JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Cross-Linking Proteins by Laccase-Catalyzed Oxidation: Importance Relative to Other Modifications

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Laccase-catalyzed oxidation was able to induce intermolecular cross-links in β-lactoglobulin, and ferulic acid-mediated laccase-catalyzed oxidation was able to induce intermolecular cross-links in α-casein, whereas transglutaminase cross-linked only α-casein. In addition, different patterns of laccase-induced oxidative modifications were detected, including dityrosine formation, formation of fluorescent tryptophan oxidation products, and carbonyls derived from histidine, tryptophan, and methionine. Laccase-catalyzed oxidation as well as transglutaminase induced only minor changes in surface tension of the proteins, and the changes could not be correlated to protein cross-linking. The presence of ferulic acid was found to influence the effect of laccase, allowing laccase to form irreducible intermolecular cross-links in β-lactoglobulin and resulting in proteins exercising higher surface tensions due to cross-linking as well as other oxidative modifications. The outcome of using ferulic acid-mediated laccase-catalyzed oxidation to modify the functional properties of proteinaceous food components or other biosystems is expected to be highly dependent on the protein composition, resulting in different changes of the functional properties.

KEYWORDS: Surface tension; ferulic acid; dityrosine; formylkynurenine; carbonyls; *Trametes* spec. laccase

INTRODUCTION

Laccase (EC 1.10.3.2) is a multicopper oxidase that catalyzes the formation of phenoxyl radicals and water by one-electron oxidation of phenolic hydroxyl groups with oxygen as electron acceptor. The enzyme catalyzes the oxidation of *o*- and *p*-diphenols, aminophenols, and phenol-containing polymers, such as lignin, that strengthens the cell walls in wood (*1*). The laccase-catalyzed oxidation of lignin involves radicals as important intermediates and results in depolymerizations of the three-dimensional lignin structure as well as polymerization (*2, 3*).

Laccase is also able to catalyze the oxidation of proteins resulting in both cross-linking and fragmentation (*4, 5*). The cross-linking of tyrosine-containing peptides by laccase has been shown to proceed via tyrosyl radicals that form primarily isodityrosine and also a small amount of dityrosine bonds (*6*). The formation of disulfide bonds by oxidation of cysteines (C) into cystine (C−C) is another way of cross-linking proteins, which laccase is capable of inducing in flour dough using ferulic acid as a mediator (*7, 8*). Fragmentation of proteins has been observed, for example, during laccase-catalyzed oxidation of meat proteins (*9*), and it was suggested that the fragmentation involved protein radicals formed by laccase (*10*).

The oxidation of proteins by radicals can generally lead to many different types of oxidative modifications in addition to fragmentations and cross-linking.

Because radicals are formed in proteins treated with laccase, it is likely that the laccase-catalyzed oxidation of proteins can result in several amino acid modifications besides fragmentations and formation of cross-links. Thus, using laccase for targeted engineering of proteins depends on whether a prediction of the laccase-induced modifications of a given protein (with a known structure and amino acid composition) can be given.

Laccase has been considered for use in food systems (*11, 12*), where it may change rheological properties of proteinaceous foods (*13*). One such rheological property is the surface tension of a protein, which is an important functional property of food proteins, as it governs the stability against phase separations in food systems such as dairy goods, baked goods, ice cream, meat, and mayonnaise (*14*−*16*). The surface tension is correlated to the spatial structure of the protein and negatively to the

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10.1021/jf801234v CCC: $40.75
Published on Web 11/20/2008

hydrophobicity of the protein (*17, 18*). As cross-linking and oxidation of proteins can alter both the spatial structure and the hydrophobicity of a protein, it must be expected that laccase-catalyzed oxidation can also change the surface pressure of proteins.

In this study we have analyzed the laccase-catalyzed oxidation of three food-related proteins with different structures and amino acid compositions. The proteins α-casein, β-lactoglobulin, and bovine serum albumin (BSA) were exposed to laccase-catalyzed oxidation and subsequently examined to detect polymerization of the proteins and oxidative changes to individual amino acids. The amino acids that were examined for modification in this study was tryptophan, which is easily oxidized to *N*′-formylkynurenine and further to kynurenine (*19, 20*); methionine, which is easily oxidized to sulfoxide or sulfone, where cleavage of the thioether bond also can take place, resulting in an aldehyde side chain (*21, 22*); and histidine, which can similarly be oxidized to a carbonyl-containing derivative, 2-oxohistidine (*23*).

The presence of mediators such as ferulic acid has been reported to enhance the effects of laccase-catalyzed oxidation of proteins (*24*). Thus, the reactions were performed in the presence and absence of ferulic acid to show the effect of a mediator and how this effect is related to the protein structure. The cross-linking enzyme, transglutaminase, which is common in commercial use and forms cross-links between the amide group of glutamine and the amine group of lysine by a nonredox mechanism, was included in the experiments for comparison of cross-linking abilities. The potential to change functional properties in the proteins was monitored through the measurement of surface tension.

MATERIALS AND METHODS

The substrate proteins used were α-casein (85% purity, mixture of α_{S1}-casein and α_{S2}-casein, Sigma-Aldrich, St. Louis, MO), β-lactoglobulin A (90% purity, Sigma-Aldrich), and BSA (98% purity, Calbiochem, Merck KGaA, Darmstadt, Germany), and the laccase mediator was ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, Fluka, Sigma-Aldrich, St. Louis, MO).

Laccase from *Trametes (Coriolus) versicolor* (Juelich Fine Chemicals GmbH, Jülich, Germany) was a lyophilized powder with 1% enzyme, and it had an enzyme activity measured to 300 U/g (1U = 1 μmol of catechol/min). Transglutaminase from *Streptoverticillium mobaraense* (Activa MP, Ajinomoto Foods, Hamburg, Germany) contained 1% enzyme in lactose/maltodextrin and had an enzyme activity of 100 U/g (1U = 1 μmol of hydroxymate/min).

