified β-lactoglobulin with molecular mass 18 481 Da; (A) vanillic acid modified α-lactalbumin with molecular mass 14 557 Da; (B) vanillic acid modified β-lactoglobulin with molecular mass 18 798 Da.
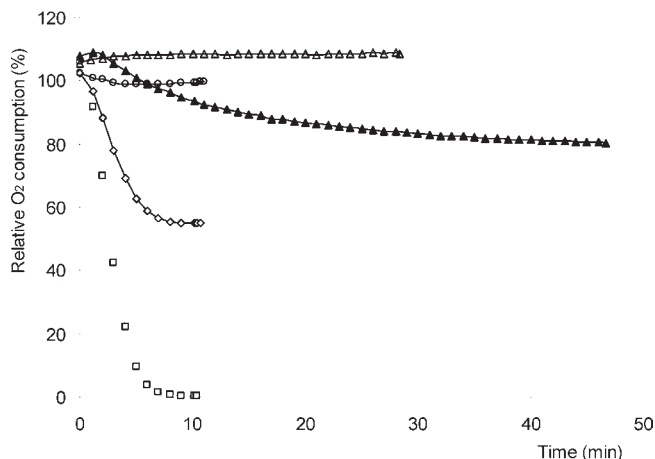

Figure 3. Oxygen consumption measurement of laccase reaction of unmodified WPI with free vanillic acid and modified WPI. 2.5 nkat/mg laccase was added into WPI with 2 mM (□), 0.2 mM (◇), and 0.02 mM (○) free vanillic acid and 10 nkat/mg laccase was added into WPI alone (△) and Van-WPI (▲).

The modified WPI (Van-WPI) had two similar peaks, but with higher molecular masses, that is, 14 557 and 18 798 Da as compared to the unmodified WPI. From the spectra, it can be seen that α-lactalbumin seemed to be more prone to the vanillic acid modification since a larger increase in molecular mass was detected compared to the modified β-lactoglobulin. The average number of lysine residues that were modified was calculated by dividing the difference between the molecular mass of WPI and Van-WPI by the mass of vanillic acid. According to this calculation, on average 2.9 of 12 lysine residues in α-lactalbumin and 2.1 of 16 lysine residues in β-lactoglobulin were modified by vanillic acid.

