JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 9231−9237 **9231**

Enzyme-Aided Modification of Chicken-Breast Myofibril Proteins: Effect of Laccase and Transglutaminase on Gelation and Thermal Stability

R. LANTTO,*,† E. PUOLANNE,‡ N. KALKKINEN,§ J. BUCHERT,† AND K. AUTIO†

VTT Biotechnology, Post Office Box 1500, 02044 VTT, Finland, Food Technology, Post Office Box 66, 00014 University of Helsinki, Helsinki, Finland, and Institute of Biotechnology, Post Office Box 65, 00014 University of Helsinki, Helsinki, Finland

The effect of laccase and transglutaminase (TG) on cross-linking, gelation, and thermal stability of salt-soluble chicken-breast myofibril proteins was investigated at pH 6. Both enzymes modified the protein pattern detected by SDS−PAGE. Identification of proteins by peptide mass mapping showed that myosin heavy chain (MHC) and troponin T were the most affected proteins. These proteins faded or disappeared as a function of the incubation time with both enzymes on SDS−PAGE. The molecular weight of actin was not, however, affected by either enzyme. The effects that the enzymes had on the gel formation of chicken-breast myofibrils were studied in 0.35 and 0.60 M NaCl solutions at 3% protein content and a constant temperature of 40 °C by using a small deformation viscoelastic measurement. TG substantially increased the storage modulus (G′) of 3% protein in 0.35 M NaCl. Without the enzymes, gelation was insignificant in 0.35 M NaCl. The increased solubility of the proteins at 0.60 M NaCl intensified gelation with TG. G′ increased 32 and 64% at dosages of 10 and 100 nkat of TG, respectively. Also, laccase increased G′ of the gel in 0.60 M salt concentration. However, a high laccase dosage decreased the magnitude of G′ below the control level. Differential scanning calorimetric (DSC) measurements indicated slightly reduced myosin heat stability after TG pretreatment and increased actin heat stability with both enzymes. Maximum transition temperatures did not alter with either enzyme.

KEYWORDS: Chicken myofibril proteins; protein modification; cross-linking; transglutaminase; laccase; gelation; storage modulus; isothermal heating; heat stability

1. INTRODUCTION

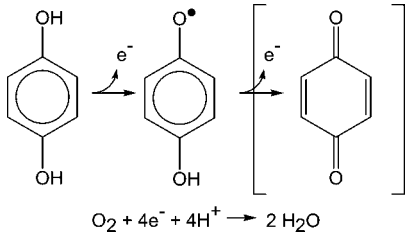
Myofibril proteins are responsible for the formation of a gel network and thus greatly affect the textural characteristics of meat products. In addition to the type and quantity of proteins, their solubility, pH, and temperature affect gel formation and the viscoelastic properties of the gel (*1*, *2*). Myofibril proteins undergo unfolding and aggregation before they can form a three-dimensional protein network. Denaturation of myofibril proteins can be monitored by endothermic transitions and development of the protein network by a viscoelastic measurement of storage (G′) and loss (G″) moduli. The effects of pH, protein concentration, and heating conditions on unfolding, aggregation, and gelation of poultry muscle myofibril proteins have been thoroughly reviewed (*1*, *3*). The structure formation and water-holding capacity (WHC) of meat systems are greatly dependent upon pH values. Above the average isoelectric point (approximately pH 5.0) of the muscle proteins, the WHC increases (*4*) and intact myofibrils in meat swell as a function of increasing pH. According to Lesiów and Xiong (*5*), gelling of isolated chicken myofibril proteins is optimal at pH 6. In isolated myofibrils pH-dependent swelling leads to protein extraction. According to Xiong and Brekke (*6*), the extractability of salt-soluble chicken-breast myofibril proteins improved visibly when the pH was changed from 5.75 to 6.00.

In addition to the pH, the functional properties of meat products are enhanced by adding salt to the system. NaCl plays an essential role in the swelling of myofibrils and the solubilization of myofibril proteins. Cl^- ions penetrate the myofilaments and cause them to swell (*4*). Cl^- ions result in an increased negative net charge, causing repulsive forces between the molecules of of the filament shaft and consequently swelling or disintegration of the filament. The negative net charge of myofilaments attracts positive ions close to the filament surfaces. The Na^+ cloud thus formed around the myofilaments results in local concentration changes (*7*, *8*) leading to an increased osmotic pressure inside the myofibrils and thus a swelling of the matrix. Offer and Trinick (*9*) have shown that increasing the NaCl concentration up to 1 M increases swelling. However, myofibrils do not swell indefinitely because of resistance forces,

* To whom correspondence should be addressed Telephone: +358-20-722-4411. Fax: +358-20-722-7071. E-mail: raija.lantto@vtt.fi.
† VTT Biotechnology.
‡ Food Technology, University of Helsinki.
§ Institute of Biotechnology, University of Helsinki.

10.1021/jf051602a CCC: $30.25
Published on Web 10/12/2005

Scheme 1. Laccase-Catalyzed Oxidation of a Diphenol



e.g., van der Waals forces and covalent cross-links in the protein (*10*). A comprehensive review on the effect of NaCl on meat was recently published by Ruusunen and Puolanne (*8*).

Enzymes that stabilize proteins by forming additional covalent cross-links may be used to fabricate meat products with better texture despite low salt or low protein content. Currently, transglutaminases (TG, glutaminylpeptide:amine γ-glutamyltransferase, EC 2.3.2.13) that originated from the *Streptomyces* bacterium genus are the only commercially available enzymes for cross-linking proteins and for improving the texture of meat products. The potential of TG has been intensively studied in meat processing to improve texture (*11*) and gelling (*12*). In cooked products, consistency can be positively affected by TG (*13*), although with a concomitant increase in cooking loss (*13*). TG catalyzes an acyl transfer reaction between a protein- or peptide-bound glutamine residue and a primary amine, which is an ϵ-amino group of a lysine residue in a protein system. This covalent ϵ (γ-glutamyl) lysine isopeptide bond can be formed inter- or intramolecularly in actin and myosin (*14*), actomyosin (*15*), and collagen (*16*). The effects of TGs in food protein matrixes have been reviewed by, e.g., Kuraishi et al. (*17*).