All other chemicals were of analytical grade from Sigma (St. Louis, MO). All solutions were made up in 50 mM phosphate buffer (pH 6.8, ionic strength = 0.16 M, adjusted with NaCl).

Enzymatic Incubation. Substrate proteins were dissolved to a concentration of 3 mg/mL and treated at room temperature for 24 h with 0.2 mg/mL (60 mU/mL) laccase in the presence and absence of 1 mM ferulic acid in open containers with agitation. Control samples without enzyme were similarly incubated for 24 h at room temperature. Transglutaminase is used as positive control. Substrate proteins were treated with 0.2 mg/mL (20 mU/mL) transglutaminase for 1 h at 40 °C and subsequently for 23 h at room temperature.

SDS-PAGE. The molecular mass distributions of the modified proteins formed during the 24 h enzyme exposure were analyzed by SDS-PAGE (12.5% and Tris-HCl polyacrylamide gel, Bio-Rad, Hercules, CA) under reducing and nonreducing conditions, using dithiothreitol to reduce disulfide bonds. Subsequent staining was performed with Coomassie Brilliant Blue according to the method of Laemmli (*25*). Molecular mass standards of 14−200 kDa (Bio-Rad, Hercules, CA) were used for molecular mass estimations.

Dityrosine Measurements. According to the method by Østdal et al. (*26*), dityrosine was detected by using acid hydrolysis of the cross-linked proteins followed by high-performance liquid chromatography (HPLC) separation and identification. HCl was added to samples to a concentration of 6 M; the samples were then flushed with argon, hydrolyzed overnight (105 °C), and subsequently neutralized with 6 M NaOH. Hydrolyzed samples (20 μL) were injected onto an HPLC column (Microsorb 100-5 C-18, 250 × 4.6, Varian Chromatographic Systems, Walnut Creek, CA), equilibrated with 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55), with a flow of 1 mL/min. Chromatographic separation was performed on an HPLC system consisting of a 9012 HPLC pump connected to a 9100 autosampler and a 9075 fluorescence detector (Varian). The used excitation wavelength was 283 nm, and the emitted fluorescence intensity was detected at 410 nm. Dityrosine was quantified using a standard curve made from a dityrosine standard prepared according to the method of Nomura et al. (*27*).

Analysis of *N*′-Formylkynurenine. *N*′-Formylkynurenine, the oxidation product of tryptophan (*19*), was detected as described by Pirie (*28*) and measured on a Luminescence Spectrometer LS 50 B (Perkin-Elmer, Waltham, MA). Bandwidths of 5 nm were used for excitation and emission slits. The excitation wavelength was 330 nm. The fluorescence intensity was monitored in the wavelength range of 350−550 nm. The emission peak observed with a maximum at 434 nm is taken as evidence of *N*′-formylkynurenine being present in the sample.

Detection of Carbonyl Formation. According to a method developed by Dalsgaard et al. (*29*), carbonyl formation in proteins was detected using acid hydrolysis and 2,4-dinitrophenylhydrazine (DNPH) derivatization followed by reverse phase HPLC−electrospray−ionization mass spectrometry separation and identification.

HCl was added to samples to a concentration of 6 M; the samples were then flushed with argon, hydrolyzed overnight (105 °C), freeze-dried to remove acid, and finally redisolved in 10 mM DNPH solution. After incubation for 15 min at room temperature, the samples were transferred to a Whatman Mini-UniPrep vial with a pore size of 0.2 μm. Twenty microliters of sample was injected onto a reverse phase C18 column (218TP5215, 15 × 2 × 1 mm i.d., 5 μm particle size) from Vydac. Chromatographic separation of the samples was performed at a flow rate of 0.20 mL/min. The column was equilibrated for 10 min with 0.1% trifluoroacetic acid (TFA, solvent A) before injection of a sample. The samples were eluted from the 20 °C thermostated column by applying a linear gradient of solvent B (80% acetonitrile, 0.1% TFA) within the time schedule of 2−10 min, 40%; 15 min, 50%; 45−50 min, 100% solvent B. Analyses were performed on an Agilent (Waldbronn, Germany) HPLC series 1100 composed of a model G1312A binary pump, a model G1379A micro vacuum degasser, a model G1327A thermostated autosampler, a model G1316A thermostated column compartment, a model G1315B diode array detector, and a model G2707DA LC/MSD SL detector fitted with a model G1948A electrospray source. UV signals were recorded at 370 and 280 nm with a bandwidth of 4 nm and a reference fixed at 800 nm. Mass spectra of samples were recorded simultaneously by applying SCAN (from *m*/*z* 50 to 600 Da) and SIM (ions with *m*/*z* 209 and 298 Da) in positive modes. The acquisition parameters were as follows: fragmentor, 100 V; gain, 1.0 EMV; and step size, 0.20. Nitrogen was used as drying gas at a flow rate of 13 L/min and as nebulizing gas at a pressure of 60 psig (413.7 kPa) and a temperature of 300 °C. A potential of 3000 V was used on the capillary.

Identification of carbonyl groups in tryptophan (*m*/*z* 209 Da), histidine (*m*/*z* 298 Da), and methionine (*m*/*z* 298 Da) was performed according to their mass as well as their retention times of 6.5, 15.2, and 15.8 min, respectively. Quantification of carbonyl groups in the different amino acid residues was performed according to the area under the chromatographic peak with UV detection at 370 nm.