Laccase-Catalyzed Cross-Linking of Vanillic Acid Modified WPI. Vanillic acid modified WPI contains methoxyphenol side groups that are reactive toward laccase and therefore is expected to have an improved cross-linkability compared to the unmodified WPI. The reactivity of Van-WPI with laccases was studied using oxygen consumption measurement and SDS-PAGE electrophoreses.
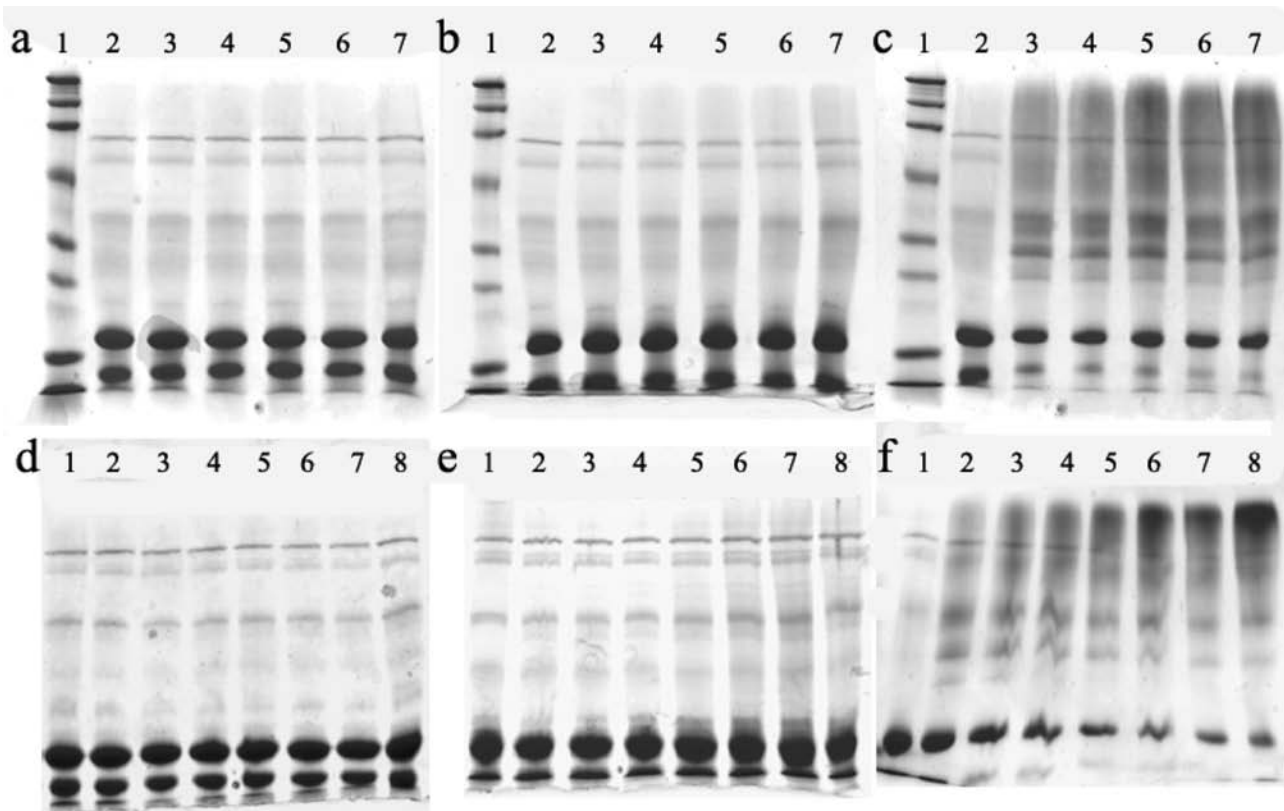
Figure 4. SDS-PAGE of the unmodified and modified WPI after laccase reaction. The cross-linking of WPI and vanillic acid modified WPI (Van-WPI) was performed at a laccase dosage of 2.5 nkat/mg (a−c) and 10 nkat/mg (d−f). (a) WPI with 2.5 nkat/mg laccase; (b) WPI with 2 mM free vanillic acid and 2.5 nkat/mg laccase; (c) Van-WPI with 2.5 nkat/mg laccase. (a−c) lane 1, prestained SDS-PAGE standard proteins of 206.3, 118.1, 97.3, 54.8, 37.7, 29.4, 17.5, and 6.7 kDa (BIO-RAD); lane 2, cross-linked sample at 0 h; lane 3, cross-linked sample at 2 h; lane 4, cross-linked sample at 4 h; lane 5, cross-linked sample at 8 h; lane 6, cross-linked sample at 12 h; lane 7, cross-linked sample at 24 h. (d) WPI with 10 nkat/mg laccase; (e) WPI with 2 mM free vanillic acid and 10 nkat/mg laccase; (f) Van-WPI with 10 nkat/mg laccase. (d−f) lane 1, cross-linked sample at 0 h; lane 2, cross-linked sample at 10 min; lane 3, cross-linked sample at 20 min; lane 4, cross-linked sample at 30 min; lane 5, cross-linked sample at 1 h; lane 6, cross-linked sample at 2 h; lane 7, cross-linked sample at 4 h; lane 8, cross-linked sample at 12 h.

The conventional approach to improve cross-linking by adding free phenolic compound to the unmodified protein was used as a comparison.

Oxygen consumption measurements were performed to study the radical formation stage of the laccase-catalyzed cross-linking reaction (Figure 3). For the samples containing unmodified WPI and free vanillic acid, an increasing amount of oxygen was consumed depending on the concentration of vanillic acid: in the presence of 2 mM vanillic acid, all the oxygen in the reaction vial was consumed in 10 min, 47% of oxygen was consumed in the sample with 0.2 mM vanillic acid, and for a mixture with 0.02 mM vanillic acid only 3% oxygen was consumed in the same time. The unmodified WPI solution without addition of free vanillic acid did not show consumption of oxygen although the reaction was carried out with a higher laccase dosage of 10 nkat/mg for a longer time. The oxidation of Van-WPI was limited when 2.5 nkat/mg laccase was used (data not shown). When a higher dosage of 10 nkat/mg laccase was used, about 29% of the oxygen in the vial was consumed within 50 min.

