JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Formation of Protein−Oligosaccharide Conjugates by Laccase and Tyrosinase

EMILIA SELINHEIMO,* PIRITTA LAMPILA, MAIJA-LIISA MATTINEN, AND JOHANNA BUCHERT

VTT Technical Research Centre of Finland, P.O. Box 1000, Espoo FIN-02044 VTT, Finland

Proteins and certain carbohydrates contain phenolic moieties, which are potential sites for modification of the function of the biopolymers. In this study, the capability of two different fungal oxidative enzymes, laccase from *Trametes hirsuta* (ThL) and tyrosinase from *Trichoderma reesei* (TrT), to catalyze formation of hetero-cross-linking between tyrosine side chains of α-casein and phenolic acids of hydrolyzed oat spelt xylan (hOSX) was studied. Formation of reaction products was followed by size exclusion chromatography (SEC), fluorescence spectroscopy, and SDS-PAGE, using specific staining methods for proteins and protein−carbohydrate conjugates. ThL and TrT were observed to differ significantly in their ability to catalyze the formation of protein−carbohydrate conjugates or the linking of the small molecular weight phenolic compounds to α-casein. The efficiency of these enzymes to directly cross-link protein also differed notably. TrT was able to cross-link α-casein more efficiently than ThL. ThL-catalyzed casein cross-linking was significantly enhanced by ferulic acid, *p*-coumaric acid, and also hOSX. The main reaction products by ThL appeared to be phenolic acid-bridged α-caseins. Indications of hetero-cross-link formation between α-casein and hOSX by both oxidative enzymes could be visualized by glycoprotein-specific staining in the SDS-PAGE analysis, although ThL was observed to be more effective in the heteroconjugate formation than TrT.

KEYWORDS: Laccase; tyrosinase; protein; xylan; phenolic acids; cross-linking

INTRODUCTION

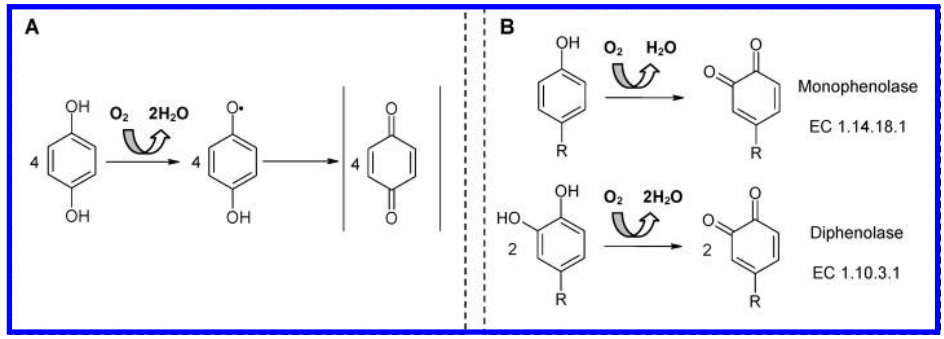
Enzymes, which are able to functionalize biopolymers, are potential tools for the production of value-added end-products. Modification of biopolymers by enzymes, which can generate covalent linkages, enables incorporation of novel functions and altered structural properties to the polymers (*1–3*). Phenolic moieties of biopolymers, such as proteins, polysaccharides, and lignin, can be oxidized by enzymes or chemicals, and the oxidation reactions can lead to cross-linking of the biopolymers (*4, 5*). Tyrosinases and laccases are enzymes capable of oxidizing various phenolic compounds. These enzymes are copper-containing proteins, which oxidize their substrates by shuttling electrons from the substrate to molecular oxygen. The reactions catalyzed by these oxidative enzymes may result in cross-linking of polymers. Tyrosinases are mono-oxygenases and bifunctional enzymes, which catalyze ortho-hydroxylation of monophenols and subsequent oxidation of *o*-diphenols to quinones (**Scheme 1A**) (*6*). Quinones can further react nonenzymatically to produce mixed melanins and heterogeneous polymers. Tyrosine side chains in proteins can be oxidized by tyrosinase, and lysyl, tyrosyl, cysteinyl, and histidinyl moieties have been reported to react further with tyrosinase-oxidized tyrosine residues (*5, 7–10*). Laccases typically show wider substrate specificity than tyrosinases, oxidizing, for instance, monophenols, polyphenols, different substituted phenols, diamines, and aromatic amines and thiols (*11, 12*). Laccase-catalyzed cross-linking is based on radical formation and the further reactions of the radicals, which can undergo nonenzymatic reactions, including polymerization, hydration, and disproportionation (**Scheme 1B**) (*11, 13*). Laccase is reported to cross-link arabinoxylan and pectin via biopolymer-conjugated ferulic acid (*14–17*). Laccase has also been found to oxidize the amino acids, tyrosine and cysteine, to catalyze peptide polymerization (*18*), and to cross-link certain proteins (*19–21*).

Cross-linking enzymes can be exploited to improve the technological functionality of different food biopolymers, such as proteins from cereals, milk, and soy, and carbohydrates, which contain phenolic acids (*22*). Besides the homo-cross-linking of proteins or polysaccharides, the hetero-cross-linking of proteins with polysaccharides has applications in the food industry. There is great interest, for instance, in the generation of protein−carbohydrate conjugates for stabilization of foams and emulsions (*23, 24*). Soluble egg white−galactomannan protein−polysaccharide conjugates, prepared through Maillard reactions between the ε-amino groups in the protein and the reducing carbonyl residue in the polysaccharide, have been reported to have very good emulsifying properties (*24*). β-Lactoglobulin emulsion gels have been produced with the help of transglutaminase (*25*), and Kato

* Author to whom correspondence should be addressed (telephone +358 20 722 7135; fax +358 20 722 7071; e-mail Emilia.Selinheimo@vtt.fi).

10.1021/jf0730791 CCC: $40.75
Published on Web 04/19/2008

Scheme 1. Catalytic Mechanisms of Laccase (**A**) and Tyrosinase (**B**)



et al. (*26*) reported that enzyme-cross-linked ovomucin−α_{s1}-casein conjugates could have better emulsifying properties than pure α_{s1}-casein. Tyrosinase-mediated grafting of peptides and silk proteins with the amine-containing polysaccharide chitosan has been studied by Åberg et al. (*2*) and Freddi et al. (*3*), respectively. Peroxidases, on the other hand, have been reported to be able to conjugate ferulic acid and feruloylated AX to tyrosine-containing peptides or to form conjugates between β-casein and arabinoxylans (*27*–*29*).

The aim of this study was to elucidate the suitability of laccase and tyrosinase for heteroconjugate formation between proteins and carbohydrates and grafting of phenolic acids to proteins, using α-casein, ferulic acid (FA), *p*-coumaric acid (*p*-CA), and hydrolyzed oat spelt xylan (hOSX) as model compounds.

MATERIALS AND METHODS

Enzymes. Laccase (ThL) was produced by the white-rot fungus *Trametes hirsuta* and purified as described by Rittstieg et al. (*30*). ThL activity was determined as described by Niku-Paavola et al. (*31*), using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) as substrate. Tyrosinase (TrT) was obtained from the filamentous fungus *Trichoderma reesei*, produced and purified as previously described (*32*). Activity of TrT was measured as described by Robb (*33*), using 15 mM 3,4-dihydroxy-L-phenylalanine (L-dopa) as substrate. Xylanase from *Bacillus subtilis* (BsX) was from Danisco A/S (Brabrand, Denmark), and xylanase activity was determined using birch glucuronoxylan (Roth) as substrate (*34*).

Substrates. Bovine milk α-casein was obtained from Calbiochem (CN Biosciences Inc., San Diego, CA). FA, *p*-CA, and oat spelt xylan (OSX) were obtained from Sigma. The phenolic acids of OSX were analyzed by HPLC (Millipore Waters, Bedford, MA), using a Hypersil BDS-C18 column (Agilent Technologies, Palo Alto, CA) according to the method described by Mattila et al. (*35*).

Depolymerization of OSX by BsX. Depolymerization of OSX was performed using a BsX dosage of 2 nkat mg^{-1} of OSX. OSX was suspended in 0.1 M sodium phosphate buffer (pH 7.0), and the hydrolysis was performed at ambient temperature for 24 h under constant magnetic stirring. Enzyme was inactivated by heating the reaction mixture for 10 min at 95 °C, and the remaining insolubles were removed from the hydrolysate by centrifugation. Depolymerization of OSX was analyzed by thin layer chromatography (TLC) and size exclusion chromatography (SEC) analyses (SEC specifications are described below). TLC (silica gel, Merck, Nottingham, U.K.) was carried out with ethyl acetate, acetic acid, and water (3:2:2, v/v/v) as the solvent. Xylans were stained with a solution of orcin 0.2% (w/w), H_2SO_4 10%, EtOH 80% (v/v), and the sheet was incubated at 105 °C for 10 min.

Treatments with Oxidative Enzymes. ThL and TrT treatments of α-casein, FA, *p*-CA, and hydrolyzed hOSX were performed in a volume of 1 or 2 mL, in 0.1 M sodium phosphate buffer (pH 7.0), at room temperature for 20 h. Reactions were terminated by incubating the reaction mixtures at 95 °C for 10 min. ThL reactions of α-casein and the α-casein and hOSX mixtures contained 0.15, 1.5, or 15 nkat mg^{-1} of α-casein and the corresponding TrT reactions contained 0.005, 0.05, or 0.5 nkat mg^{-1} of α-casein. Alternately, 0.15, 1.5, or 15 nkat of ThL and 0.005, 0.05, or 0.5 of nkat TrT mL^{-1} of substrate solution were added to hOSX to provide enzymatic activity equivalent to that with 10 mg mL^{-1} of α-casein. FA and *p*-CA were added either in four portions at 10 min intervals after the addition of the enzyme or in one portion before the addition of the enzyme. Control incubations without enzyme addition were performed for all substrate mixtures. The substrate concentrations used for the enzymatic reactions are summarized in **Table 1**.