Oxidation by chemical agents is known to induce protein polymerization (*18*) and protein unfolding (*19*), thus affecting the texture and stability of protein gels. ϵ-Amino groups of lysine residues are oxidized to carbonyls, and SH groups of cysteine residues are cross-linked to disulfides (*20*). Enzyme-aided oxidation of proteins is also an attractive approach. Polyphenol oxidases, lipoxygenase, and enzymes acting on sulfhydryl groups and disulfide bonds (*21*, *22*) are all oxidative enzymes capable of catalyzing covalent cross-links in proteins.

Laccases (EC 1.10.3.2) are radical-forming polyphenol oxidases. They catalyze the oxidation of various phenol compounds, simultaneously reducing molecular oxygen to water. Laccases are mainly found in plants and fungi, and their role in nature and their most common application are in the synthesis of lignin (*23*, *24*). As shown in **Scheme 1**, laccase catalyzes an electron-transfer reaction of a diphenol to form an oxygen-centered free radical. The mechanism of laccase-catalyzed oxidation of tyrosine-containing peptides has been proposed (*25*) to proceed via generation of radicals in the hydroxyl group of the phenolic ring, with concomitant generation of a semiquinone and rapid delocalization of the radical into the different positions of the aromatic ring. Isodityrosine bonds were formed when hydroxyl and tyrosyl radicals located in different molecules reacted with each other. The capability of laccases to oxidize a wide variety of phenolic and other substrates has raised interest in studying their application to different sources of protein (*26*−*30*). Heldt-Hansen (*30*) has reported on the *Myceliophthora thermophila* laccase-induced decrease of free thiol groups in a gluten dough, indicating oxidation of available cysteine residues to disulfide bridges. However, there is no comprehensive view about which amino acid residues may act as substrates for laccases and between which amino acids laccases are capable of catalyzing a covalent bond. At present, the reaction mechanism of protein modification as a consequence of laccase-catalyzed oxidation and radical formation is poorly understood. However, it should be emphasized that, e.g., ionizing radiation of proteins in solution involves the formation of radical species from proteins and water (*31*). It is also known that at least oxygen radicals derived from water also cause protein fragmentation and amino acid modifications in addition to polymerization and aggregation (*32*). On the basis of the current knowledge on the mechanism of laccase catalysis, it appears probable that the laccase-catalyzed radicals act in a similar way.

The aim of the present study was to elucidate the potential of laccase in modifying chicken-breast muscle myofibril proteins. Also, the consequences of the enzyme-aided modification on gel formation and the thermal stability of chicken-breast meat myofibrils were studied. The effects of laccase were compared to those of a well-known TG.

2. MATERIALS AND METHODS

2.1. Enzymes. Laccase from a filamentous fungus *Trametes hirsuta* has been purified to a high degree of purity and previously characterized at VTT Biotechnology (*33*). Laccase activity was assayed according to Leonowicz and Grzywnowicz (*34*) at pH 6 using 0.5 mM syringaldazine (Sigma Chemical Co., St. Louis, MO) as the substrate. TG was fractionated free of maltodextrin from the commercial TG product Activa WM purchased from Vesantti Oy (Helsinki, Finland). Purification was carried out by cation-exchange chromatography. Before chromatography, the enzyme preparation was dissolved in a 30 mM sodium-acetate buffer, with the pH adjusted to 5.5, and filtered through a Whatman GF/A glass fiber filter (Whatman International Ltd., Maidstone, U.K.). The enzyme solution was applied to a CM Sepharose FF (Pharmacia BP 252, Pharmacia, Uppsala, Sweden) column and eluted with a buffer gradient of 30 mM to 500 mM sodium-acetate at pH 5.5 and a flow rate of 6 mL/h. After concentration by ultrafiltration (Prep/Scale-TFF, cut off 10 000, Millipore, Bedford, MA), the TG activity (*35*) of the preparation was determined using 0.04 M *N*-carbobenzoxy-L-glutaminyl-glycine (Sigma) as the substrate at pH 6. Endoproteinase activity was assayed at pH 7 using azurine cross-linked casein (Megazyme International Ireland Ltd., Bray, Co., Wicklow, Ireland) as the substrate. Enzyme activities are expressed as nanokatals. A single nanokatal is defined as the amount of enzyme activity that converts 1 nmol/s of the substrate used in the assay conditions.

2.2. Myofibril Proteins. Commercial chicken-breast muscles excised 3−4 days postmortem were trimmed free of visible fat and connective tissue and cut into pieces of about 2 cm^2. Myofibrils were isolated according to Xiong and Brekke (*36*). Instead of the isolation buffer (0.1 M NaCl, 50 mM sodium-phosphate buffer at pH 7.4, 5 mM EDTA, and 1 mM NaN_3), only a 50 mM sodium-phosphate buffer at pH 7.4 and 0.1 M NaCl was used. Phenylmethylsulfonyl fluoride (0.1 mM, Sigma) was added to inactivate any possible endogenous proteinase activity of the myofibril preparation. Salt-soluble proteins (SSP) were extracted from the isolated myofibrils in 1 M NaCl (*36*). The myofibril suspensions and SSP were stored in liquid nitrogen. The protein concentration was determined according to Lowry et al. (*37*).

2.3. Enzyme Treatments. *2.3.1. SSPs.* For SDS−PAGE analysis, the SSPs were suspended to a concentration of 3 mg/mL in the 50 mM sodium-phosphate buffer at pH 6. NaCl was added to the buffer to obtain a Na^+ concentration of 0.60 M. The protein suspension was tempered to the treatment temperature prior to enzyme addition. Laccase (50 and 200 nkat) and TG (10 and 100 nkat) were dosed according to their measured activity per gram of protein. Protein suspensions were incubated at 40 °C in the presence of the enzymes for up to 24 h.