Determination of Ferulic Acid Monomers and Dimers. The method used to determine ferulic acid and dimers of ferulic acid was based on the HPLC method described by Andreasen et al. (*30*) Before analysis, the samples were freeze-dried and redissolved in methanol/water (80:20, v/v), filtered through a 0.45 μm Minisart SPR 25 filter (Sartorious, Goettingen, Germany), and transferred directly into 4 mL HPLC vials. The samples were analyzed by analytical HPLC (Shimadzu, Kyoto, Japan) using a photodiode array detector (La Chrom,

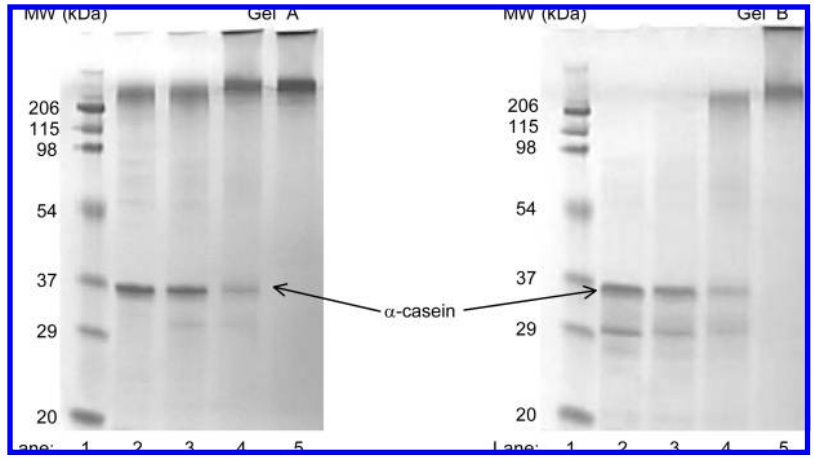

Figure 1. SDS-PAGE gel of α-casein under nonreducing (gel **A**) and reducing (gel **B**) conditions. Volumes of 10 μL, containing 15 μg of protein, were loaded in the gel. Lane 1, marker; lane 2, native α-casein; lane 3, α-casein treated with laccase (24 h); lane 4, α-casein treated with laccase in the presence of ferulic acid (24 h); lane 5, α-casein treated with transglutaminase (24 h).

L-7450, Merck, Darmstadt, Germany) operating at 280 and 320 nm. Separations were performed at 35 °C on a reverse phase C18 column (Purospher STAR-RP-18 endcapped, 5 μm, 250 × 4.6 mm, Merck) by gradient elution with solvent A, 0.5% TFA in water, and solvent B, 100% methanol. The elution profile was 0−25 min, 5−30% B linearly; 25−30 min, 30% B; 30−60 min, 30−50% B linearly; 60−70 min, 50−90% B linearly; 70−100 min, 90−100% B linearly; and 100−110 min, 100% B. The flow rate was 1 mL/min, and the injection volume was 20 μL. Ferulic acid was identified by spiking with an authentic ferulic acid standard and from spectral data. The dimers of the ferulic acids were tentatively identified by their spectral data. Ferulic acid and the dimers were quantified using an external standard curve of ferulic acid, constructed by concentrations from 2 to 20 μg/mL. The standard curve showed linearity over the tested concentrations with a correlation coefficient of $r^2 = 0.997$.

Surface Tension Measurements. The surface tension of cross-linked and native proteins was measured in triplicate at 25 ± 1 °C using a Sigma 701 Tensiometer (KSV Instruments Ltd., Helsinki, Finland), according to a method by Hammershøj et al. (*31*) A platinum/iridium Wilhelmy plate (19.6 × 10.0 × 0.1 mm) was attached to a microbalance and positioned in the center of a 46 mm diameter glass vessel. Both the glass vessel and the Wilhelmy plate were rinsed with deionized water and ethanol, and the Wilhelmy plate was burned to glow with a Bunsen burner between measurements. The measuring conditions were 2 mm wetting depth, pull speed of 0.25 mm/s, 2 s stabilization time at the surface, and 5 s integration time. The surface tension was recorded continuously for 9500 s after the protein solution had been applied with a thin pipet below the surface of 20 mL of double-deionized water. The surface pressure of the applied protein, which forms a monolayer at the air−water interface, was determined as the reduction in surface tension as compared to the double-deionized water before protein was added. The surface pressure caused by the enzymes alone, and laccase in combination with ferulic acid has been subtracted from the values found for the enzyme-treated proteins.

Initially, the purity of the double-deionized water was checked by measuring the surface tension to 72.55 ± 0.55 mN/m.

Statistical Analysis. Significant differences are determined using Student's *t* test with a significance level of 0.05. Deviations in figures are shown as standard deviations calculated from three repetitions.

RESULTS

SDS-PAGE. Possible fragmentations or formation of intermolecular cross-links by the laccase-catalyzed oxidation of α-casein, β-lactoglobulin, and BSA were characterized by SDS-PAGE analysis, at reducing and nonreducing conditions to distinguish oligomerization or aggregates formed by reducible disulfide bonds.

The S1 (23 kDa) and S2 (25 kDa) components of α-casein were not distinguished by the SDS-PAGE analysis, but were seen as one band below 37 kDa (**Figure 1** gel **A**, lane 2, and gel **B**, lane 2). In SDS-PAGE, α-casein is known to migrate as if it has a higher mass (*32*). In addition to the α-casein band, a reducible aggregate with a mass above 206 kDa was present (**Figure 1**, compare gel **A**, lane 2, and gel **B**, lane 2); this may be formed between α-casein and different protein impurities by heat induction.

Laccase treatment did not fragment or cross-link α-casein (**Figure 1**, gel **A**, compare lanes 2 and 3, and gel **B**, compare lanes 2 and 3). However, when ferulic acid was present during laccase treatment, irreducible cross-linking was observed as a band above 206 kDa (**Figure 1**, compare gel **A**, lanes 3 and 4, and gel **B**, lanes 3 and 4). The adduct in this band appeared to have a slightly higher mass than the reducible aggregate originally present. In addition, a reducible aggregate with a molecular weight too high to enter into the gel was also observed.

Transglutaminase treatment resulted in α-casein polymerization, similar to the laccase and ferulic acid treatment except that the polymerization was irreducible.