Table 1. Summary of the Substrate Concentrations in the Mixtures of α-Casein, *p*-CA, FA, and hOSX[a]

α-casein (mg mL^{-1})	hOSX (mg mL^{-1})	FA (mg mL^{-1})	*p*-CA (mg mL^{-1})
10			
10		1[b]	
10		1[c]	
10			1[b]
10			1[c]
10	2		
5			
5	10		
	10		
1			
1	20		
	20		

[a] Three different enzyme dosages were tested. ThL was added to the substrate mixtures as 0.15, 1.5, and 15 nkat per mg of α-casein and TrT as 0.005, 0.05, and 0.5 nkat per mg of α-casein. For the hOSX alone, dosing of enzymes was as nkat per mL of hOSX solutions, to provide enzymatic activity equivalent to the enzyme doses with 10 mg mL^{-1} α-casein. [b] Added in four portions. [c] Added in one portion.

Oxygen Consumption Measurements. To study the oxidation efficiency of ThL and TrT on the selected substrates, the enzymatic reactions were monitored with a single-channel oxygen meter (Precision Sensing GmbH, Regensburg, Germany), at room temperature in a closed and fully filled vial (1.8 mL). The oxidation rate (mg L^{-1} s^{-1}) was calculated from the linear part of the oxygen consumption curve.

SDS-PAGE Analysis. Effects of enzymes on the cross-linking of α-casein in the presence of FA, *p*-CA, and hOSX were studied by SDS-PAGE analysis according to the method of Laemmli (*36*), using ready-made 12% separating Tris-HCl gels (Bio-Rad, Richmond, CA) and prestained SDS-PAGE Standards (Broad Range catalog no. 161-0318, Bio-Rad). For protein visualization the gels were stained with Coomassie Brilliant Blue (Serva Blue R, Serva Electrophoresis GmbH, Heidelberg, Germany). For detection of the carbohydrate−protein conjugates the gels were further stained by Schiff's reagent (Sigma) after removal of the Coomassie Brilliant Blue dye by destaining the gels with 40% (v/v) ethanol and 10% (v/v) acetic acid. In the glycoprotein-specific staining, periodic acid oxidizes carbohydrates to aldehydes, which react with Schiff's reagent (a mixture of pararosaniline and sodium metabisulfite), releasing a pararosaniline adduct and staining the glyco-containing proteins pink (*37*).

Size Exclusion Chromatography. Samples were analyzed by SEC using an ÄKTA Purifier liquid chromatography system (Amersham

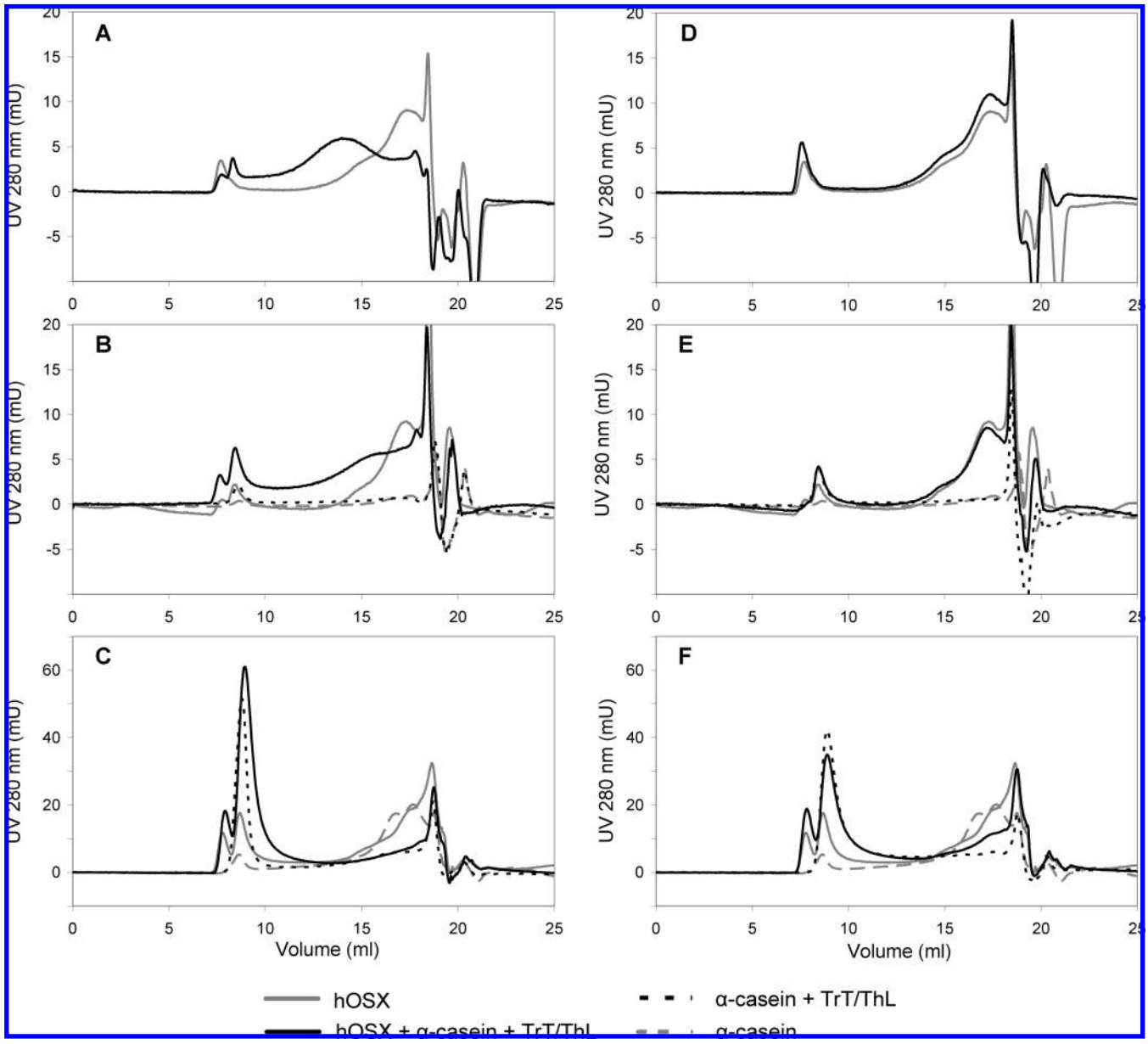


Figure 1. Size exclusion chromatograms for α-casein and hOSX after treatment with ThL and TrT. Reactions with hOSX alone (**A**, **D**), α-casein−hOSX mixtures 1−20 mg mL^{-1} (**B**, **E**), and α-casein−hOSX mixtures 5−10 mg mL^{-1} (**C**, **F**), treated with ThL 15 nkat mg^{-1} (**A**−**C**) and TrT 0.5 nkat mg^{-1} (**D**−**F**).

Pharmacia Biotech, Uppsala, Sweden). Prior to the injection to the SEC column, the samples were filtered through a 0.45 μm membrane (Syringe Driven Filter unit, 25 or 4 mm, Millex-HA, Millipore). The chromatographic analyses were performed by injection of 100 μL of sample into a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden), with 0.1 M sodium phosphate, 1% (w/v) SDS, and 0.15 M NaCl (pH 7.0) as eluent, at the flow rate of 0.5 mL min^{-1}, at ambient temperature. SDS was added to the eluent to prevent protein self-aggregation. Elute was monitored with a UV detector at 280 nm. The void volume of the column (8.4 mL) was determined with blue dextran (2000 kDa). Fractions of 0.5 mL were collected and further analyzed by fluorescence spectroscopy (Varioskan™, Thermo Electron Corp., Vantaa, Finland).

Fluorescence Spectroscopy. Fluorescence measurements were performed with Varioskan equipment using SkanIT software version 2.2.1 (Thermo Electron Corp.) on 96-well black microtiter plates (Thermo Electron Corp.), using the following excitation and emission wavelengths ($\lambda_{ex}/\lambda_{em}$), 274/303, 284/417, 318/410, and 330/430 nm, specific for Tyr, di-Tyr, FA, and *p*-CA, respectively.

RESULTS

Depolymerization of OSX by BsX. Because the intact OSX was mostly insoluble in the conditions in which the enzymatic reactions were performed, OSX was hydrolyzed with BsX to decrease its molecular weight. The hydrolysis efficiency and the product size were determined by TLC and SEC. The hydrolysis of OSX by BsX rendered OSX water-soluble, and the OSX hydrolysate eluted in SEC as a broad pattern around 13–18 mL (**Figure 1**). According to the TLC analysis, a wide variety of OSX oligomers was present after the hydrolysis (data not shown).