2.3.2. Myofibrils. For viscoelastic measurements, isolated myofibrils were suspended to a concentration of 40 mg/mL of the 50 mM sodium-phosphate buffer at pH 6, having a Na^+ concentration of 0.35 or 0.60 M. The protein suspension was briefly tempered to 40 °C prior to enzyme addition. Enzymes were dosed per gram of protein according

to the measured activity. The enzyme dosages used were 100, 200, and 1000 nkat/g of protein for laccase and 10 and 100 nkat/g of protein for TG.

For differential scanning calorimetric (DSC) measurements, isolated myofibrils were suspended to a concentration of 70 mg/mL in the 50 mM sodium-phosphate buffer at pH 6, having a Na^+ concentration of 0.60 M. The suspension was briefly tempered to 40 °C prior to enzyme addition. Enzyme treatments of 1 and 3 h at 40 °C were carried out prior to the DSC measurement. The enzyme dosages used were 200 nkat of laccase/g of protein and 100 nkat of TG/g of protein.

2.4. SDS−PAGE. Changes in molecular weight and intensity of SSP bands caused by the enzymes as a function of time at 40 °C were analyzed by SDS−PAGE according to Laemmli (*38*). Ready-made polyacrylamide gels (12%, Tris-HCl) (Bio-Rad, Hercules, CA) were used. Protein bands were visualized by staining with Coomassie Brilliant Blue (R350, Pharmacia) and compared with molecular-weight markers (Prestained SDS−PAGE standards, broad range, Bio-Rad).

2.5. Protein Identification by Peptide Mass Fingerprinting and Peptide Fragment Ion Analysis. Coomassie Brilliant Blue stained protein bands of interest were excised from the polyacrylamide gel and "in-gel" digested essentially as described by Shevchenko et al. (*39*). Proteins were reduced with dithiothreitol and alkylated with iodoacetamide before digestion with trypsin (sequencing-grade-modified trypsin, V5111, Promega) supplied by BioFellows (Helsinki, Finland). The recovered peptides were, after desalting, subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI−TOF) mass spectrometric analysis. The MALDI−TOF mass spectra for mass fingerprinting and the MALDI−TOF/TOF mass spectra for identification by fragment ion analysis were acquired using an Ultraflex TOF/TOF instrument (Bruker-Daltonik GmbH, Bremen, Germany). Protein identification with the generated data was performed using Mascot Peptide Mass Fingerprint and MS/MS Ion Search programs (http://www.matrixscience.com).

2.6. Viscoelastic Measurements. The effect of NaCl and the enzymes on the gel-forming ability of the myofibril suspension was measured using a Bohlin VOR rheometer (Bohlin Reologi Ab, Lund, Sweden) in an oscillatory mode to determine the storage modulus (G′) and loss modulus (G″) during heating at a constant temperature of 40 °C. The rheometer was equipped with a high-temperature cell and a plate−plate measuring geometry (PP25HT). The gap between plates was 20 mm, with the strain at 10^{-3}, and the fixed frequency was 1 Hz. The strain used was in the linear viscoelastic region. Samples of 1.2 g were placed between the measuring probes. Silicone oil was applied on the exposed edges of the sample to prevent drying. The samples were equilibrated at the desired temperature for 10 min prior to measuring. The samples were sheared in an oscillatory mode at a constant frequency of 1 Hz throughout the measurement. Two replicates were run for all samples.

2.7. Differential Scanning Calorimetry. Changes in thermal stability of the enzymatically cross-linked myofibril proteins were measured using a Mettler Toledo DSC820 differential scanning calorimeter (Greifensee, Switzerland). Myofibrils with and without enzyme treatment (70 mg of protein/mL) of about 16 mg of protein were weighed into aluminum capsules, tempered first for 10 min at 40 °C, and then heated from 40 to 100 °C at a scan rate of 10 °C/min. Samples were analyzed in duplicate.

2.8. Statistical Analysis. Multivariate analysis and Tukey's *b* test were used to compare the mean values and to determine the significance of difference between rheological measurements at two different salt concentrations and between treatments in which enthalpies and transition temperatures were analyzed by DSC. Values were considered to be significantly different at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effects of the Enzymes on the Myofibril Proteins. The major modifications of the myofibril proteins caused by laccase and TG (**Figures 1−3**) were tentatively determined by comparing the relative mobilities and staining intensities of the enzyme-treated protein on SDS−PAGE gels to those treated otherwise similarly but without enzyme addition (**Figure 1**). Six distinct bands were visible on SDS−PAGE when the proteins were incubated without added enzymes (**Figure 1**). A clear effect of the enzymes was the intensified precipitation of protein out of the solution because of the enzymes during the treatments. Intensified precipitation occurred already in the beginning of the treatment with both TG dosages. With laccase, the precipitation exceeded that of the control after 3 h of treatment with the higher dosage and after 24 h of treatment with the lower dosage. Precipitation indicated the decreasing solubility of proteins, apparently because of polymerization and/or novel intramolecular bonds catalyzed by these enzymes. Precipitates observed after 3 h of treatment in the laccase-treated and all control samples dissolved in the SDS−PAGE sample buffer. After 24 h of treatment, precipitation had intensified in the laccase-treated samples compared to the control. Reduction of the disulfide bridges in the presence of 3% β-mercaptoethanol, denaturizing of the protein in the presence of 1% SDS, and heating dissolved the precipitates of the TG-treated SSP samples only partially. In the laccase-treated (50 and 200 nkat/g, 24 h) samples, a precipitate was also seen after heating the sample in the SDS−PAGE sample buffer. The insolubility of the precipitates still present in the SDS−PAGE sample buffer indicates that covalent bonds other than disulfide bridges had formed between/in the protein molecules. These insoluble precipitates were not loaded onto the gels.