The extent of laccase-catalyzed cross-linking of Van-WPI was studied using SDS-PAGE electrophoresis (Figure 4). Unmodified WPI, a mixture of WPI and free vanillic acid, and Van-WPI were treated with different dosages of laccase and monitored for different times. The efficiency of cross-linking of unmodified WPI was very low: a little increase of intensity of dimers of the

unmodified α-lactalbumin and β-lactoglobulin at molecular mass around 29.4 kDa and 37.7 kDa was observed, indicating a slight cross-linking of the unmodified WPI by laccase after 24 h (lane 7, panel a). Addition of free vanillic acid improved the cross-linking to a small extent as slightly more dimers of α-lactalbumin and β-lactoglobulin were formed (lanes 4−7, panel b). However, most of the unmodified WPI stayed non-cross-linked, and increasing the enzyme dosage barely improved the cross-linking (panels d and e). In contrast, at an enzyme dosage of 2.5 nkat/mg, the majority of vanillic acid modified α-lactalbumin was cross-linked after 2 h as can be seen from lane 3 in panel c. Vanillic acid modified β-lactoglobulin took a longer time to be cross-linked. A decrease of intensity of the band of vanillic acid modified β-lactoglobulin was observed, although uncross-linked monomers still could be detected after 24 h. The molecular mass of the cross-linked proteins increased with duration of the reaction, ranging from 29 kDa to over 206 kDa as was detected in the top of each lane (panel c). At a higher dosage of laccase, the cross-linking of the Van-WPI was greatly accelerated (panel f). After 30 min, almost all the vanillic acid modified α-lactalbumin and nearly half of the modified β-lactoglobulin was already heavily cross-linked. With a longer reaction time, after 4 h most of the Van-WPI was extensively cross-linked, assembling at the top of the lane (lane 7, panel f).

The SDS-PAGE gel and oxygen consumption results suggested that the method to improve the cross-linking by adding free
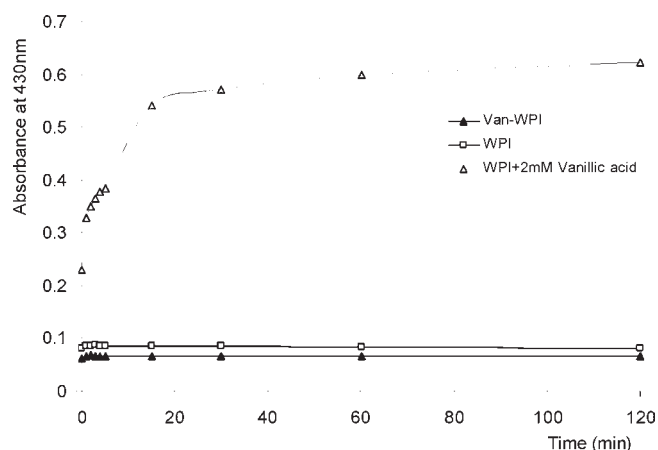
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Figure 5. Color formation during the laccase reaction. 2.5 nkat/mg laccase was added into WPI (□), a mixture containing WPI and 2 mM free vanillic acid (△), and Van-WPI(▲). The color formation was followed by plotting the absorbance at wavelength 430 nm against time.

Table 1. Emulsifying Activity of WPI and Van-WPI at Different pH Values by Measuring the Mean Particle Diameter from Volume Distribution[a]

	pH 5	pH 6	pH 7
WPI (μm)	ND	0.84 ± 0.07	0.72 ± 0.13
Van-WPI (μm)	3.1 ± 0.4	1.0 ± 0.0	0.93 ± 0.12

[a] ND: Not determined; phase separation took place right after the pre-emulsification process.

vanillic acid made a quick oxidation of the free phenolic compounds by laccase, while the nonenzymatic reaction during which the free radicals mediated the cross-linking of the protein was much delayed as it took a long time for the free radicals to reach the tyrosine residues inside the protein structure, and the radicals formed in unmodified WPI may not be able to react with radicals in other molecules due to steric hindrance. The major reaction occurred in this "vanillic acid mediated" system was the oxidation and self-polymerization of vanillic acid, which proved by the change in color (Figure 5). In comparison, the polymerization of Van-WPI was proved to occur simultaneously with the radical formation as a small amount of the Van-WPI was already cross-linked during the first 10 min, and by the end of the radical formation stage the Van-WPI was extensively polymerized (Figures 3 and 4).