Analysis of Phenolic Acids of OSX. The availability of phenolic acids of OSX for enzymatic oxidation was verified by oxygen consumption measurements, and the identity of phenolic acids was determined by HPLC (data not shown). FA and *p*-CA were the only phenolic acids detected by HPLC analysis. OSX contained 140 ± 10 and 100 ± 10 μg g^{-1} of OSX-bound FA

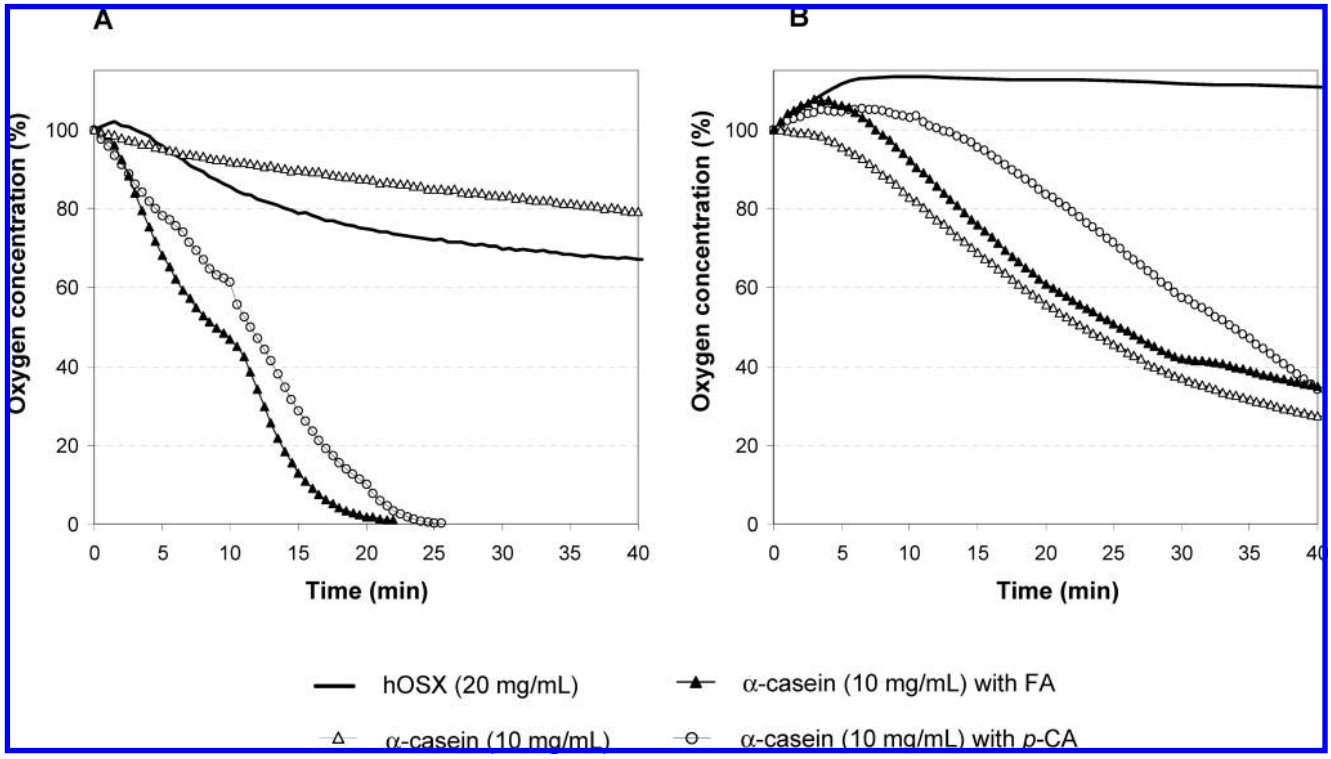


Figure 2. Consumption of oxygen as a function of time by ThL (**A**) and TrT (**B**): reactions in the presence of α-casein (10 mg mL^{-1}), hOSX (20 mg mL^{-1}), α-casein (10 mg mL^{-1}) with FA, and α-casein (10 mg mL^{-1}) with *p*-CA. FA and *p*-CA were added at four 10 min intervals to reach a final concentration of 1 mg mL^{-1}. Dosing of enzymes: ThL 15 nkat mg^{-1} and TrT 0.5 nkat mg^{-1}.

Table 2. Oxygen Consumption Rates for α-Casein and hOSX and Mixtures of α-Casein and Phenolic Acids in the Presence of ThL and TrT[a]

substrates (mg mL^{-1})				enzyme dosages (nkat/mg of α-casein)		oxygen consumption (mg L^{-1} s^{-1} × 10^4)	
α-casein	FA	*p*-CA	hOSX	ThL	TrT	ThL	TrT
10				0.15	0.05	0.2	4.7
10				1.5	0.5	1.1	13.9
10	1[b]			0.15	0.05	3.3	5.3
10	1[b]			1.5	0.5	11.6	11
10		1[b]		0.15	0.05	2.5	5.5
10		1[b]		1.5	0.5	9.7	17.6
			20[c]	0.15	0.05	1.1	nd[d]
			20[c]	1.5	0.5	3.1	0.2
			10[c]	0.15	0.05	0.8	nd
5			10	0.15	0.05	0.6	2.7
5			10	1.5	0.5	nd	6.4
5				0.15	0.05	nd	3.3
5				1.5	0.5	nd	11.1
1			20	0.15	0.05	1.0	1.4
1			20	1.5	0.5	nd	1.9
1				0.15	0.05	nd	1.7
1				1.5	0.5	nd	3.0
10			2	0.15	0.05	0.6	5.0
10			2	1.5	0.5	nd	15.1

[a] Oxygen consumption rates were calculated from the linear part and the deepest slope of the oxygen consumption curves. [b] Added in four portions to reach the concentration of 1 mg mL^{-1}. [c] Dosing as nkat per mL of hOSX solution, to provide enzymatic activity equivalent to the enzyme doses with 10 mg mL^{-1} α-casein. [d] Not determined.

and *p*-CA, respectively. Oxygen consumption measurements demonstrated that ThL consumed oxygen in the presence of hOSX (**Figure 2**).

Activity of ThL and TrT on Model Substrates. The activity of ThL and TrT on different substrates and substrate mixtures was studied by oxygen consumption measurements (**Table 2** and **Figure 2**). It should be noted, on the basis of the reaction stoichiometry, that oxidation of one phenolic molecule by laccase requires only one-fourth of an O_2 molecule (**Scheme 1A**), whereas in the case of tyrosinase, one monophenolic molecule needs one oxygen molecule to be able to catalyze a monophenol to a quinone (**Scheme 1B**). Therefore, laccase needs 4 times less oxygen to oxidize monophenolic compounds than tyrosinase. ThL oxidized both α-casein and hOSX, and the oxygen consumption rate was higher when FA and *p*-CA were included in the reaction mixture (**Table 2**). However, the activity of ThL on mixtures of hOSX and α-casein was similar to that with hOSX or α-casein alone. TrT, on the other hand, oxidized α-casein readily, but on the basis of the oxygen consumption curves, addition of FA seemed to decrease the reaction rate to some extent (**Figure 2B**). However, FA did not notably affect on the maximal oxygen consumption rate with α-casein (**Table 2**). The presence of *p*-CA slightly increased the maximum reaction rate with α-casein, although a lag period was observed (**Figure 2B**). TrT was not able to oxidize hOSX alone. However, the maximum oxidation rate for α-casein by TrT decreased and the lag period increased, when higher concentrations of hOSX were present (**Table 2**) (data on lag intervals not shown).

Cross-linking of α-Casein by ThL and TrT. ThL and TrT catalyzed direct cross-linking of α-casein, as indicated by SDS-PAGE (**Figure 3**) and SEC (**Figures 4** and **5**) analyses. The cross-linking efficiency increased as a function of enzyme dosage. Fluorescence spectroscopy measurements from SEC fractions, using $\lambda_{ex}/\lambda_{em}$ parameters specific for Tyr, showed the reaction of Tyr residues (**Figures 4** and **5**) and the subsequent formation of di-Tyr adducts (data not shown). For efficient cross-linking, a higher enzyme dosage of ThL was needed compared to the dosage of TrT. A ThL dosage of 1.5 nkat mg^{-1} seemed to result mainly in the intra-cross-linking of α-casein rather than inter-cross-linking, as higher molecular weight products were not observed, but changes in the α-casein elution were observed

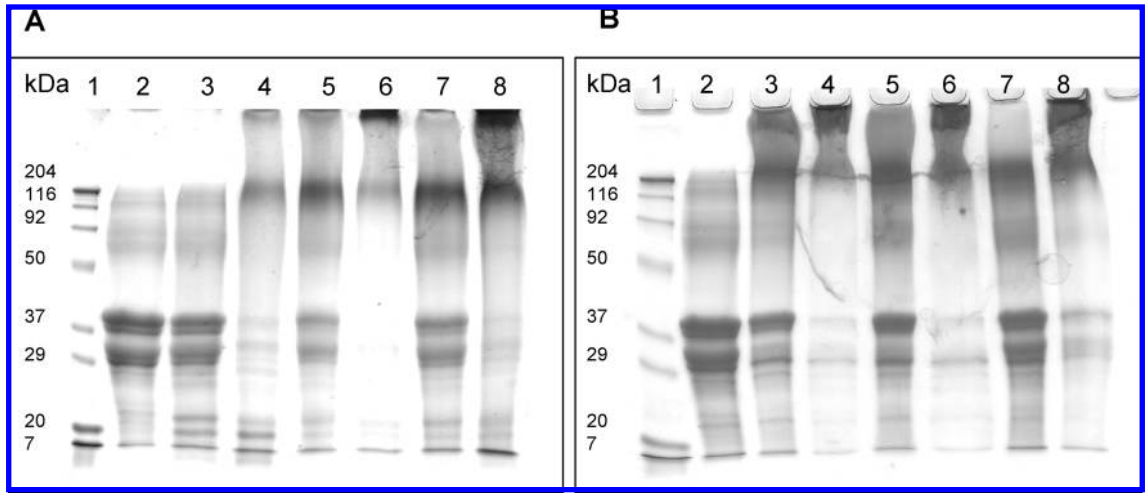


Figure 3. SDS-PAGE gel showing cross-linking of α-casein by ThL (**A**) and TrT (**B**) in the presence of FA and *p*-CA. Lanes: 1, molecular weight marker; 2, α-casein; 3, α-casein + ThL (1.5 nkat mg^{-1}) or TrT (0.05 nkat mg^{-1}); 4, α-casein + ThL (15 nkat mg^{-1}) or TrT (0.5 nkat mg^{-1}); 5, α-casein + FA + ThL (1.5 nkat mg^{-1}) or TrT (0.05 nkat mg^{-1}); 6, α-casein + FA + ThL (15 nkat mg^{-1}) or TrT (0.5 nkat mg^{-1}); 7, α-casein + *p*-CA + ThL (1.5 nkat mg^{-1}) or TrT (0.05 nkat mg^{-1}); 8, α-casein + *p*-CA + ThL (15 nkat mg^{-1}) or TrT (0.5 nkat mg^{-1}). α-Casein (10 mg mL^{-1}) and FA or *p*-CA were added at four 10 min intervals to reach a final concentration of 1 mg mL^{-1}. Dosing of enzymes is given as nkat per mg of α-casein.

in the SEC chromatogram (**Figure 3A**) and also a reduction of Tyr residues in α-casein was detectable (**Figure 4A2**). When α-casein was incubated with higher dosage of ThL (15 nkat mg^{-1}), both high and low molecular weight products were observed, whereas with the ThL dosage of 1.5 nkat mg^{-1}, only the low molecular weight degradation products were detected (**Figure 3A**, lanes 3 and 4). Corresponding low molecular weight degradation products were not detected in any of the TrT reactions.