A similar series of experiments was carried out with β-lactoglobulin (18 kDa) (**Figure 2**). What must be a reducible dimer was observed around 35 kDa after the laccase-catalyzed oxidation of β-lactoglobulin (**Figure 2**, compare gel **A**, lane 3, and gel **B**, lane 3). The laccase-catalyzed oxidation of β-lactoglobulin in the presence of ferulic acid caused a smear of the β-lactoglobulin band toward higher masses. In addition, the laccase-catalyzed oxidation of β-lactoglobulin in the presence of ferulic acid resulted in an irreducible adduct appearing as a smeared band with a slightly higher mass than the dimer observed in the absence of ferulic acid. Also, a faint and equally smeared appearance of an irreducible adduct at a mass exceeding 3 times the mass of β-lactoglobulin appeared after laccase-catalyzed oxidation of β-lactoglobulin in the presence of ferulic acid (**Figure 2**, gel **A**, lane 4, and gel **B**, lane 4). Transglutaminase was not able to cross-link β-lactoglobulin (**Figure 2**, gel **A**, lane 5, and gel **B**, lane 5).

Neither the laccase-catalyzed oxidation nor the treatment of BSA (66 kDa) with transglutaminase showed any cross-linking or fragmentation in the SDS-PAGE analysis (**Figure 3**). However, after laccase-catalyzed oxidation of BSA in the presence of ferulic acid, an adduct that could be a BSA dimer was observed above 100 kDa. Interestingly, the adduct could be observed only at reducing conditions (**Figure 3**, compare

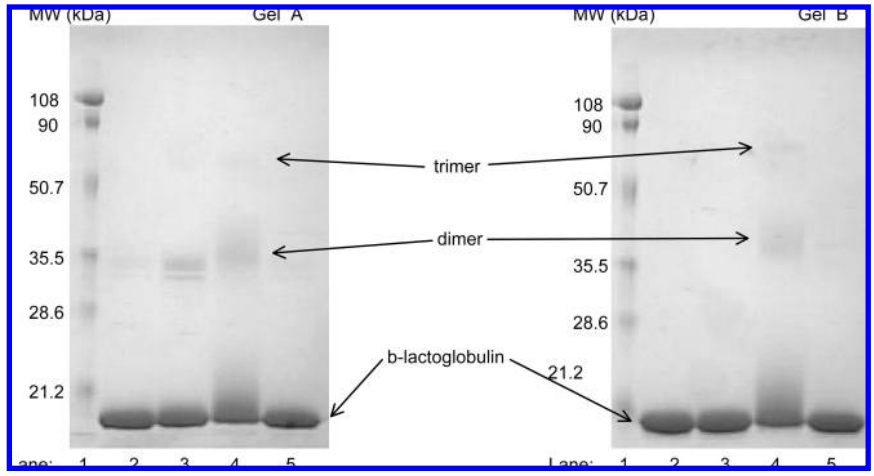

Figure 2. SDS-PAGE gel of β-lactoglobulin under nonreducing (gel **A**) and reducing (gel **B**) conditions. Volumes of 30 μL containing 45 μg of protein were loaded in the gel. Lane 1, marker; lane 2, native β-lactoglobulin; lane 3, β-lactoglobulin treated with laccase (24 h); lane 4, β-lactoglobulin treated with laccase in the presence of ferulic acid (24 h); lane 5, β-lactoglobulin treated with transglutaminase (24 h).

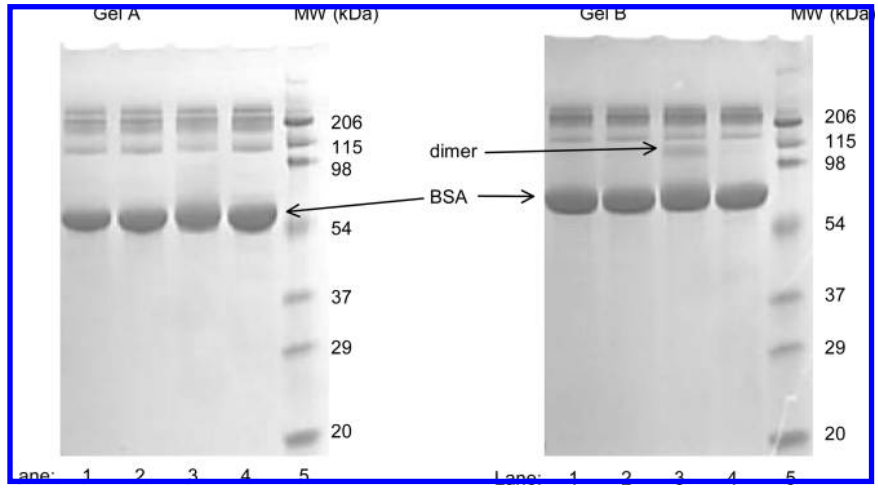

Figure 3. SDS-PAGE gels of BSA under nonreducing (gel **A**) and reducing (gel **B**) conditions. Volumes of 10 μL containing 15 μg of protein were loaded in the gel. Lane 1, native BSA; lane 2, BSA treated with laccase (24 h); lane 3, BSA treated with laccase in the presence of ferulic acid (24 h); lane 4, BSA treated with transglutaminase (24 h); lane 5, marker.

gel **A**, lane 3, and gel **B**, lane 3). Also, an irreducible smear of the BSA band toward higher masses was seen, which is similar to what was observed with β-lactoglobulin.

Dityrosine. Upon laccase-catalyzed oxidation of the three proteins, dityrosine was formed only in α-casein (**Figure 4**). The detected dityrosine corresponded to <0.16% of the theoretically available tyrosines in α-casein, which was the highest dityrosine formation detected, suggesting that dityrosine formation is a minor reaction pathway. The presence of ferulic acid during the laccase-catalyzed oxidation of α-casein resulted in the formation of less dityrosine. In β-lactoglobulin, dityrosine was formed only in the presence of ferulic acid, whereas oxidation of BSA caused no dityrosine formation.

Even smaller but still statistically significant amounts of dityrosine were found when all three proteins were treated with transglutaminase.