Color Formation during the Cross-Linking Reaction. The color formation during the laccase reactions with unmodified and modified WPI was studied, and the results are shown in Figure 5. A mixture of 3 mg/mL unmodified WPI and 2 mM vanillic acid had a very slight orange color when free vanillic acid was dissolved, while the other two samples without the free vanillic acid stayed colorless. Treated with laccase, a strong orange color was formed within 15 min in the mixture of WPI and vanillic acid which could be monitored as a strong absorbance increase at wavelength of 430 nm. No color formation was observed in WPI or Van-WPI after laccase treatment, and the absorbance remained below 0.1 within 2 h.

Effect of the Cross-Linking on the Emulsifying Properties of Van-WPI. The volume-weighted geometric mean particle diameter of freshly made emulsions was determined as an indication of emulsifying activity of WPI and Van-WPI. For both the unmodified WPI and Van-WPI, better emulsifying activity was found with increasing pH (Table 1). The emulsifying activity of the unmodified WPI was slightly better than that of modified WPI at pH 6 and pH 7, resulting in a smaller mean particle size. At pH 5, the unmodified WPI could not be emulsified as the oil and water phases separated immediately after the stirring type homogenization. The vanillic acid modified WPI showed an improved emulsifying activity over the unmodified WPI at pH 5. In theory, the vanillic acid modification changes the isoelectric point (pI) of WPI, since positively charged

lysine residues are converted into neutral side groups. The pI of Van-WPI was not significantly changed; the pI of the vanillic acid modified α-lactalbumin was shifted from 4.9 to 4.6 and from 4.9 to 4.8 for β-lactoglobulin according to the method of pI calculation described by Ma et al.[8] This small decrease of pI could explain the slightly improved emulsifying activity of Van-WPI at pH 5.

The effect of post-emulsification cross-linking on the stability of emulsions was studied by comparing the particle size distribution before and after the laccase treatment. Since addition of free vanillic acid caused collapse of emulsions (data not shown) and it did not make significant improvement of cross-linking (Figure 4), the unmodified WPI emulsion was prepared without vanillic acid and compared with the Van-WPI emulsion for the emulsion stability study.

The laccase-catalyzed post-emulsification cross-linking was performed on emulsions made of WPI and Van-WPI at pH 6, and an improved shelf life was observed for the cross-linked emulsion of Van-WPI (Figure 6). For the emulsion made of WPI, 62.8% of the particles had a diameter of less than 1 μm, and the presence of relatively larger particles from 1 to 28.7 μm was also detected. Laccase treatment for 24 h did not improve emulsion stability, and probably due to the addition of enzyme the laccase treated emulsion of WPI was shown to be slightly less stable than the one without enzyme treatment. The volume-weighted geometric mean particle diameter of a fresh WPI emulsion was 0.84 μm, and after 72 h the particle size significantly increased to 1.54 μm for the emulsion without laccase and 2.11 μm for the laccase treated one (panels a and b, Figure 6). The fresh emulsion made of Van-WPI contained 43.2% of particles less than 1 μm and the larger particles ranged from 1 to 4.4 μm. The average particle size of the fresh emulsion was determined to be 1.02 μm. Without treatment with laccase, the average particle size of the Van-WPI emulsion increased to 1.37 μm and a small peak was observed from the particle distribution curve, representing the generation of large particles sized from 10 to 37.9 μm during storage. The laccase treated emulsion of Van-WPI exhibited the best stability among all the samples. The mean particle diameter increased from 1.02 to 1.05 μm after 72 h. Despite the fact that a small amount (0.8%) of particles that were less than 1 μm had become slightly larger during the storage, the overall particle size stayed within the range from 0.1 to 4.8 μm, and no larger particles were observed (panels c and d, Figure 6).