Coupling of FA and *p*-CA to α-Casein by ThL and TrT. The role of phenolic acids in the cross-linking of α-casein was investigated by adding FA and *p*-CA to the reaction mixtures either in four portions after enzyme additions or in one portion before. In the ThL-catalyzed reactions, when α-casein was incubated in the presence of FA and *p*-CA, the formation of high molecular weight products was clearly enhanced as compared to the reactions with α-casein alone (**Figure 3A**, lanes 5–8). FA was more efficient in inducing α-casein cross-linking than *p*-CA, presumably because ThL can more readily oxidize FA than *p*-CA, as detected by the oxygen consumption tests and also previously reported by Selinheimo et al. (*39*). On the basis of the SEC chromatograms, the phenolic acids were consumed in the enzyme-catalyzed reactions and linked to α-casein, detected as a significant increase in the absorbance of the reaction products. Fluorescence measurements ($\lambda_{ex274}/\lambda_{em303}$) from the SEC fractions also showed that the amount of free tyrosine side chains decreased in the reactions (**Figure 4B**2). Furthermore, increase in the intensity of fluorescence at $\lambda_{ex318}/\lambda_{em410}$ suggested that FA was linked to the protein during the reaction (data not shown).

The influence of phenolic acids on protein cross-linking in the TrT-catalyzed reactions was different from that observed with ThL. TrT can oxidize *p*-CA but not FA (*32*). Hence, FA was not consumed in the reactions. However, when a high concentration of FA was used at the beginning of the reaction, cross-linking of α-casein was hindered (data not shown). The TrT-catalyzed reactions of α-casein in the presence of *p*-CA were found to vary, depending on the enzyme dosage. In the incubations with the two lowest enzyme dosages (0.005 and 0.05 nkat mg^{-1}), *p*-CA blocked α-casein cross-linking as detected in the SDS-PAGE analysis (**Figure 3B**, lanes 3 and 4 compared to lanes 6 and 7). However, with the highest enzyme dosage (0.5 nkat mg^{-1}), *p*-CA seemed to also induce formation of high molecular weight products, although, at the same time, the overall cross-linking of α-casein subunits was reduced to some extent (**Figure 3B**). Conjugation of *p*-CA to the polymerized α-casein subunits could be detected by fluorescence spectroscopy ($\lambda_{ex330}/\lambda_{em430}$) from the SEC fractions (data not shown). Fluorescence measurements at $\lambda_{ex274}/\lambda_{em303}$ from the SEC fractions (**Figure 5C2**) showed that the amount of unreacted Tyr side chains in the cross-linked α-casein and α-casein with *p*-CA were rather similar, suggesting that *p*-CA quinones formed in the TrT catalysis reacted with other amino acid side chains of α-casein rather than Tyr. Furthermore, only a small amount of *p*-CA reacted in the incubation, on the basis of the SEC analysis. There was no clear explanation for the low *p*-CA consumption by TrT, but it may have resulted from the consumption of all oxygen in the reaction solution or from the inhibition of tyrosinase activity by the reaction products.

Enzyme-Aided Coupling of hOSX to α-Casein. The ability of ThL and TrT to catalyze heteroconjugate formation between α-casein and hOSX was studied with three different concentration ratios of α-casein-hOSX mixtures: 10 to 2 mg mL^{-1}, 5 to 10 mg mL^{-1}, and 1 to 20 mg mL^{-1}.

When hOSX alone was incubated with ThL and TrT, higher molecular weight products were detected in SEC analyses by ThL (**Figure 1A**), but not by TrT (**Figure 1D**). The results were in accordance with the oxygen consumption measurements, which showed that only ThL was able to oxidize hOSX (**Figure 2**).

When the mixtures of α-casein and hOSX were incubated with ThL, an increase in the formation of high molecular weight compounds was detected in SEC (**Figure 1B**,**C**) and SDS-PAGE (**Figure 6A**) analyses. Furthermore, a double peak appeared in the SEC chromatogram at 8.4 mL (a shoulder prior to the main peak). A similar peak could also be observed in the ThL reactions with hOSX without any α-casein addition. However, in the α-casein−hOSX reactions, the double peak gave more intense absorbance than that obtained in the hOSX reactions. This double peak was never detected in reactions with α-casein alone and could therefore indicate the formation of α-casein−hOSX conjugates. Furthermore, the products eluting in SEC around

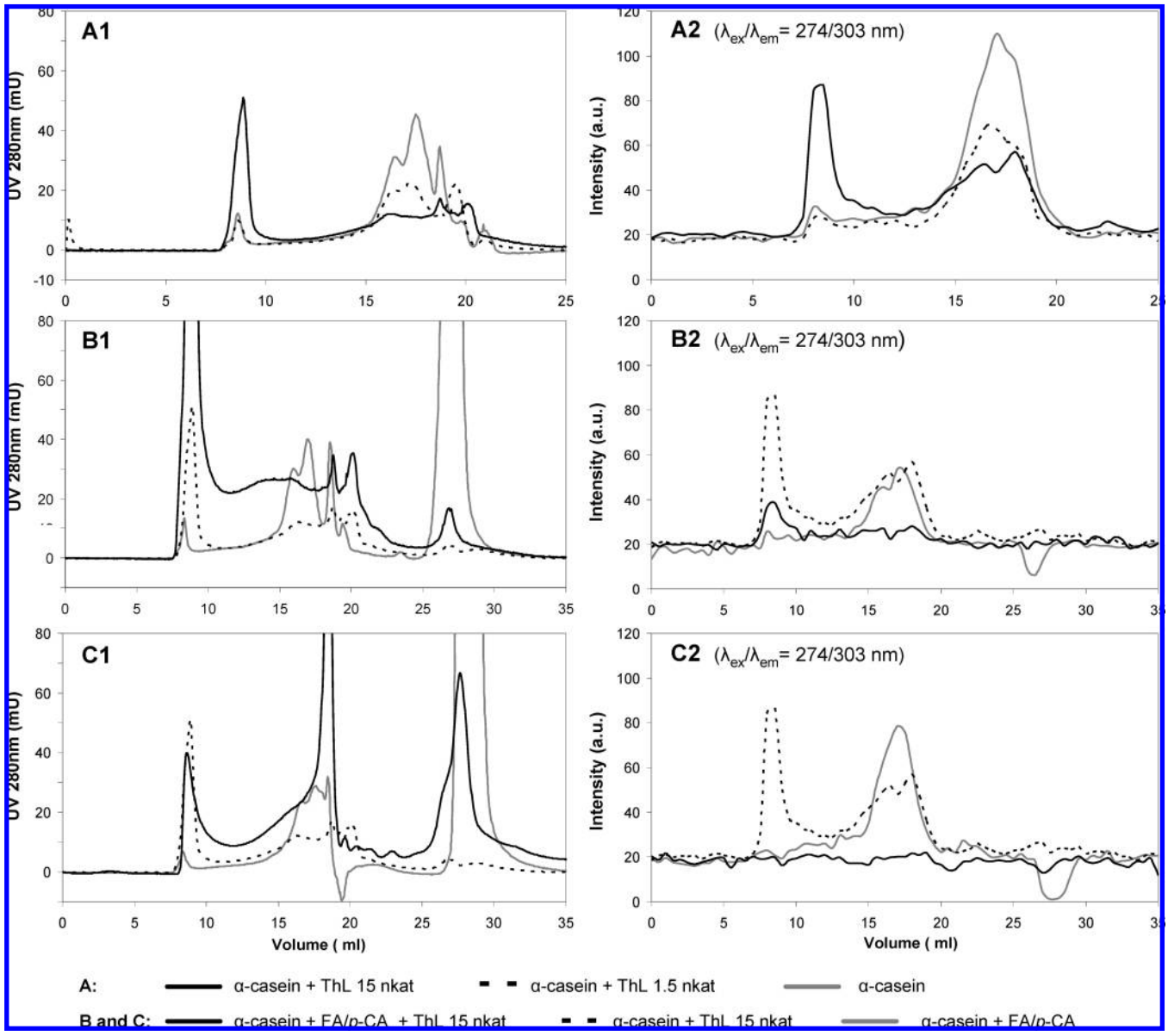


Figure 4. Size exclusion chromatograms for α-casein and phenolic acids after treatments with ThL: reactions with (**A**) α-casein (10 mg mL^{-1}), (**B**) with α-casein (10 mg mL^{-1}) in the presence of FA (1 mg mL^{-1}), and (**C**) with α-casein (10 mg mL^{-1}) in the presence of *p*-CA (1 mg mL^{-1}). A1, B1, and C1 show UV spectra (280 nm), and A2, B2, and C2 show Tyr-specific fluorescence spectra ($\lambda_{ex274nm}/\lambda_{em303nm}$) of the reaction mixtures. Dosing of ThL: 15 and 1.5 nkat mg^{-1} (**A**) and 15 nkat mg^{-1} (**B**, **C**).