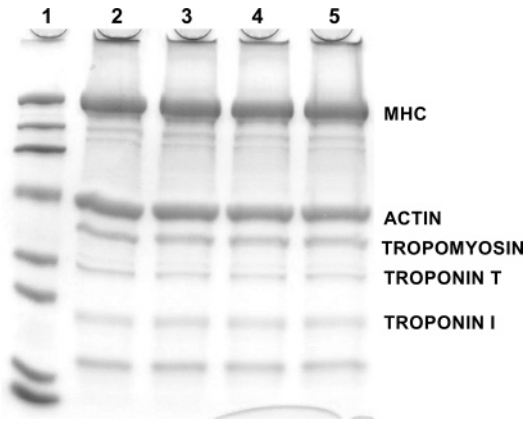


Figure 1. SDS–PAGE of SSP treated without added enzymes. Samples drawn from different time points (lane 2, at time 0; lane 3, after 1 h; lane 4, after 3 h; and lane 5, after 24 h) of the treatment. Lane 1, molecular-weight marker (209, 124, 80, 49, 35, 29, 21, and 7 kDa). Treatment conditions: 50 mM sodium-phosphate buffer at pH 6, protein concentration of 3 mg/mL, 0.60 M Na^+, at 40 °C. 20 μg of protein (9 μL) were loaded/lane.

The molecular changes catalyzed by laccase are shown in **Figure 2**. Using either dosage of laccase (50 or 200 nkat/g), the protein bands were not visibly changed after 3 h of treatment. After 24 h of treatment, clear precipitation was observed with both laccase dosages. On SDS−PAGE, the intensity of the myosin heavy chain (MHC, ∼200 kDa) was reduced (parts **a** and **b** of **Figure 2**). Apparently, polymerized MHC had precipitated out of the solution to some extent. Furthermore, the troponin T band (∼30 kDa) was found to disappear (**Figure 2b**). An extra band of <21 kDa appeared after 3 h of treatment with the higher dosage of laccase (200 nkat/g) (**Figure 2b**), indicating protein degradation probably because of the action of free radicals during the prolonged treatment. Reduction of strength in a wheat dough system as a function of the laccase dosage was recently observed by Selinheimo et al. (*40*). Another but less obvious explanation could be the increased mobility of an intramolecularly modified protein on SDS−PAGE. Emerging and fading protein bands detected on SDS−PAGE were

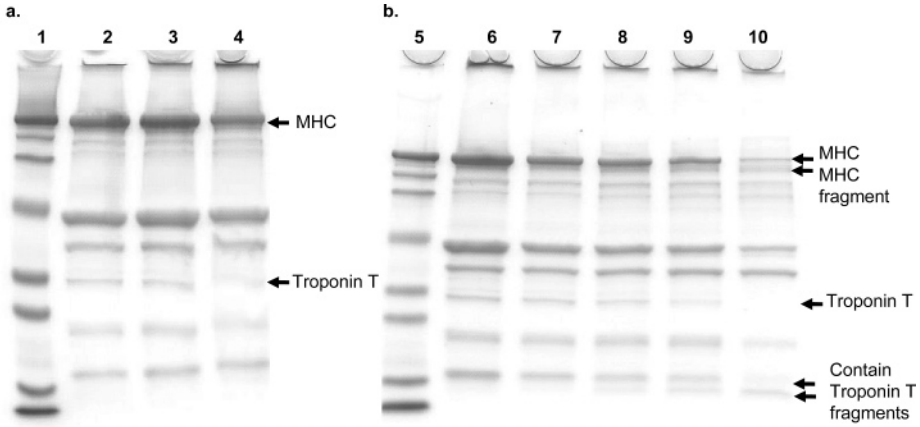


Figure 2. SDS–PAGE of SSP treated with (a) 50 nkat/g and (b) 200 nkat/g of laccase. Samples drawn from different time points of the treatment with 50 nkat of laccase/g (lane 2, after 1 h; lane 3, after 3 h; and lane 4, after 24 h). Samples drawn from different time points of the treatment with 200 nkat of laccase/g (lane 6, at time 0; lane 7, after 1 h; lane 8, after 3 h; lane 9, after 6 h; and lane 10, after 24 h). Lanes 1 and 5, molecular-weight marker (209, 124, 80, 49, 35, 29, 21, and 7 kDa). Treatment conditions: 50 mM sodium-phosphate buffer at pH 6, protein concentration of 3 mg/mL, 0.60 M Na^+, at 40 °C. A total of 9 μL of the soluble fraction was loaded/lane.

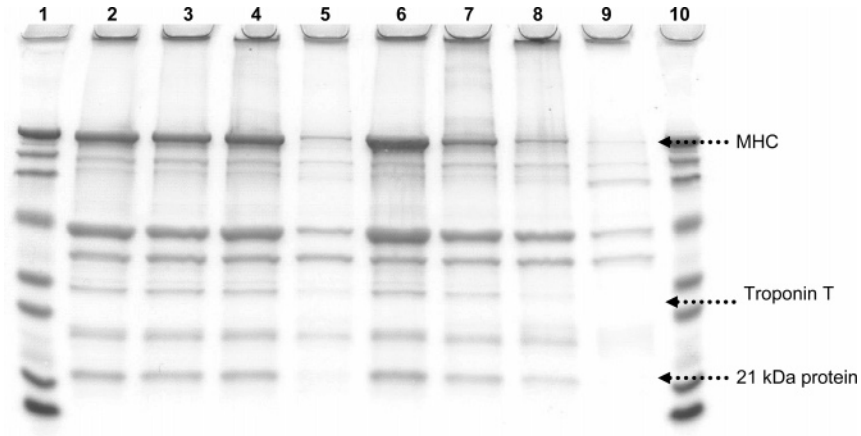


Figure 3. SDS–PAGE of SSP treated with 10 nkat/g (lanes 2–5) and 100 nkat/g of TG (lanes 6–9). Samples drawn from different time points of the treatment with 10 nkat of TG/g (lane 2, at time 0; lane 3, after 3 h; lane 4, after 6 h; and lane 5, after 24 h). Samples drawn from different time points of the treatment with 100 nkat of TG/g (lane 6, at time 0; lane 7, after 3 h; lane 8, after 6 h; and lane 9, after 24 h). Lanes 1 and 10, molecular-weight marker (209, 124, 80, 49, 35, 29, 21, and 7 kDa). Treatment conditions: 50 mM sodium-phosphate buffer at pH 6, protein concentration of 3 mg/mL, 0.60 M Na^+, at 40 °C. A total of 9 μL of the soluble fraction was loaded/lane.