The increased particle size suggested the aggregation of oil droplets in the non-cross-linked emulsions. Confocal microscopy was used to get a direct vision of droplet behavior. For fresh emulsions made of both unmodified and modified WPI, the oil droplets were homogeneously distributed and the diameter of most of the oil droplets was less than 1 μm despite the presence of a small number of bigger droplets (panels a and d, Figure 7). Coalescence was found to occur during the storage of emulsions without cross-linking as large oil droplets were detected after 72 h
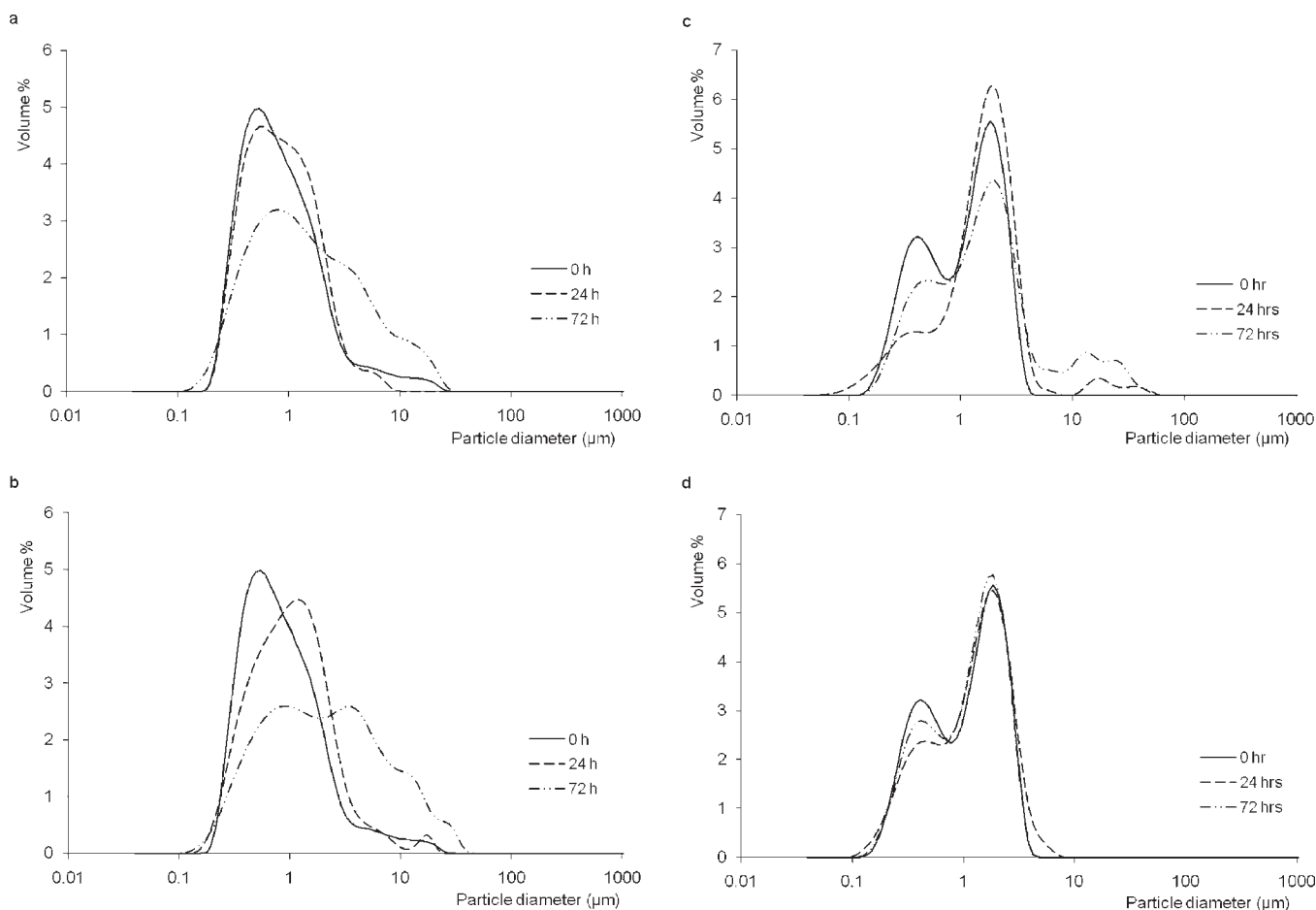
Figure 6. Effect of cross-linking on particle size distribution of emulsions. The particle size distribution of freshly made emulsions and emulsions stored at room temperature was measured after 24 and 72 h storage. The volume-weighted geometic mean particle diameter (d33) was calculated. (a) Emulsion made of the unmodified WPI without laccase treatment; (b) laccase-treated emulsion made of the unmodified WPI; (c) emulsion made of the Van-WPI without laccase treatment; (d) laccase-treated emulsion made of the Van-WPI. The experiment was repeated three times. The standard deviation of the particle diameter of the fresh emulsions was less than 10% of the mean value and around 20% for the aged emulsions. However, the trend of size change was consistent.