13–15 mL from α-casein−hOSX reactions were probably the homo-cross-linked hOSX oligomers as seen in **Figure 1A**. These products were not detected in the ThL-treated α-casein−hOSX mixtures with ratios of 10 to 2 mg mL^{-1} (data not shown) and 5 to 10 mg mL^{-1} (**Figure 1C**). In the ThL-treated α-casein−hOSX mixture with a 1 to 20 mg mL^{-1} ratio, some intermediate size products were still present, probably due to the high hOSX concentration, which could lead to the partial homo-cross-linking of hOSX (**Figure 1B**). The enhancement of α-casein cross-linking in presence of hOSX was marked. Especially, with the ThL dosage of 1.5 nkat mg^{-1} hardly any direct α-casein cross-linking was observed; however, when hOSX was added to the reaction, cross-linking of α-casein clearly occurred (**Figure 6A**). Furthermore, the degradation of α-casein protein subunits seemed to decrease slightly in the presence of hOSX, as also observed in the ThL incubations of α-casein with FA and *p*-CA.

When the α-casein−hOSX mixture with a 1 to 20 mg mL^{-1} ratio was incubated with TrT, the formation of high molecular weight products was slightly enhanced (**Figure 1E**). However, when compared to the reactions without hOSX, the cross-linking of α-casein subunits was blocked as shown in the SDS-PAGE gel (**Figure 6D**, lanes 3 and 4 compared to lanes 7 and 8). When a higher concentration of hOSX was added to α-casein (10 to 5 mg mL^{-1}), a double peak similar to that observed in ThL reactions appeared in the SEC chromatogram (**Figure 1F**). However, both SEC and SDS-PAGE analyses showed that reactivity of α-casein was slightly reduced in the presence of hOSX compared to the control reactions (data not show). The double peak was not very clearly detectable in SEC with the α-casein−hOSX mixture of 1 to 20 mg mL^{-1}, and only a small shoulder was observed prior to the main product peak.

The heteroconjugate formation between α-casein and hOSX by ThL and TrT was further studied by SDS-PAGE with glycoprotein-specific staining (**Figure 6B**,**D**). The glycoprotein-specific staining did not stain α-casein or hOSX alone (**Figure 6B**,**D**, lanes 8–10). The color reaction, indicating the presence

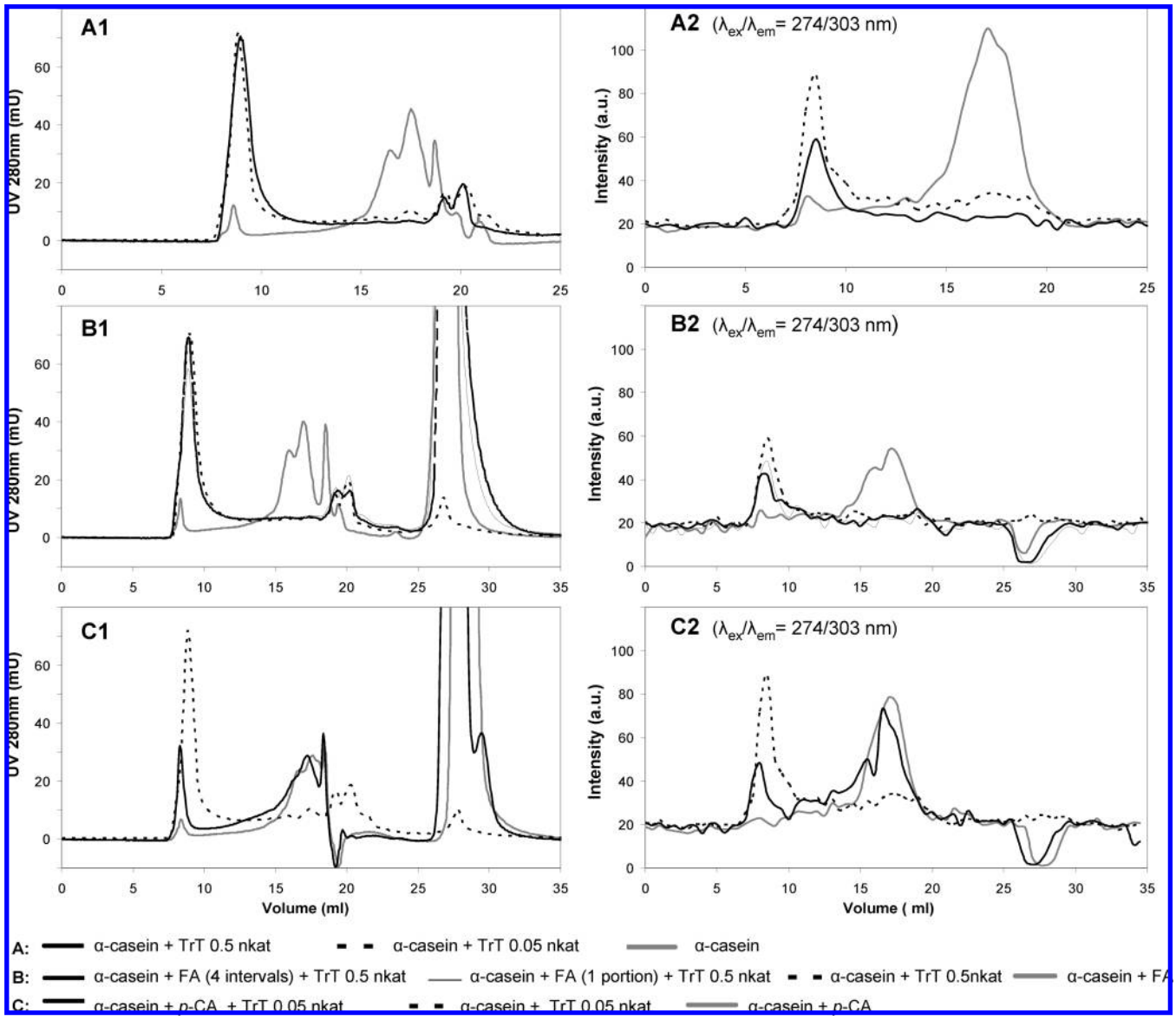


Figure 5. Size exclusion chromatograms for α-casein and phenolic acids after treatments with TrT: reactions with 10 mg mL^{-1} α-casein (**A**), with 10 mg mL^{-1} α-casein in the presence of 1 mg mL^{-1} FA (**B**), and with 10 mg mL^{-1} α-casein in the presence of 1 mg mL^{-1} *p*-CA (**C**). A1, B1, and C1 show UV (280 nm), and A2, B2, and C2 show Tyr-specific fluorescence $\lambda_{ex274nm}/\lambda_{em303nm}$. FA was added either at four intervals or in one portion (**B**). Dosing of TrT: 0.5 and 0.05 nkat mg^{-1} (**A**), 0.5 nkat mg^{-1} (**B**), and 0.05 nkat mg^{-1}(**C**).

of carbohydrates, occurred only in those lanes including samples with both α-casein and hOSX (**Figure 6B**,**D**, lanes 6 and 7). Although the control, that is, α-casein with hOSX with no enzyme, also showed some staining, an increase in the color intensity was detectable when either ThL or TrT was added to the reaction. With α-casein−hOSX mixtures of 5 to 10 mg mL^{-1} and 10 to 2 mg mL^{-1} ratios, the formation of heteroconjugation between proteins and carbohydrates was not clearly detected with the glycoprotein-specific staining method, most presumably because of the low concentration of hOSX.

DISCUSSION

Hetero-cross-linking of biopolymers by enzymatic means is an attractive tool to tailor the functional properties of the polymers. By enzymatic heteroconjugation of proteins and carbohydrates or functionalization of polymers with specific compounds, production of novel food formulations can be achieved. Because proteins and certain carbohydrates contain phenolic moieties, enzymes that are able to catalyze oxidation of the phenolic compounds, with subsequent formation of covalent conjugates, are interesting tools in a view of generating cross-linked structures of the biopolymers. Laccase and tyrosinase are enzymes that have potential in catalyzing these kinds of conjugation reactions as they can use various mono- and polyphenolic compounds as substrates. For instance, through covalent linking of plant-derived carbohydrates or small molecular weight bioactive phenolic compounds to meat or milk proteins, it could be possible to produce foods with altered textural or nutritional properties. In the present work, the ability of laccase and tyrosinase to catalyze the heteroconjugation between α-casein and hOSX, as well as between α-casein and phenolic acids was evaluated. The objective was to elucidate whether phenolic acids, ferulic acid, *p*-coumaric acid, and oat-derived xylan could be covalently linked to milk protein α-casein by the oxidative enzymes.