***N′*-Formylkynurenine.** *N′*-formylkynurenine is a tryptophan oxidation product, which gives distinct fluorescence spectra that can be used to monitor the oxidation of tryptophan (*28*). The fluorescence emission spectrum obtained from laccase-treated α-casein strongly indicates the formation of formyl kynurenine (**Figure 5A**). No indication of *N′*-formylkynurenine was observed with transglutaminase treatment of α-casein.

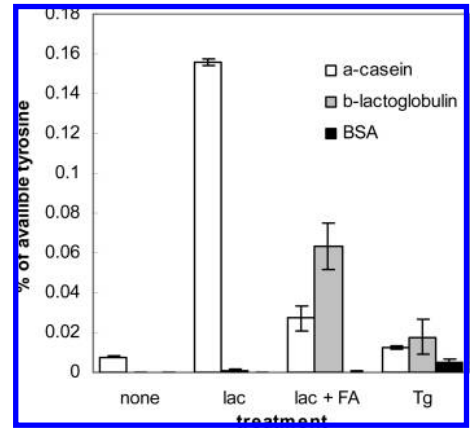

Figure 4. Formation of dityrosine in α-casein, β-lactoglobulin, or BSA after a 24 h reaction with laccase (lac) in the presence or absence of 1 mM ferulic acid (FA) or with transglutaminase (Tg).

Formation of *N′*-formylkynurenine by the laccase-catalyzed oxidation of β-lactoglobulin could not be observed from the fluorescence spectra (**Figure 5B**). The higher intensity observed after laccase-catalyzed oxidation of β-lactoglobulin can be explained as the sum of intensities observed in the spectra measured from the protein and the enzyme separately.

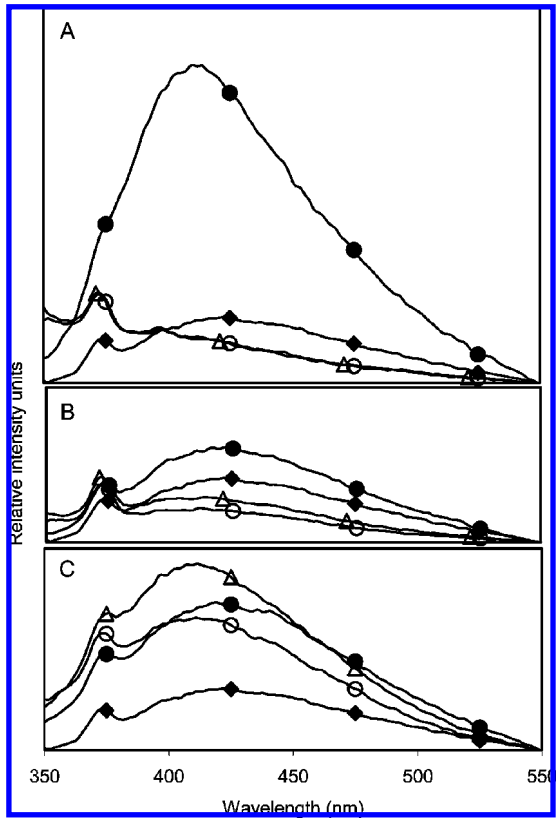

Figure 5. Fluorescence spectra showing *N*′-formylkynurenine in α-casein (**A**), β-lactoglobulin (**B**), and BSA (**C**) when treated with laccase (●) or transglutaminase (△) or with no enzyme (○) for 24 h. Fluorescence spectra showing *N*′-formylkynurenine in laccase (◆) are also included.

Table 1. Content of Relevant Amino Acid in Analyzed Proteins

	Y	W	H	M	C	C−C	R	phosphorylation
α_{S1}-casein	10	2	5	5			6	9
α_{S2}-casein	12	2	3	4	2	6	12	
β-lactoglobulin	4[a]	2[b]	2	4	5	2	3	
BSA	20[b]	2	17	4	35	17	23	

[a] Amino acids are located in the protein core at a pH of 6.8. [b] Amino acids are located partly in the core and partly on the surface of the protein. Adapted from Swiss-prot: http://au.expasy.org/sprot.

BSA incubated without enzyme gave a strong fluorescence spectrum very similar to that of *N*′-formylkynurenine (**Figure 5C**). The relatively low amount of tryptophan in BSA (**Table 1**) suggests this originates elsewhere than from preoxidation of tryptophan residues in BSA. The laccase-catalyzed oxidation of BSA had only a minor effect on the fluorescence spectrum, suggesting that no *N*′-formylkynurenine was formed by the oxidation. Attempts were also made to examine the effect of ferulic acid by fluorescence spectroscopy. Unfortunately, ferulic acid shows an emission spectrum that closely resembles that of *N*′-formylkynurenine, except for quenching of the Raman peak from water with emission maxima at 370 nm. Thus, it was not possible to conclude if more or less *N*′-formylkynurenine was formed in the presence of ferulic acid.

Carbonyl Formation. The presence of the carbonyl compounds kynurenine, 2-oxohistidine, and a methionine-derived carbonyl compound was analyzed in the native and cross-linked proteins after DNPH derivatization by a HPLC-based method according to the method of Dalsgaard et al.(*29*). All three native proteins contained tryptophan-, methionine-, and histidine-derived carbonyl compounds (**Figure 6**). The laccase-catalyzed oxidation of α-casein and BSA did not lead to statistically