(panels b, c, and e, Figure 7). Due to stronger attractive interaction, the droplet concentration around these large particles was higher than other regions with smaller droplets. For the laccase-treated Van-WPI emulsion, the droplets stayed almost the same size as in the fresh sample, indicating better storage stability against coalescence. The emulsion droplets were homogeneously distributed, and no concentration effect indicating flocculation as observed for the non-cross-linked samples was found (panel f, Figure 7). The increased particle size in the non-cross-linked emulsions significantly accelerated the creaming of droplets. As a result of this, clarification was visually observed at the bottom of these samples and the large oil droplets on the top eventually merged together into a separate oil layer with light yellow color (Figure 8).

■ DISCUSSION

Laccase Catalyzed Cross-Link Formation. The exact mechanism of laccase-catalyzed cross-linking of proteins is not completely understood so far. The only known mechanism is that cross-linking of tyrosine-containing proteins or peptides is primarily based on formation of isodityrosine bonds, with a small amount of dityrosine and disulfide bonds. Isodityrosine bonds are formed between hydroxyl and tyrosyl radicals that are located

in different protein molecules; therefore, the accessibility of tyrosine residues in protein is expected to be very important for its susceptibility to a laccase-catalyzed reaction.[27,34] Actually, tyrosine alone or in short peptides can be directly oxidized by laccase with subsequent formation of cross-links,[27] while in proteins that have a compact structure internal tyrosine residues may not be directly accessible for the enzyme. Phenolic acids, being small molecules that can easily interact with both the enzyme and the protein substrates, are able to enhance the cross-linking either by transferring electrons to protein which can be cross-linked via radical reactions or by directly bridging the connection between two tyrosine residues from protein.

The modified WPI could be efficiently cross-linked by laccase, whereas when free vanillic acid was used with the unmodified WPI; the main reaction seemed to be polymerization of vanillic acid by itself. The vanillic acid mediated the cross-linking reaction by transferring electrons and creating reactive radicals in the protein. It is also possible that some vanillic acid was incorporated into the cross-linked protein. The role of phenolic acid as a bridging agent in cross-linking had been proved by Mattinen et al.[27] Another study on laccase-catalyzed cross-linking of β-lactoglobulin with ferulic acid speculated the incorporation of ferulic acid in the cross-linked structure.[29] The introduction of

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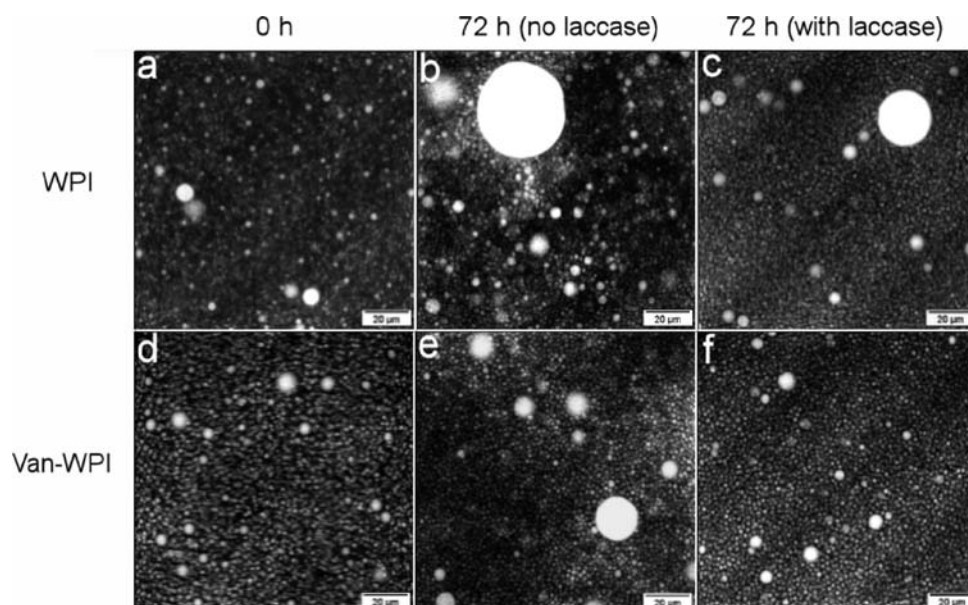dx.doi.org/10.1021/jf103591p |*J. Agric. Food Chem.* 2011, 59, 1406–1414