identified by in-gel digestion, peptide mass fingerprinting, and peptide fragment analysis of the resulting tryptic peptides. Extensive laccase treatment caused the appearance of an obscure protein band slightly smaller than MHC. According to mass analysis, this band was identified as an MHC fragment. A fragment of troponin T was identified from the small protein band, <21 kDa. Also, the ~21-kDa band present already at the beginning of the treatments contained fragments of troponin T.

Molecular changes catalyzed by TG are shown in **Figure 3**. After 3 h of treatment, no change in the protein pattern was observed when the lower dosage (10 nkat/g) of TG was used (**Figure 3a**). With the higher dosage (100 nkat/g), the intensity of the MHC band began to decrease after 3 h of treatment (**Figure 3b**). The troponin T band (~30 kDa) also began to disappear. After 24 h of treatment, the protein pattern was drastically changed with both enzyme dosages. MHC, troponin T, and the ~21-kDa band had disappeared, and other protein bands had faded. In addition, the precipitate in the SSP suspension disappeared.

Under the given conditions and with the enzyme dosages used, the following detectable electrophoretic changes were observed: (1) progressive disappearance of the MHC ~200-kDa band and (2) of the troponin T ~30-kDa band with both enzymes and (3) the appearance of a <21-kDa band with laccase. The SDS−PAGE results show that MHC and troponin T were the myofibril proteins most susceptible to the action of both laccase and TG. On the other hand, actin was less modified in the presence of TG and not perceivable in the presence of laccase.

Laccases have generally been distinguished from tyrosinases because of their inability to oxidize tyrosine (*41*). However, because of the phenolic structure of its side chain, tyrosine is susceptible to oxidation, at least by some laccases. A recent study by Mattinen et al. (*25*) showed that *Trametes hirsuta* laccase is capable of catalyzing the formation of an ether-type covalent bond between two aromatic carbons of tyrosine residues in a small peptide. Cysteine is also reported to be a substrate, at least for *Myceliophthora maltophila* laccase (*30*). Shotaro (*28*) reported polymerization of bovine serum albumin by a laccase from *Pycnoporus coccineus*. The higher susceptibility of MHC than actin with laccase can at least partially be explained by the different availabilities of suitable amino acid residues, as well as by the size and shape of these two proteins. In regard to activity because of laccase, these proteins contain different amounts of free thiol groups. Chicken-breast muscle myosin contains ~43 thiol groups, 42 of which are located on

the outer parts of the molecule (*42*) and thus are readily available for intermolecular disulfide bonding. Actin contains only ~5 thiol groups (*12*). However, potential laccase-aided disulfide bridging does not explain the reactivity of myosin detected by SDS−PAGE. The amount of tyrosine residues is 3.1 and 5.6 g/16 g of N in myosin and actin, respectively (*43*). Thus, the amount of tyrosine does not explain the different sensitivities of these proteins to the action of laccase detected by SDS−PAGE either.

TG is an enzyme with a significant ability to modify various food proteins via covalent cross-linking. It has been reported to introduce cross-links to the myofibril proteins actin and myosin (*12*), actomyosin of turkey (*44*) and beef (*15*), and chicken myosin heavy chain (*45*). Myosin reacting readily with TG contains high amounts of glutamic acid/glutamine and lysine (*43*), which are the amino acid residues acting as counterparts in TG-catalyzed isopeptide bonds. Presumably, the rodlike shape and better accessibility of the target amino acids explain the observed susceptibility of myosin to cross-linking with both enzymes studied.

In addition to polymerization, the action of laccase in a mixture of the myofibril proteins also resulted in protein fragmentation when extensive treatment times and enzyme dosages were used. Free radicals formed in the laccase reaction may have caused fragmentation of the most susceptible proteins, i.e., myosin and troponin T. It is well-known that, e.g., ionizing radiation in biological systems involves formation of oxygen radicals derived from water as well as radical species derived from DNA and/or protein (*31*). At least oxygen radicals are known to cause various chemical changes in proteins, i.e., aggregation and polymerization, but also protein fragmentation (*31*, *32*, *46*). There is no definite reason that free radicals formed in the laccase catalysis would not affect protein in a similar way as radicals formed because of ionizing radiation.

3.2. Gel Formation. The G′ and G″ of the samples were monitored at a constant temperature of 40 °C during 3 h of treatment. At the lower ionic concentration (0.35 M) and without enzymes, the G′ increase of the myofibril gel was very weak (62.5 Pa) because of the limited solubility of the myofibril proteins and probably because of too low of a protein concentration (**Figure 4**). When the salt concentration was increased to 0.60 M but without enzymes, the G′ increase ($p < 0.05$) after 3 h of measurement was 1720 Pa (**Figure 5**). This result was expected because actin and myosin, which are responsible for gel formation, are salt-soluble. In 0.60 M NaCl, these proteins are practically solubilized. Solubilization increases the flexibility and mobility of the proteins and thus facilitates the interaction of the functional groups necessary for gel formation.