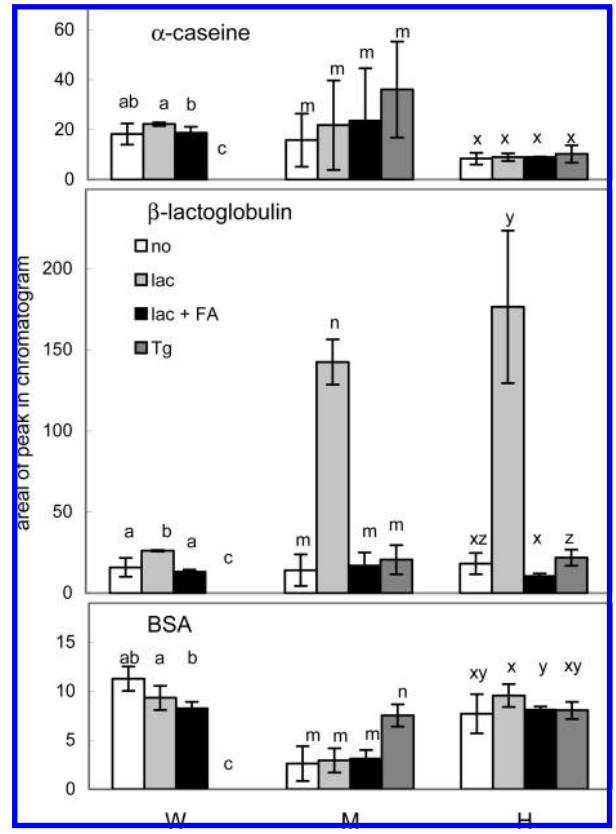

Figure 6. Carbonyl groups derived from tryptophan (W), methionine (M),and histidine (H) by enzymatic treatments of α-casein, β-lactoglobulin,and BSA for 24 h with laccase (lac), laccase in the presence of ferulic acid (lac + FA), or transglutaminase (Tg). For each protein, different letters indicate a significant difference in the amount of carbonyl groups detected.

significant changes in the levels of the three types of carbonyl groups, and inclusion of ferulic acid in the reaction mixture also had no effect. In laccase-treated β-lactoglobulin, high amounts of methionine and histidine carbonyl groups and small amounts of tryptophan carbonyls were formed. However, when ferulic acid was present, none of the carbonyls were formed. As transglutaminase is not an oxidative enzyme, carbonyl formation was not expected in the three proteins upon treatment with transglutaminase; still, the carbonyl formation was measured with surprising results. The tryptophan-derived carbonyl group kynurenine, initially found in all three proteins, disappeared in the three proteins after treatment with transglutaminase, and treatment with transglutaminase also caused an unexpected increase in the methionine-derived carbonyl content of BSA.

Ferulic Acid Monomers and Dimers. The fate of ferulic acid during the laccase-catalyzed oxidation of the proteins was studied by detection of ferulic acid monomers and dimers after the reaction (**Table 2**). No monomers of ferulic acid were detected in any of the experiments in which laccase was used, indicating that ferulic acid is easily oxidized by laccase. After laccase-catalyzed oxidation of ferulic acid alone, half of the ferulic acid was modified to an extent whereby it escaped detection. This is also the case after laccase-catalyzed oxidation of BSA in the presence of ferulic acid. The presence of α-casein partly prevented the modification of ferulic acid to undetectable substances, as more ferulic acid dimers could be detected, whereas the presence of β-lactoglobulin seems to cause modification of more ferulic acid, whereby it escapes detection.

Table 2. Detected Amount of Ferulic Acid Monomers and Dimers (Percent of Total Ferulic Acid) after Incubation with Laccase in the Presence of Proteins

	monomers	dimers
none	nd[a]	49 ± 3
α-casein	nd	72 ± 15
β-lactoglobulin	nd	27 ± 5
BSA	nd	53 ± 2

[a] Could not be detected.

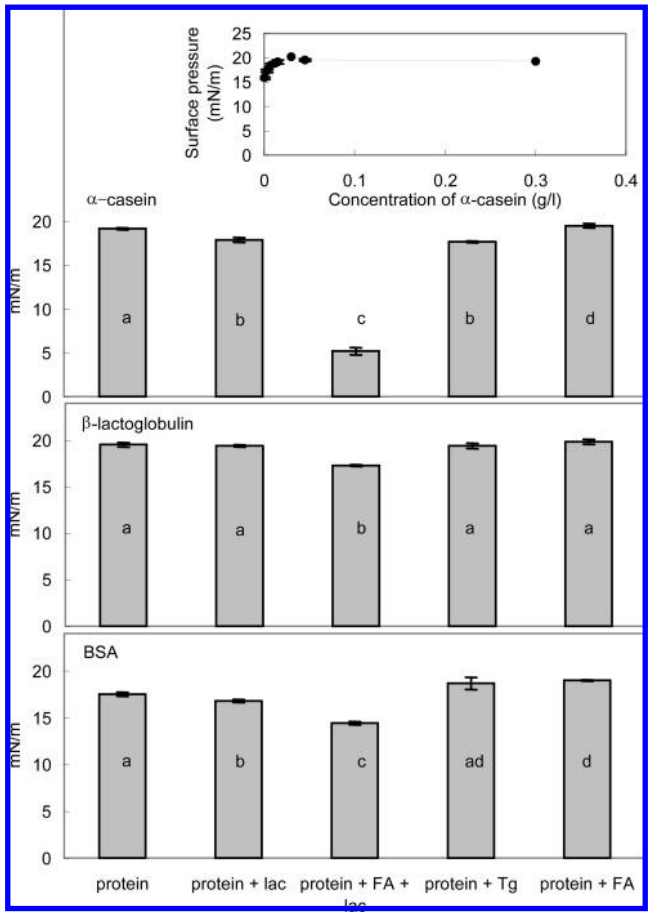

Figure 7. Surface pressure of α-casein, β-lactoglobulin, and BSA treated with laccase (lac) in the presence and absence of ferulic acid (FA) or with transglutaminase (Tg) for 24 h. Data were corrected for the effect caused by the presence of enzymes and mediator. For each protein, different letters indicate a significant difference in surface pressure. (Inset) Surface pressure of α-casein as a function of casein concentration.

Surface Tension. The amounts of protein needed to form monolayers when placed in a measuring vessel with 20 mL of buffer and allowed to spread on the 16.6 cm^2 surface were initially determined as 0.015 g/L α-casein, 0.030 g/L β-lactoglobulin, and 0.30 g/L BSA, executing surface pressures of 19.16 ± 0.11, 19.54 ± 0.23, and 17.51 ± 0.23 mN/m, respectively, for the native proteins. Larger amounts of protein did not increase the surface pressure significantly, whereas smaller amounts of protein decreased the measured surface pressure, as exemplified with α-casein in the inset of **Figure 7**.