Figure 7. CLSM images of emulsions made of the unmodified and modified WPI with or without laccase treatment. Size bar = 20 μm. Nile Red was used to stain the oil phase = bright regions. (a−c) Different emulsions made from WPI; (d−f) different emulsions made from Van-WPI.
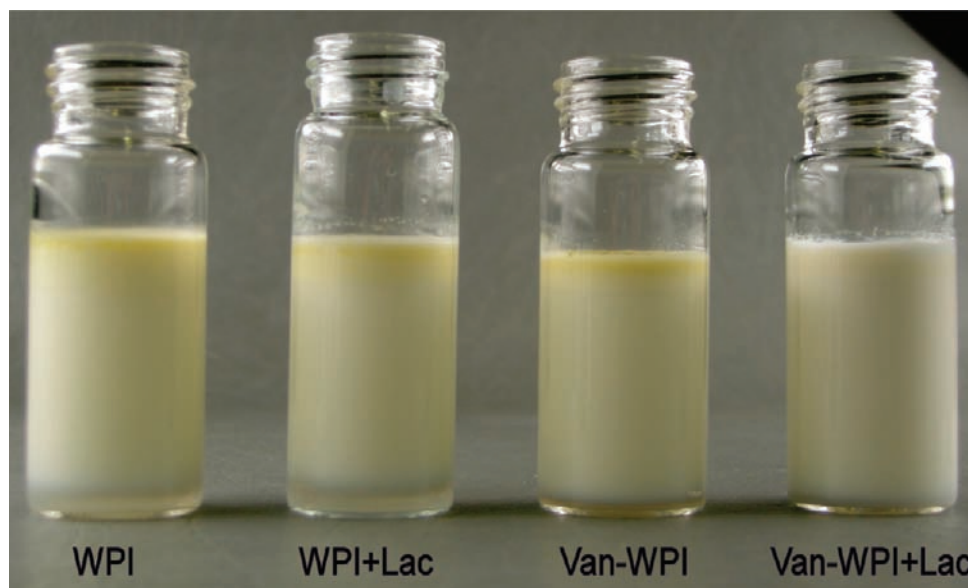

Figure 8. Visual observation of aged emulsions. The emulsion samples were stored at room temperature for 1 week. Clarification and creaming were observed, respectively, from the bottom and the top of tubes.

methoxyphenol groups onto the protein surface enhanced the laccase-catalyzed cross-linking reaction. These introduced methoxyphenol groups on the protein surface seem therefore to be accessible for laccase. Using this system, it became possible to perform the cross-linking reaction in a "phenolic-compound-free" system where the laccase enzyme seems to work directly on the protein; both the enzymatic oxidation reaction indicative for radical formation and the subsequent nonenzymatic cross-linking could be monitored.

The interaction of laccase with vanillic acid modified WPI is also interesting from a laccase substrate binding pocket architecture perspective and its natural ability to oxidize polymeric phenol containing substates. Recently determined complex structures

with a methoxy phenol compound bound in the substrate binding pocket and modeling studies show that the active site in laccases is a relatively hydrophobic cavity whose size and shape seem to vary, when laccases of different origins are compared.[35,36] From these studies, it can be seen that the para-position of the benzene ring relative to the phenolic hydroxyl group seems to be facing toward the solution. The orientation makes interactions with methoxyphenol groups incorporated in a polymeric structure such as lignin feasible. The interaction of laccase with an artificial methoxyphenol containing polymer observed in this study supports these structural observations.

The two major components of WPI, α-lactalbumin and β-lactoglobulin, have different reactivity toward the laccase

enzyme. α-Lactalbumin has been reported to be relatively more susceptible to laccase although the extent of cross-linking is still very limited.[32] The vanillic acid modification improved the reactivity of both α-lactalbumin and β-lactoglobulin. The vanillic acid modified α-lactalbumin was the preferred substrate and thoroughly cross-linked by a low dosage of laccase, while a higher dose of the enzyme was needed for extensive cross-linking of the vanillic acid modified β-lactoglobulin.