The effect of laccase on gelling was investigated at a series of enzyme dosages, i.e., 50, 100, 200, and 1000 nkat/g of protein. At the low salt concentration, no increase ($p > 0.05$) in gelling was observed with any of the laccase dosages used (**Figure 4a**). The reason for this was obviously the limited solubility of the proteins and, therefore, the insufficiency and/or inaccessibility of target sites for laccase catalysis. The ability of laccase to increase G′ occurred only when the salt concentration was raised to 0.60 M. The low laccase dosages, i.e., 50 and 100 nkat/g, were not able to enhance ($p > 0.05$) gelling, although 50 nkat/g was already enough to modify myosin detectable by SDS−PAGE (**Figure 2**). G′ was enhanced slightly ($p > 0.05$) from 1384 to 1795 Pa when a dosage of 200 nkat/g was used (**Figure 5a**). A further increase in laccase dosage caused a decrease in gel formation. After 3 h of treatment with

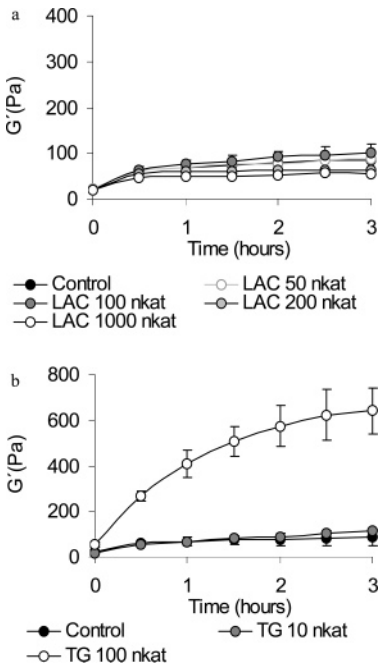


Figure 4. Storage modulus (G′) of chicken-breast myofibrils measured at a constant temperature of 40 °C. (a) Treatment with laccase and (b) TG. Treatment conditions: 4% protein, 50 mM sodium phosphate buffer at pH 6, 0.35 M Na^+.

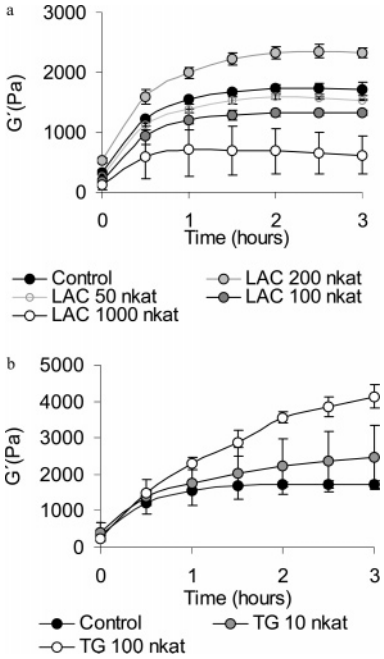


Figure 5. Storage modulus (G′) of chicken-breast myofibrils measured at a constant temperature of 40 °C. (a) Treatment with laccase and (b) TG. Treatment conditions: 4% protein, 50 mM sodium phosphate buffer at pH 6, 0.60 M Na^+.

1000 nkat/g of laccase, G′ was 888 Pa ($p > 0.05$) below the 1384 Pa control value, indicating a weakening of the gel formation.

The laccase preparation used had been purified to homogeneity, and it did not exhibit any other enzyme activities. Low laccase dosages, i.e., 50 and 100 nkat/g, were not sufficient to induce either protein polymerization or fragmentation. When the dosage was increased to the more optimal level of 200 nkat/g, polymerization apparently exceeded protein fragmentation. A further dosage increase resulted in intensified fragmentation

Table 1. Heat Transition (ΔH) and Peak Temperature (T_{max}) of the Enzyme-Treated Myofibrils

		1st transition peak (myosin)		2nd transition peak (actin)	
pretreatment[a]	pretreatment time (h)	ΔH (J/g)	T_{max} (°C)	ΔH (J/g)	T_{max} (°C)
control	1	5.93 ab (0.30)[b]	59.35 a (0.01)	0.18 a (0.05)	66.26 a (0.81)
	3	5.86 ab (0.81)	59.52 a (0.02)	0.17 a (0.05)	65.93 a (0.61)
TG	1	4.29 a (0)	58.45 a (0.13)	0.44 b (0.01)	66.69 a (0.47)
100 nkat/g	3	4.14 a (0)	58.59 a (0.28)	0.45 b (0.02)	66.74 a (0.51)
Laccase	1	6.50 b (0.71)	59.14 a (0.57)	0.48 b (0.04)	66.96 a (0.57)
200 nkat/g	3	5.79 ab (0.51)	58.71 a (0.28)	0.17 a (0.04)	66.45 a (0.64)

[a] Pretreatment conditions: 40 °C, 50 mM sodium-phosphate buffer at pH 6, 0.60 M Na^+, 70 mg protein/mL. Myofibrils were heated from 40 to 100 °C at 10 °C/min. [b] Means with different letters in the same column are significantly different at $p < 0.05$. Values in parentheses are standard deviations for the means.

leading to a severe decrease in gel formation. Arabinoxylan gels induced by laccase have been reported to have poor stability properties (*47*). Furthermore, softening of dough because of a high dosage of laccase has been reported by Selinheimo et al. (*40*). In summary, it can be concluded that laccase causes protein polymerization but also protein fragmentation. However, conditions in which covalent link formation and thus polymerization exceeds fragmentation must be optimized by empirical testing.

The effect of TG on gelling was studied with dosages of 10 and 100 nkat/g of protein. As shown in **Figure 4b**, the higher dosage of 100 nkat/g was needed for a clear increase ($p < 0.05$) of G′ when the NaCl concentration of the protein suspension was 0.35 M. At this salt concentration, G′ increased after 3 h of measurement from 62.5 to 587 Pa. When the salt concentration was increased to 0.60 M, both 10 and 100 nkat of TG enhanced G′ (**Figure 5b**). G′ increased from 1384 to 2050 Pa ($p > 0.05$) and to 3891 Pa ($p < 0.05$), respectively. The enhanced gelling with the increased salt concentration is explained by an increased solubility of the proteins and thus better accessibility of the target sites for TG. The better efficiency of TG compared to laccase is explained by the higher concentration of the target sites available for this enzyme in myosin and also in actin. A further explanation can be the synergistic effect of NaCl and TG activity, which has been studied and discussed by Kütemeyer et al. (*48*).