The surface pressure of the enzyme-treated proteins was measured on the same amount of protein needed to form a monolayer when in its native form (**Figure 7**). Small changes in the surface tension were seen after laccase treatment of α-casein and BSA. The presence of ferulic acid during the oxidation gave significantly lower surface tensions of all three proteins, and in the case of α-casein the measured surface pressure was reduced by 13.97 mN/m. Control experiments in which the proteins were mixed with ferulic acid, but without enzyme treatment, were carried out to examine if the presence of ferulic acid affected the surface pressure of the solutions. The mixtures of ferulic acid with α-casein and BSA gave small (≈1 mN/m) increases in surface tension, whereas the surface tension of β-lactoglobulin was not affected. Only α-casein gave a statistically significant, although minor, change in the surface tension due to treatment with transglutaminase.

DISCUSSION

The laccase-catalyzed oxidations of α-casein, β-lactoglobulin, and BSA resulted not only in cross-linking of the proteins but also in other types of oxidative modifications. The three proteins with very different structures and amino acid compositions (**Table 1**) gave different patterns of oxidation products, indicating that laccase-catalyzed oxidation of proteins proceeds through a number of simultaneously competing reaction pathways depending on the protein. The complex nature of protein oxidation here observed emphasizes that comparison of oxidation in different proteins will be difficult, if based on only a single analytical method.

The natural substrates of laccase are phenolic compounds. Tyrosines were therefore expected to be the prime targets in proteins, resulting in the formation of tyrosyl radicals that can lead to inter- or intramolecular protein cross-links through dityrosine bonds. The results of Østdal et al. (*33*) show that the lifetimes of lactoperoxidase-induced radicals, presumably tyrosyl radicals, formed in α-casein, β-lactoglobulin, and BSA are correlated to the degree of protein structure and inversely correlated to the amount of dityrosine formed. Consequently, tyrosyl radicals in unstructured proteins are more prone to form dityrosine than tyrosyl radicals in structured proteins. In the present study, dityrosine formation was accordingly seen, although the amount was very low, in laccase-treated α-casein with no inherent structure, and not after oxidation of the structured proteins BSA and β-lactoglobulin (**Figure 4**).

Apart from a small reduction in the surface tension observed after laccase treatment (**Figure 7**), BSA was found to be generally unaffected by laccase-catalyzed oxidation according to the analytical methods applied in the present study. Still, to explain the reduction in surface tension, some extent of modification is expected to occur in laccase-treated BSA, for example, generation of hydroperoxides (*34*). Due to the globular structure of BSA, only 25% of the tyrosine residues are considered to be solvent exposed (*35*), thus being available for oxidation by laccase. The globular structure has also been shown to stabilize radicals in the interior of the protein (*36*). Such interior radicals can be formed by transfer of oxidation equivalents from oxidizable amino acids, closer to the surface of the protein, thus protecting these amino acids from oxidative damages (*37*). This ability to stabilize radicals in the interior of the protein is consistent with the absence of oxidative modifications upon laccase treatment of BSA on any of the examined amino acids.

In α-casein, all amino acids are accessible to laccase and are therefore potential oxidation targets. Although laccase-catalyzed oxidation of α-casein led to formation of dityrosine, the amount is minute. Also, at the used enzyme dosage no cross-linking of the protein was observed by SDS-PAGE (**Figure 1**). Using a higher enzyme dosage, cross-linking of α-casein has recently been reported (*4*). Assuming that tyrosine is indeed the prime target of oxidation, it must be concluded that intermediate tyrosyl

radicals generated will form products other than dityrosine or that the radical is transferred to other amino acids. Tryptophan is a likely candidate, as *N′*-formylkynurenine is detected in laccase-treated α-casein, and tryptophan also is among the most easily oxidized amino acids (*22*).

β-Lactoglobulin contains fewer tyrosine residues than α-casein, and the higher degree of structure makes some amino acids inaccessible to laccase. It has been reported that only one of the four tyrosine residues present in *β*-lactoglobulin is exposed to solvent, and only at pH above 7 (*38*), and that only one of the two tryptophans is exposed to solvent (*39, 40*). Whereas the structure of *β*-lactoglobulin might prevent tyrosine residues from forming dityrosine, the laccase-catalyzed oxidation of *β*-lactoglobulin instead results in a more extensive oxidation of tryptophan to the carbonyl kynurenine as well as the formation of carbonyls located on histidine and methionine (**Figure 6**). Carbonyl formation was detected on only these three amino acids, but it is likely that carbonyls could also be formed on other easily oxidized amino acids, for example, arginine (*41*).

As the formation of carbonyls is observed only in *β*-lactoglobulin where tyrosine is unavailable, this indicates that tyrosine is the primary target for laccase-catalyzed oxidation.

From the surface pressure measurements, it can be seen that laccase introduces changes in both α-casein and BSA, which reduces their surface tension, but not in *β*-lactoglobulin (**Figure 7**). By comparison of the results from the SDS-PAGE analysis (**Figures 1** and **3**), it is evident that intermolecular cross-linking cannot be the cause of this reduction, as neither α-casein nor BSA shows detectable cross-linking. This leaves other possible explanations such as reduction of flexibility in α-casein due to intermolecular cross-links, decreased hydrophobicity caused by modifications other than cross-linking (*15, 42, 43*), or altered diffusion behavior due to the mere presence of laccase to account for the observed reduction in surface pressure.

The oxidation of tryptophan and methionine in *β*-lactoglobulin is expected to make the protein more hydrophilic, resulting in a lowered surface pressure (*43*), but this was not seen (**Figure 7**). This suggests that the oxidation of *β*-lactoglobulin distorts the protein conformation slightly, leading to exposure of some hydrophobic core residues (*44*) or reducing its flexibility and thus counteracting the expected effect on surface pressure of a more hydrophilic protein.