The conventional way of using free phenolic acids as mediator/bridging agents usually leads to a colored product due to the formation of quinone components in the laccase-catalyzed oxidation reaction. Color formation by laccase-catalyzed oxidation of phenolic compounds has already been utilized to synthesize colorants.[37] However, in cases where phenolic compounds are present to serve the purpose of cross-linking of protein, a colored byproduct might be regarded as an undesirable side effect. So far, most of the relevant studies were based on a phenolic-acid-aided system in order to make laccase work on proteins, and color formation seemed to be inevitable in such a system. The vanillic acid modification of WPI facilitated the laccase-catalyzed cross-linking by directly creating sites for phenoxy radical formation in the protein. The protein-bound radical polymerization resulted in noncolored product. This could broaden the use of laccase in cross-linking applications where color formation is unwanted.

Emulsifying Properties of Van-WPI. The observed improvement of stability of the laccase-treated Van-WPI emulsion would be mainly due to the enhancement of interfacial viscoelastic properties. Cross-linking between the adsorbed protein and free protein molecules in the water phase could increase the length and thickness of the protein layer on the droplet surface and therefore provide stronger steric repulsive force against coalescence in collision. On the other hand, the cross-linking between proteins in the continuous phase may play a role in preventing the emulsion sample from creaming. Previous literature had reported the effect of cross-linking on viscosity of high concentration protein solution.[32] In an emulsion system with high protein content, the extensively cross-linked proteins are expected to form a network that decreases the rate of upward movement of oil droplets and thus slows down the creaming process. In our study where the protein concentration was relatively low, although the cross-linked Van-WPI in the continuous phase could not be enough to form a well-shaped network, a further extended protein structure may also increase of viscosity to some extent and limit the movement of oil droplets better than non-cross-linked proteins.

It is theoretically possible that the cross-links can also be formed between proteins that are adsorbed on adjacent oil droplets, resulting in flocculation. The effect of cross-linking on emulsion stability is largely dependent on the extent of the reaction and the conformation of the cross-linked proteins. Huge extended protein polymers formed by a large extent of cross-linking could impair the stability of emulsions by facilitating droplet aggregation while a proper extent of cross-linking could enhance the emulsion stability. In our study where a complex of oligomerized proteins with different extents of cross-linking was formed (panel c, Figure 4), flocculation or coalescence was not observed in the cross-linked Van-WPI emulsion according to the particle size distribution and confocal microscopy results. Hinz et al. reported that pasteurized cream samples which had high fat content (380 g/L) were treated with transglutaminase and the particle size was stable after the cross-linking reaction despite

the condensed system where the distance between oil droplets was closer than in ours.[38] Based on the agreement between these two studies, we assumed that the cross-linking did not occur between droplets but between free proteins in the water phase or free proteins and adsorbed proteins. Actually, Færgemand et al. also speculated that the cross-linking enzyme may work more effectively on nonadsorbed protein substrates.[39] A possible explanation for this could be the electrostatic repulsion between droplets or the adsorption of proteins onto the oil surface hampered their mobility and flexibility for inter-particle cross-linking. For future study, it would be worthwhile to investigate the viscosity of the continuous phase and the thickness of the adsorbed protein layer after cross-linking treatment.

Potential Applications of the Vanillic Acid Modification. Laccase has drawn much attention nowadays due to its capability to activate and modify lignin, polymerize sugar beet pectin, or arabinoxylan via their ferulic acid moieties.[40] However, the cross-linking application of laccase is affected by the accessibility of phenolic moieties in these raw materials; for example, in lignin the reactive sites are only partially accessible toward laccase. Especially for protein substrates which do not naturally contain many phenolic groups, the efficiency of laccase-catalyzed cross-linking is very limited. In our study, the cross-linkability of protein substrate was enhanced by the vanillic acid modification which in a sense created a "protein–lignin hybrid". This could expand the utilization of laccase in the field of modification of renewable biopolymers and open ways to create new applications which consist of combinations of different renewable biopolymers. Vanillic acid modified proteins may thus become an interesting starting material for production of complex polymers for various purposes.

■ ABBREVIATIONS USED

ABTS,2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; DMF, dimethylformamide; EA, emulsifying activity; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ES, emulsion stability; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MS, mass spectrometry; O/W emulsion, oil in water emulsion; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; SDS, sodium dodecyl sulfate; sulfo-NHS, N-hydroxysulfosuccinimide; TFA, trifluoroacetic acid; WPI , whey protein isolate.

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