3.3. Changes in Thermal Stability. Dependent upon the enzymatic pretreatment, the myofibril samples showed different thermal behavior (**Table 1**). Two major endothermic transitions were observed with peak temperatures (T_{max}) at about 59 and 66 °C. These transitions were presumably due to denaturation of myosin and actin, respectively (*3*, *49*, *50*). Laccase pretreatment of 1 h increased the calorimetric enthalpy (ΔH) of the myosin transition from 5.93 to 6.50 J/g. Unlike laccase, TG reduced the ΔH to 4.29 J/g. The result indicates slightly enhanced ($p > 0.05$) stability against thermal denaturation of myosin by laccase but decreased stability ($p > 0.05$) with TG at the given treatment conditions. After 1 h of pretreatment, the ΔH of actin transition increased from the control value of 0.18 to 0.48 J/g ($p < 0.05$) and 0.44 J/g ($p < 0.05$) for laccase and TG, respectively. Extending the time from 1 to 3 h did not alter the ΔH of myosin in any pretreatment. The ΔH stood at 5.79, 4.14, and 5.86 J/g after laccase, TG, and control pretreatments, respectively. However, 3 h of pretreatment with laccase decreased the ΔH of actin (0.17 J/g) to the level of the control (0.18 J/g). Extending the TG pretreatment did not change the ΔH of actin (0.45 J/g); it remained at the same level as after 1 h of pretreatment. T_{max} for the myosin or actin transition was not affected by any pretreatment.

TG-catalyzed a change of ΔH on myosin observed in DSC (**Table 1**). An insignificant ($p > 0.05$) but clearly detected decrease in thermal stability of the TG-treated myosin may be a consequence of changes because of polymerization of the molecules to larger but less thermostable structures. A change in the thermal stability of myosin was not detected with laccase, which can possibly be explained by the less distinct or different type of reaction of myosin with laccase compared to that with TG.

Additional intramolecular bonds may lead to more stable (*21*), i.e., also more thermostable, protein structures. A significant increase ($p < 0.05$) in the ΔH of the actin transition was observed after 1 h of pretreatment with laccase, but during the extended laccase pretreatment, this effect disappeared (**Table 1**). An increase ($p < 0.05$) of the ΔH of actin detected with TG was independent of the duration of the pretreatment (**Table 1**).

On SDS−PAGE, the molecular weight of the actin band was not altered by the enzymes (**Figures 2−4**). In addition to a possible insufficiency or inaccessibility of target sites for enzymatic action, this result may also be explained by possible intramolecular cross-linking, which did not affect the mobility of the actin protein on SDS−PAGE, but may have increased its thermostability detected by DSC. An explanation of the stability increase during the first hour of the laccase pretreatment but the subsequent decrease as a function of time may be too high of an enzyme dosage, causing protein fragmentation during an extended pretreatment.

ACKNOWLEDGMENT

We thank Heljä Heikkinen, Eeva Manninen, and Elina Ahola for their skilful technical assistance.

LITERATURE CITED

(1) Lesiów, T.; Xiong, Y. L. Gelation properties of poultry myofibrillar proteins and comminuted poultry meat. Effect of protein concentration, pH and muscle type—A review. *Fleischwirtschaft Int*. **2001**, *4*, 39−44.

(2) Westphalen, A. D.; Briggs, J. L.; Lonergan, S. M. Influence of pH on rheological properties of porcine myofibrillar protein during heat induced gelation. *Meat Sci.* **2005**, *70*, 293−299.

(3) Lesiów, T.; Xiong, Y. L. Mechanism of rheological changes in poultry myofibrillar proteins during gelation. *Avian Poult. Biol. Rev.* **2001**, *12*, 137−149.

(4) Hamm, R. *Kolloidchemie des Fleisches*; Paul Parey: Berlin and Hamburg, Germany, 1972; p 222.

(5) Lesiów, T.; Xiong, Y. L. Chicken muscle homogenate gelation properties: Effect of pH and muscle fiber type. *Meat Sci*. **2003**, *64*, 399−403.

(6) Xiong, Y. L.; Brekke, C. J. Protein extractability and thermally induced gelation of properties of myofibrils isolated from pre- and postrigor chicken muscles. *J. Food Sci*. **1991**, *56*, 210−215.

(7) Offer, G.; Knight, P. The structural basis of water-holding in meat. Part 1: General principles and water uptake in meat processing. In *Developments in Meat Science*; Lawrie, R. A., Ed.; Elsevier Applied Sciences, London, U.K., 1983.