Ferulic acid was included in the reactions, as it is expected to act as a mediator of laccase-catalyzed reactions. Laccase is able to catalyze oxidation of ferulic acid, generating a ferulic acid radical, which is able to dimerize to ferulic acid dimers (*45*). After laccase treatment of ferulic acid, half of the ferulic acid was detected as dimers, demonstrating that ferulic acid is efficiently oxidized by laccase. The other half of the ferulic acid, which was not detected, may have been transformed into polymers of ferulic acid, which the applied analytical method does not detect. Also, in the presence of an oxidizable protein ferulic acid is dimerized. Thus, whereas ferulic acid can act as a mediator, it can also act as a competing substrate, which thereby complicates the reaction pathways (*46*).

The presence of ferulic acid during laccase treatment had a major effect on the laccase-catalyzed oxidation, causing intermolecular cross-linking (**Figures 1** and **2**) of α-casein and *β*-lactoglobulin, but not of BSA, which formed cross-links only upon reduction (**Figure 3**). Presumably, cross-linking forms larger and more rigid protein structures with a reduced ability to spread and cover a water/air interface, which can explain some of the reduction in protein surface pressure observed, as the reduction of surface pressure of BSA necessarily must be explained by other modifications (**Figure 7**).

The cross-linking observed in BSA by SDS-PAGE analysis exclusively at reducing conditions when ferulic acid is present (**Figure 3B**) must be caused by stabilized long-lived radicals in the interior of BSA. When the protein is denatured by reduction of its disulfide linkages, such stabilized radicals will become exposed on the surface and could dimerize intermolecularly, forming BSA dimers. As ferulic acid is necessary for this dimerization, it must be involved either in the formation of radicals or in facilitating radical stabilization in the interior of the protein. The ferulic acid radical produced by laccase treatment should, due to its low molecular weight, be able to penetrate into the globular structure of BSA and initiate radical reactions at amino acids that are unreachable by laccase.

The laccase-catalyzed irreducible cross-linking observed by SDS-PAGE analysis in α-casein and *β*-lactoglobulin (**Figures 1** and **2**) in the presence of ferulic acid may be dityrosine bonds (**Figure 4**), although tyrosyl radicals can also dimerize and form isodityrosine bonds. It has previously been shown that isodityrosine bonds are likely to be responsible for laccase-catalyzed cross-linking of peptides (*6*).

A remarkably high level of carbonyl groups in *β*-lactoglobulin was formed after laccase-catalyzed oxidation. However, inclusion of ferulic acid in the laccase-catalyzed oxidation of *β*-lactoglobulin resulted in the formation of dityrosine bonds and a lack of carbonyl formation. This resembles the modifications that were found in α-casein in the absence of ferulic acid, suggesting that ferulic acid must have acted as a mediator transporting radicals or oxidation equivalents to less accessible amino acids in *β*-lactoglobulin, whereby all amino acids become accessible as is the case in α-casein. In addition, the dityrosine formation must be accompanied by structural changes of *β*-lactoglobulin, allowing tyrosine residues located in the protein core to dimerize.

When *β*-lactoglobulin and BSA were treated with laccase in the presence of ferulic acid (**Figures 2** and **3**), the bands of the protein showed a smear toward higher masses. It is possible that ferulic acid has become added to amino acids in the proteins. This may explain the smaller level of ferulic acid monomers and dimers found after oxidation of *β*-lactoglobulin. Incorporation of ferulic acid has previously been observed in laccase-catalyzed oxidation of tyrosine-containing small peptides. If this is also the case in proteins, ferulic acid not only acts as a mediator of oxidation but can also become incorporated into the protein structure of especially structured proteins. Incorporation of ferulic acid into BSA could also influence the proteins' ability to stabilize radicals in the interior, which resulted in dimerization of the protein upon denaturation by reduction.

The enzyme transglutaminase was included in this study of laccase for comparison. In contrast to the laccase/ferulic acid system, which was also able to form oligomers of the structured protein *β*-lactoglobulin, transglutaminase was able to cross-link only the unstructured protein α-casein, and likewise it was the only protein for which treatment with transglutaminase could affect the surface pressure significantly. Reports state that transglutaminase, which is otherwise an efficient cross-linking enzyme, is not able to cross-link *β*-lactoglobulin or BSA (*47*). The disappearance of the small amount of kynurenine measured by HPLC mass spectrometry in the three proteins after treatment with transglutaminase shows that transglutaminase is able to modify carbonyl groups on tryptophan. No literature has been encountered that explains the fate of kynurenine after treatment with transglutaminase, but kynurenine contains an amide group

that might participate in the transglutaminase-catalyzed transamidation that causes cross-linking. The carbonylation of methionine observed in BSA after treatment with transglutaminase remains unexplained, as the known action of transglutaminase is unable to induce such a modification.

Whereas the impact of transglutaminase on the macroscopic properties of a protein exemplified by surface tension correlates directly to cross-linking, the impact of laccase-catalyzed oxidation seems to be governed by cross-linking and other oxidative modifications alike. Thus, the use of laccase for targeted engineering of proteins will depend on whether a thorough understanding of its actions in proteinaceous systems is developed. In this study, model systems each containing a single protein were used. However, food systems as well as other biosystems often contain complex mixtures of several proteins, and the outcome of laccase-catalyzed oxidations in such systems is therefore expected to be highly dependent on the actual system. With the development of such an understanding, laccase holds potential to challenge the position of the cross-linking enzyme transglutaminase due to its ability to cross-link both unstructured and structured proteins.

ACKNOWLEDGMENT

We thank Ulla Kidmose for analysis of the ferulic acid monomers and dimers.

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Received for review April 18, 2008. Revised manuscript received August 22, 2008. Accepted October 16, 2008.

JF801234V