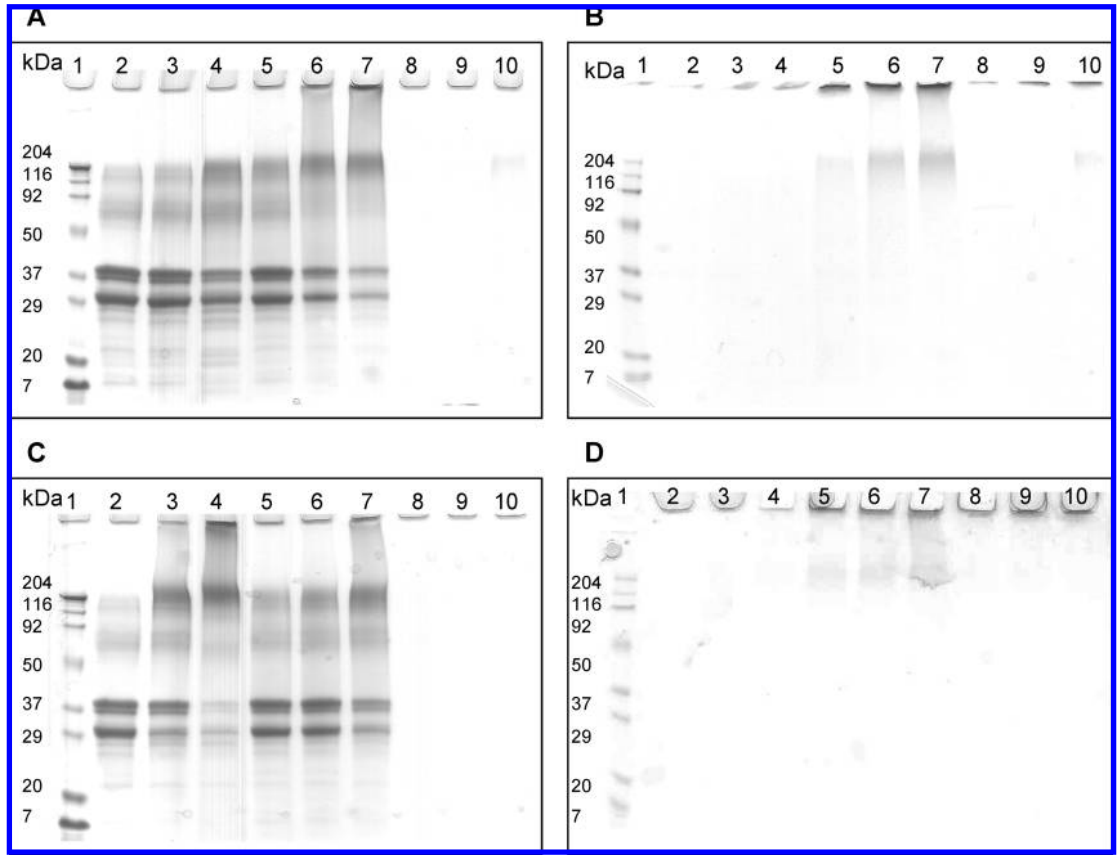


Figure 6. SDS-PAGE gels with protein (**A**, **C**) and glycoprotein-specific staining (**B**, **D**). ThL-catalyzed reactions with α-casein and/or hOSX are shown in **A** and **B** and TrT-catalyzed reactions in **C** and **D**. Lanes: 1, molecular weight marker; 2, α-casein; 3, α-casein + ThL (1.5 nkat mg^{-1}) or TrT (0.05 nkat mg^{-1}); 4, α-casein + ThL (15 nkat mg^{-1}) or TrT (0.5 nkat mg^{-1}); 5, α-casein−hOSX mixture; 6, α-casein−hOSX mixture + ThL (1.5 nkat mg^{-1}) or TrT (0.05 nkat mg^{-1}); 7, α-casein−hOSX mixture + ThL (15 nkat mg^{-1}) or TrT (0.5 nkat mg^{-1}); 8, hOSX; 9, hOSX + ThL (1.5 nkat mg^{-1}) or TrT (0.05 nkat mg^{-1}); 10, hOSX ThL (15 nkat mg^{-1}) or TrT (0.5 nkat mg^{-1}). Concentrations of α-casein and hOSX were 1 and 20 mg mL^{-1}, respectively. Dosing of enzymes is given per mg of α-casein.

Laccase. ThL was directly able to cross-link α-casein. Previously laccase has been reported to cross-link specific milk and cereal proteins (*19–21, 38*). The ThL-mediated α-casein cross-linking was clearly enhanced in the presence of exogenous phenolic acids, as well as in the presence of hOSX, which contains FA and *p*-CA. The results suggest that the phenolic acids acted as bridging agents in the cross-linking of α-casein proteins. The proposed mechanism is that ThL-mediated cross-linking occurred via the oxidation of the phenolic acids and of Tyr side chains in the protein, with subsequent radical reactions and heteroconjugate formation (**Figure 7A**).

On the basis of the fluorescence spectroscopy measurements, the amount of free Tyr residues decreased, when α-casein was treated with ThL. There were also indications that FA and *p*-CA were grafted to α-casein. The results suggest that FA- and *p*-CA-derived phenoxyl radicals reacted with the Tyr residues of α-casein. Hetero-cross-linking of α-casein and hOSX could have occurred via the hOSX-bound phenolics by the same radical-mediated means, as proposed for FA and *p*-CA reactions. Previously, Mattinen et al. (*18*) have shown that the ThL-catalyzed reactions with FA and the tripeptide Gly-Leu-Tyr may lead to the formation of FA−Tyr conjugates. Peroxidases have also been reported to catalyze FA−Tyr conjugation (*27, 28*). Moreover, it has been observed by Mattinen et al. (*39*) that ThL can also oxidize tryptophan and cysteine, although clearly less efficiently than Tyr. Then again, oxidation of FA has been reported to lead to the oxidation of free SH groups in protein (*40, 41*). Hence, the oxidation reactions of these amino acid residues might also have participated in protein cross-linking and conjugation reactions with phenolic acids. Phenolic acids might also have acted as mediators in the ThL-catalyzed reactions. It is known that certain low molecular weight compounds oxidized by laccase act as redox mediators, oxidizing compounds that theoretically are not substrates of laccase because of their low redox potential (*42, 43*). It has also been proposed that mediators could also circulate and act on specific sites of a hypothetical substrate, which would be inaccessible to the enzyme itself (*42, 43*).

Furthermore, in the ThL-catalyzed reactions in which FA, *p*-CA, and hOSX were conjugated to α-casein, less degradation of α-casein proteins occurred, in comparison with reactions lacking phenolic acids or hOSX. Although the finding suggests that the heteroconjugation synergistically induces protein cross-linking and blocks degradation, there is no clear explanation for the result. However, the low molecular weight products in the ThL reactions presumably resulted from radical-mediated protein degradation (*11–13*), which has been previously reported for chicken breast myofibril proteins by Lantto et al. (*44*).

Tyrosinase. Tyrosinase has been reported to cross-link cereal, milk, and meat proteins (*38, 45–47*), and efficient cross-linking of α-casein was also observed in the present study. However, in contrast to the increase in α-casein cross-linking in ThL reactions, the presence of FA, *p*-CA, or hOSX inhibited α-casein cross-linking in the TrT-catalyzed reactions. Previously, Thalmann and Lötzbeyer (*48*) reported that lysozyme, α-lactalbumin, and β-lactoglobulin proteins were cross-linked by tyrosinase

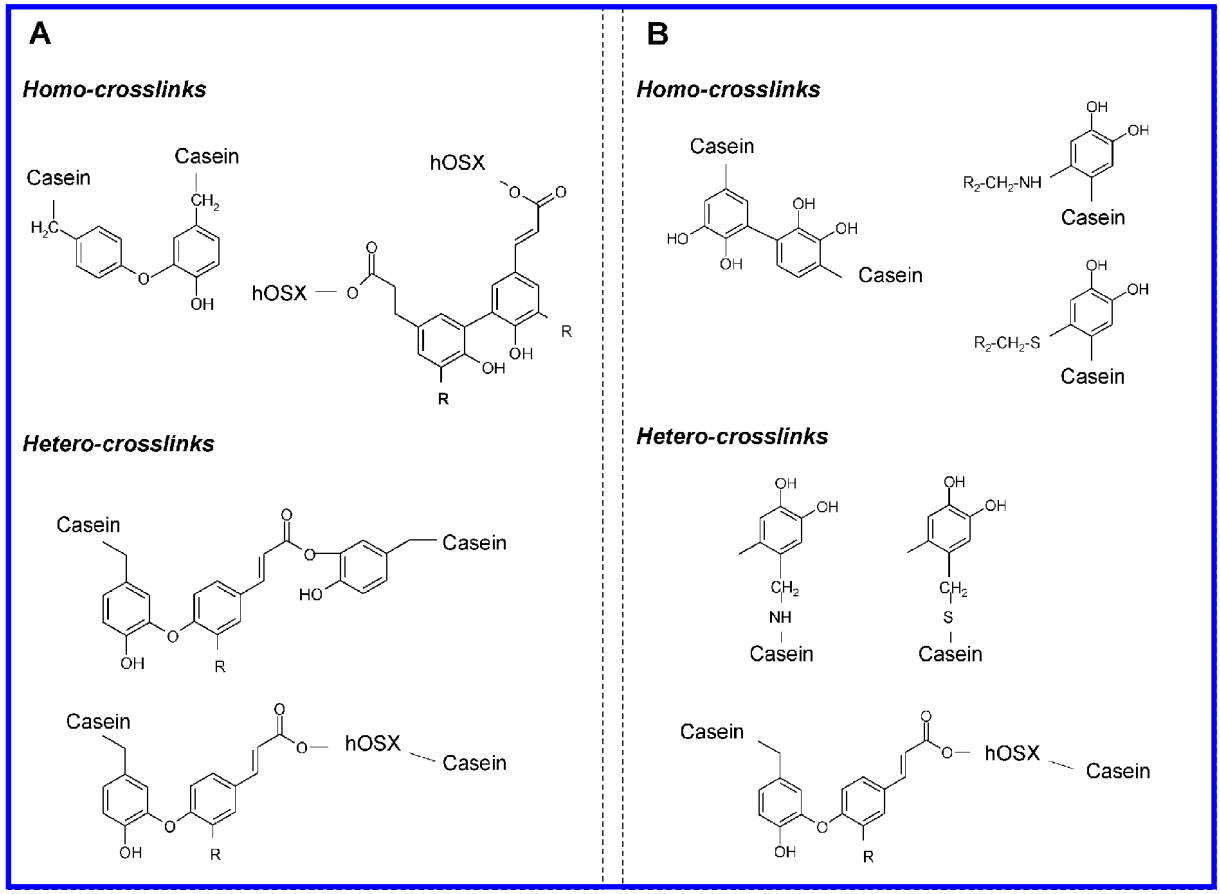


Figure 7. Schematic presentation of the possible cross-links formed in α-casein (homo-cross-links) and between α-casein and *p*-CA or FA (hetero-cross-links), and between α-casein and hOSX (hetero-cross-links) by ThL (**A**) and TrT (**B**). R in the aromatic ring structure represents either hydrogen or a methoxy group.

from *Agaricus bisporus* predominantly in the presence of low molecular weight phenolic compounds. However, in this study the cross-linking efficiency of TrT seemed to be reduced when phenolic acids were added to the reaction mixture. Similarly, Selinheimo et al. (*45*) observed that addition of diphenolic L-dopa hindered α-casein cross-linking by TrT. The mechanism by which phenolic compounds blocked protein cross-linking in TrT reactions is not clear, but it may be related to the different affinity of TrT toward Tyr side chains of protein and phenolic acids.

The inhibition mechanism of FA in α-casein cross-linking could be competitive, because FA could have acted as a substrate analogue, as also proposed by Cheng et al. (*49*). Although not oxidized by TrT, FA may have higher affinity to the active site of TrT than the Tyr side chains of α-casein. The hydroxyl group of FA may also have acted as a nucleophile, with subsequent grafting of FA to the Tyr-derived quinones in α-casein (*50*) and, thus, blocking the further conjugation of Tyr-derived quinones to the other reactive side chains of the protein. *p*-CA, similarly to FA, may also have competed with the Tyr of α-casein in the active site of TrT, if *p*-CA has a higher affinity to the active site. However, *p*-CA was found to both hinder and enhance α-casein cross-linking, depending on the enzyme dose. It should be noted that in the tyrosinase-catalyzed reactions there is typically a lag period prior to the oxidation reaction of monophenols. Resting tyrosinase enzyme consists of 85–90% of the so-called *met*-form, which can perform only the catalysis of diphenols, and only 10–15% of enzyme is in the so-called *oxy*-form, which is also capable of catalyzing monophenol oxidation (*51*). During the lag period, the *oxy*-form of tyrosinase is generated from the *met*-form by an indirect diphenol accumulation in the catalysis. After the enzyme is totally transferred to the *oxy*-form, the rate of monophenol oxidation reaches the maximum (*48, 53*). Therefore, the monophenols studied in this work, *p*-CA and Tyr side chains of protein, can be oxidized only by the *oxy*-form of tyrosinase, and a lag period is, thus, expected. On the basis of the observations, in particular from the oxygen consumption assay, protein-bound Tyr seemed to expedite the conversion of TrT from the *met*-form to the *oxy*-form, whereas *p*-CA retarded this transformation. It has been hypothesized that if a tyrosinase has a high affinity for a monophenolic substrate, the *met*-tyrosinase becomes saturated by the substrate (*54*). Therefore, the transformation from the *met*-form to the *oxy*-form would be delayed, as most of the enzyme would be in the so-called dead-end complex (*54*). This might partly explain the observed decrease in α-casein cross-linking in the presence of *p*-CA. Moreover, if TrT preferred *p*-CA over Tyr, the overriding oxidation of *p*-CA could have hindered the oxidation of the tyrosyl residues in α-casein, leading primarily to the *p*-CA-related catalysis, instead of protein cross-linking. However, because quinones have been reported to react with the nucleophilic side chains of proteins or amino acids, such as sulfhydryl, amine, amide, indole, and imidazole substituents (*5, 7–10*), the reactive quinones resulting from *p*-CA oxidation could have reacted nonenzymatically to these reactive side groups in α-casein, thus blocking the sites for further cross-linking of α-casein subunits (**Figure 7B**). Indications that grafting of *p*-CA to protein had occurred were detected in the SEC and the fluorescence analyses. With high TrT dosage, *p*-CA was also found to induce high molecular weight protein

formation, suggesting that *p*-CA also acted as a bridging agent in α-casein cross-linking as in the ThL reactions. A similar hypothesis was previously made by Thalmann and Lötzbeyer (*48*).

As with FA and *p*-CA, α-casein cross-linking by TrT was also blocked in the presence of hOSX. However, hetero-cross-link formation between α-casein and hOSX was detected, even though hOSX was not oxidized by TrT. Even though TrT can oxidize *p*-CA, it may not be able to oxidize OSX-bound *p*-CA, for instance, because of steric hindrance. Because TrT was not able to oxidize hOSX, TrT-catalyzed heteroconjugate formation was rather unexpected. However, the hOSX-bound phenolic acids may have had functioned as nucleophiles and reacted with the Tyr-derived quinones of α-casein, leading to the hetero-conjugation (*50*) (**Figure 7B**).

In conclusion, both ThL and TrT were able to catalyze oxidative conjugation of α-casein and carbohydrates. However, there were notable differences between the oxidative enzymes in their ability to catalyze hetero-cross-link formation. Radical- and quinone-mediated protein cross-linking differed, as indicated by enhancement of cross-linking by phenolic acids with ThL, but inhibition with TrT. Glycoprotein-specific staining of SDS-PAGE separated products was suitable for the detection of hetero-cross-linked structures, although relatively high hOSX concentrations were needed. To achieve adequate and efficient heteroconjugation by the oxidative enzymes, studying different kinds of phenolic substrates and biopolymers and optimizing the reaction conditions are prerequisite. Furthermore, characterization of the physicochemical and physiological properties of the heterobiopolymers and identification of the chemistry of the linkages of the hetero-cross-links should enable a range of potential substrates for each enzyme and the benefits of ThL and TrT to be identified.

ABBREVIATIONS USED

AX, arabinoxylan; BsX, *Bacillus subtilis* xylanase; di-Tyr, dityrosine; FA, ferulic acid; L-Tyr, L-tyrosine; OSX, oat spelt xylan; hOSX, hydrolyzed oat spelt xylan; *p*-CA, *p*-coumaric acid; L-dopa, 3,4-dihydroxy-L-phenylalanine; SEC, size exclusion chromatography; TLC, thin layer chromatography; ThL, *Trametes hirsuta* laccase; TrT, *Trichoderma reesei* tyrosinase.

ACKNOWLEDGMENT

The skillful technical assistance of Airi Hyrkäs is acknowledged.

LITERATURE CITED

(1) Chen, T.; Small, D. A.; Wu, L.-Q.; Rubloff, G. W.; Ghodssi, R.; Vazquez-Duhalt, R.; Bentley, W. E.; Payne, G. F. Nature-inspired creation of protein−polysaccharide conjugate and its subsequent assembly onto a patterned surface. *Langmuir* **2003**, *19*, 9382–9386.

(2) Åberg, C. M.; Chen, T. H.; Olumide, A.; Raghavan, S. R.; Payne, G. F. Enzymatic grafting of peptides from casein hydrolysate to chitosan. Potential for value-added byproducts from food-processing wastes. *J. Agric. Food Chem.* **2004**, *52*, 788–793.

(3) Freddi, G.; Anghileri, A.; Sampaio, S.; Buchert, J.; Monti, P.; Taddei, P. Tyrosinase-catalyzed modification of *Bombyx mori* silk fibroin: grafting of chitosan under heterogeneous reaction conditions. *J. Biotechnol.* **2006**, *125*, 281–294.

(4) Lule, S. U.; Xia, W. Food phenolics, pros and cons: a review. *Food Rev. Int.* **2005**, *21*, 367–388.

(5) Bittner, S. When quinones meet amino acids: chemical, physical and biological consequences. *Amino Acids* **2006**, *30*, 205–224.

(6) Lerch, K. Neurospora tyrosinase: structural, spectroscopic and catalytic properties. *Mol. Cell. Biochem.* **1983**, *52*, 125–138.

(7) McDowell, L. M.; Burzio, L. A.; Waite, J. H.; Schaefer, J. Rotational echo double resonance detection of cross-links formed in mussel byssus under high-flow stress. *J. Biol. Chem.* **1999**, *274*, 20293–20295.

(8) Ito, S.; Kato, T.; Shinpo, K.; Fujita, K. Oxidation of tyrosine residues in proteins by tyrosinase. *Biochem. J.* **1984**, *222*, 407–411.

(9) Burzio, L. A. Cross-linking in adhesive quinoproteins: studies with model decapeptides. *Biochemistry* **2000**, *39*, 11147–11153.

(10) Takasaki, S.; Kawakishi, S. Formation of protein-bound 3,4-dihydroxyphenylalanine and 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine as new cross-linkers in gluten. *J. Agric. Food Chem.* **1997**, *45*, 3472–4375.

(11) Thurston, C. The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19–26.

(12) Claus, H. Laccases and their occurrence in prokaryotes. *Arch. Microbiol.* **2003**, *179*, 145–150.

(13) Kersten, P. J.; Kalyanaraman, B.; Hammel, K. E.; Reinhammar, B.; Kirk, T. K. Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. *Biochem. J.* **1990**, *268*, 475–480.

(14) Figueroa-Espinoza, M. C.; Morel, M. H.; Surget, A.; Rouau, X. Oxidative cross-linking of wheat arabinoxylans by maganese peroxidase. Comparison with laccase and horseradish peroxidase. Effect of cysteine and tyrosine gelation. *J. Sci. Food Agric.* **1999**, *79*, 460–463.

(15) Figueroa-Espinoza, M. C.; Rouau, X. Oxidative cross-linking of pentosans by a fungal laccase and horseradish peroxidase: mechanism of linkage between feruloylated arabinoxylans. *Cereal Chem.* **1998**, *75*, 259–265.

(16) Labat, E.; Morel, M. H.; Rouau, X. Effect of laccase and ferulic acid on wheat flour dough during mixing. *Cereal Chem.* **2000**, *77*, 823–828.

(17) Labat, E.; Morel, M. H.; Rouau, X. Effect of laccase and maganase peroxidase on wheat gluten and pentosans during mixing. *Food Hydrocolloids* **2001**, *15*, 47–52.

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

(19) Færgemand, M.; Otte, J.; Qvist, K. B. Cross-linking of whey proteins by enzymatic oxidation. *J. Agric. Food Chem.* **1998**, *46*, 1326–1333.

(20) Shotaro, Y. Method for cross-linking protein by using enzyme. U.S. Patent 6,121,013, 1999.

(21) Mattinen, M.-L.; Hellman, M.; Permi, P.; Autio, K.; Kalkkinen, N.; Buchert, J. The effect of protein structure on laccase-catalyzed protein oligomerization. *J. Agric. Food Chem.* **2006**, *54*, 8883–8890.

(22) Buchert, J.; Selinheimo, E.; Kruus, K.; Mattinen, M.-L.; Lantto, R.; Autio, K. Cross-linking enzymes in food processing. In *Novel Enzyme Technology for Food Applications*; Rastall, R., Ed.; Woodhead Publishing: Cambridge, U.K., 2007; 336 pp.

(23) Ganzevles, R. A.; Zinoviadou, K.; van Vliet, T.; Cohen Stuart, M. A.; de Jongh, H. H. J. Modulating surface rheology by electrostatic protein/polysaccharide interactions. *Langmuir* **2006**, *22*, 10089–10096.

(24) Kato, A.; Minaki, K.; Kobayashi, K. Improvement of emulsifying properties of egg white proteins bythe attachment of polysaccharide through maillard reaction in a dry state. *J. Agric. Food Chem.* **1993**, *41*, 540–543.

(25) Dickinson, E. Enzymatic crosslinking as a tool for food colloid rheology control and interfacial stabilization. *Trends Food Sci. Technol.* **1997**, *8*, 334–339.

(26) Kato, A.; Wada, T.; Kobayashi, K.; Seguro, K.; Motoki, M. Ovomucin-food protein conjugates prepared through the transglutaminase reaction. *Agric. Biol. Chem.* **1991**, *55*, 1027–1031.

(27) Oudgenoeg, G.; Hilhorst, R.; Piersma, S. R.; Boeriu, C. G.; Gruppen, H.; Hessing, M.; Voragen, A. G. J.; Laane, C. Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *J. Agric. Food Chem.* **2001**, *49*, 2503–2510.

(28) Oudgenoeg, G.; Dirksen, E.; Ingemann, S.; Hilhorst, R.; Gruppen, H.; Boeriu, C. G.; Piersma, S. R.; Van Berkel, W. J. H.; Laane, C.; Voragen, G. J. Horseradish peroxidase-catalysed oligomerization of ferulic acid on a template of a tyrosine-containing tripeptide. *J. Biol. Chem.* **2002**, *277*, 21332–21340.

(29) Boeriu, C. G.; Oudgenoeg, G.; Spekking, W. T. J.; Berendsen, L. B. J. M.; Vancon, L.; Boumans, H.; Gruppen, H.; Van Berkel, W. J. H.; Laane, C.; Voragen, A. G. J. Horseradish peroxidase-catalyzed cross-linking of feruloylated arabinoxylans with β-casein. *J. Agric. Food Chem.* **2004**, *52*, 6633–6639.

(30) Rittstieg, K.; Suurnäkki, A.; Suortti, T.; Kruus, K.; Guebitz, G.; Buchert, J. Investigations on the laccase-catalyzed polymerization of lignin model compounds using size-exclusion HPLC. *Enzyme Microb. Technol.* **2002**, *31*, 403–410.

(31) Niku-Paavola, M.-L.; Karhunen, E.; Salola, P.; Raunio, V. Ligninolytic enzymes of the white-rot-fungus *Phlebia ferulic*. *Biochem. J.* **1988**, *254*, 877–884.

(32) Selinheimo, E.; Saloheimo, M.; Ahola, E.; Westerholm-Parvinen, A.; Kalkkinen, N.; Buchert, J.; Kruus, K. Production and characterization of a secreted, C-terminally processed tyrosinase from the filamentous fungus Trichoderma reesei. *FEBS J.* **2006**, *273*, 4322–4335.

(33) Robb, D. A. Tyrosinase. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press: Boca Raton, FL, 1984; Vol. 2, pp 207–240.

(34) Bailey, M. J.; Biely, P.; Poutanen, K. Interlaboratory testing methods for assay of xylanase activity. *J. Biotechnol.* **1992**, *23*, 257–270.

(35) Mattila, P.; Pihlava, J.-M.; Hellström, J. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *J. Agric. Food Chem.* **2005**, *53*, 8290–8295.

(36) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(37) Zacharius, R. M.; Zell, T. E.; Morrison, J. H.; Woodlock, J. J. Glycoprotein staining following electrophoresis on acrylamide gel. *Anal. Biochem.* **1969**, *30*, 148–152.

(38) Selinheimo, E.; Kruus, K.; Buchert, J.; Autio, K. Elucidating the mechanism of laccase and tyrosinase in wheat breadmaking. *J. Agric. Food Chem.* **2007**, *55*, 6357–6365.

(39) Mattinen, M.-L.; Hellmann, M.; Permi, P.; Autio, K.; Kalkkinen, N.; Buchert, J. Effect of Protein Structure on Laccase-Catalyzed Protein Oligomerization. *J. Agric. Food Chem.* **2006**, *54*, 8883–8890.

(40) Figueroa-Espinoza, M-C.; Morel, M.-H.; Rouau, X. Effect of lysine, tyrosine, cysteine, and glutathione on the oxidative cross-linking of feruloylated arabinoxylans by a fungal laccase. *J. Agric. Food. Chem.* **1998**, *46*, 2583–2589.

(41) Labat, E.; Morel, M. H.; Rouau, X. Effects of laccase and ferulic acid on wheat flour doughs. *Cereal Chem.* **2000**, *77*, 823–828.

(42) Bourbonnais, R.; Paice, M. G.; Freiermuth, B.; Bodie, E.; Borneman, S. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl. Environ. Microbiol.* **1997**, *63*, 4627–4632.

(43) Camarero, S.; Ibarra, D.; Martínez, A. T.; Romero, J.; Gutiérrez, A.; del Río, J. C. Paper pulp delignification using laccase and natural mediators. *Enzyme Microb. Technol.* **2007**, *40*, 1264–1271.

(44) Lantto, R.; Puolanne, E.; Kalkkinen, K.; Buchert, J.; Autio, K. Enzyme-aided modification of chicken breast myofibril proteins: effect of laccase and transglutaminase on gelation and thermal stability. *J. Agric. Food Chem.* **2005**, *53*, 9231–9237.

(45) Selinheimo, E.; NiEidhin, D.; Steffensen, C.; Nielsen, J.; Lomascolo, A.; Halaouli, S.; Record, E.; O'Beirne, D.; Buchert, J.; Kruus, K. Comparison of the characteristics of fungal and plant tyrosinases. *J. Biotechnol.* **2007**, *130*, 471–480.

(46) Lantto, R.; Puolanne, E.; Kruus, K.; Buchert, J.; Autio, K. Tyrosinase-aided protein cross-linking: effects on gel formation of chicken breast myofibrils and texture and water-holding of chicken breast meat homogenate gels. *J. Agric. Food Chem.* **2007**, *55*, 1248–1255.

(47) Mattinen, M.; Lantto, R.; Selinheimo, E.; Kruus, K.; Buchert, J. Oxidation of peptides and proteins by *Trichoderma reesei* and *Agaricus bisporus* tyrosinases. *J. Biotechnol.* **2007**, *133*, 395–402.

(48) Thalmann, C.; Lötzbeyer, T. Enzymatic cross-linking of proteins with tyrosinase. *Eur. Food Res. Technol.* **2002**, *214*, 276–281.

(49) Cheng, T.; Wenyuan, Z.; Yan, L. Aloin, cinnamic acid and sophorcarpidine are potent inhibitors of tyrosinase. *Chin. Med. J.* **2002**, *115*, 18591862.

(50) Sayre, L. M.; Nadkarni, D. V. Direct conversion of phenols to *o*-quinones by copper(I) dioxygen—questions regarding the monophenolase activity of tyrosinase mimics. *J. Am. Chem. Soc.* **1994**, *116*, 3157–3158.

(51) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. Multicopper oxidases and oxygenases. *Chem. Rev.* **1996**, *96*, 2563–2606.

(52) Cooksey, C. J.; Garratt, P. J.; Land, E. J.; Pavel, S.; Ramsden, C. A.; Riley, P. A.; Smit, N. P. Evidence of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase. *J. Biol. Chem.* **1997**, *272*, 26226–35.

(53) Land, E. J.; Ramsden, C. A.; Riley, P. A. Quinone chemistry and melanogenesis. *Methods Enzymol.* **2004**, *378*, 88–109.

(54) Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Study of stereospecificity in mushroom tyrosinase. *Biochem. J.* **1998**, *331*, 547–551.

Received for review October 19, 2007. Revised manuscript received February 18, 2008. Accepted February 20, 2008. This study has been carried out with financial support from the Research Foundation of Raisiogroup (Raisio, Finland). We also acknowledge the Finnish Funding Agency for Technology and Innovation (Tekes) for financing the project "Tailored nano-stabilizers for biocomponent interfaces".

JF0730791