(8) Ruusunen, M.; Puolanne, E. Reducing sodium intake from meat products. *Meat Sci.* **2005**, *70*, 531−541.
(9) Offer, G.; Trinick, J. On the mechanism of water holding in meat: The swelling and shrinking of myofibrils. *Meat Sci.* **1983**, *8*, 245−281.
(10) Millman, B. M.; Nickel, B. G. Electrostatic forces in muscle and cylindrical gel systems. *Biophys. J.* **1980**, *32*, 49−63.
(11) Mugumura, M.; Tsuruoka, K.; Fujino, H.; Kawahara, S.; Yamauchi, K.; Matsumura, S.; Soeda, T. Gel strength enhancement of sausages by treating with microbial transglutaminase. In *Proceeding of the 45th International Congress on Meat Science Technology*; Yokohama, Japan, 1999; Vol. 1, pp 138−139.
(12) De Backer-Royer, C.; Traoré, F.; Meunier, J. C. Polymerization of meat and soybean proteins by human placental calcium-activated factor XIII. *J. Agric. Food Chem.* **1992**, *40*, 2052−2056.
(13) Dimitrakopoulou, M. A.; Ambrosiadis, J. A.; Zetou, F. K.; Bloukas, J. G. Effect of salt and transglutaminase (TG) level and processing conditions on quality characteristics of phosphate-free, cooked, restructured pork shoulder. *Meat Sci.* **2005**, *70*, 743−749.
(14) Kahn, D.; Cohen, I. Factor XIIIa-catalyzed coupling of structural proteins. *Biochim. Biophys. Acta* **1981**, *668*, 490−494.
(15) Kim, S.-H.; Carpenter, J. A.; Lanier, T. C.; Wicker, L. Polymerization of beef actomyosin induced by transglutaminase. *J. Food Sci.* **1993**, *58*, 473−474, 491.
(16) Fernández-Díaz, M. D.; Montero, P.; Gómez-Guillén. Gel properties of collagens from skins of cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and their modification by the coenhancers magnesium sulphate, glycerol, and transglutaminase. *Food Chem.* **2001**, *74*, 161−167.
(17) Kuraishi, C.; Yamazaki, K.; Susa, Y. Transglutaminase: Its utilization in the food industry. *Food Rev. Int.* **2001**, *17*, 221−246.
(18) Srinivasan, S.; Hultin, H. O. Hydroxyl radical modification of fish muscle proteins. *J. Food Biochem.* **1995**, *18*, 405−425.
(19) Li, S. J.; King, A. J. Lipid oxidation and myosin denaturation in dark chicken meat. *J. Agric. Food Chem.* **1996**, *44*, 3080−3084.
(20) Liu, G.; Xiong, Y. L.; Butterfield, D. A. Chemical, physical, and gel-forming properties of oxidized myofibrils and whey- and soy-protein isolates. *J. Food Sci.* **2000**, *65*, 811−818.
(21) Feeney, R. E.; Whitaker, J. R. Importance of cross-linking reactions in proteins. *Adv. Cereal Sci. Technol.* **1988**, *9*, 21−46.
(22) Gerrard, J. A. Protein−protein cross-linking in food: Methods, consequences, applications. *Trends Food Sci. Technol.* **2002**, *13*, 389−397.
(23) Gianfreda, L.; Xu, F.; Bollag, J.-M. Laccases: A useful group of oxidoreductive enzymes. *Biorem. J.* **1999**, *3*, 1.25.
(24) Thurston, C. F. The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19−26.
(25) Mattinen, M.-L.; Kruus, K.; Buchert, J.; Nielsen, J. H.; Andersen, H. J.; Steffensen, C. L. Laccase-catalyzed polymerization of tyrosine-containing peptides. *FEBS J.* **2005**, *272*, 3640−3650.
(26) Færgemand, M.; Otte, J.; Qvist, K. B. Cross-linking of whey proteins by enzymatic oxidation. *J. Agric. Food Chem.* **1998**, *46*, 1326−1333.
(27) Chung, J. E.; Kurisawa, M.; Uyama, H.; Kobayashi, S. Enzymatic synthesis and antioxidant property of gelatin−catechin conjugates. *Biotechnol. Lett.* **2003**, *25*, 1993−1997.
(28) Shotaro, Y. Method for cross-linking protein by using enzyme, 2000, U.S. Patent 6,121,013.
(29) Lantto, R.; Schönberg, C.; Heine, E.; Buchert, J. Effects of laccase-mediator combinations on wool. *Text. Res. J.* **2004**, *74*, 713−717.
(30) Heldt-Hansen, H. P. Development of enzymes for food applications. In *Biotechnology in the Food Chain, New Tools, and Applications*, VTT Symposium series 177; Espoo, Finland, 1998; pp 45−55.
(31) Garrison, W. M. Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. *Chem. Rev.* **1987**, *87*, 381−398.
(32) Davies, K. J. A. Protein damage and degradation by oxygen radicals. I. General aspects. *J. Biol.* Chem. **1987**, *262*, 9895−9901.
(33) 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.
(34) Leonowicz, A.; Grzywnowicz, K. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. *Enzyme Microb. Technol.* **1981**, *3*, 55−58.
(35) Folk, J. E.; Cole, P. W. Mechanism of action of guinea pig liver transglutaminase. *J. Biol. Chem.* **1966**, *241*, 5518−5525.
(36) Xiong, Y. L.; Brekke, C. J. Changes in protein solubility and gelation properties of chicken myofibrils during storage. *J. Food Sci.* **1989**, *54*, 1141−1146.
(37) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(38) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(39) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850−858.
(40) Selinheimo, E.; Kruus, K.; Hopia, A.; Buchert, J.; Autio, K. Effects of laccase, xylanase, and their combination on the rheological properties of wheat doughs. *J. Cereal Sci.* **2005**, manuscript accepted.
(41) Mayer, A. M. Polyphenol oxidases in plants—Recent progress. *Phytochemistry* **1987**, *26*, 11−20.
(42) Smyth, A. B.; Smith, D. M.; O'Neill, E. Disulfide bonds influence the heat-induced gel properties of chicken breast muscle myosin. *J. Food Sci*. **1998**, *63*, 584−588.
(43) Belitz, H.-D.; Grosch, W. Muscle tissue: Composition and function. In *Food Chemistry*, 2nd ed.; Springer-Verlag: Berlin, Germany, 1999; pp 532−550.
(44) Akamittath, J. G.; Ball, H. R. Transglutaminase mediated polymerization of crude actomyosin refined from mechanically deboned poultry meat. *J. Muscle Foods* **1992**, *3*, 1−14.
(45) Tseng, T.-F.; Chen, M.-T.; Liu, D.-C. Purification of transglutaminase and its effects on myosin heavy chain and actin of spent hens. *Meat Sci.* **2002**, *60*, 267−270.
(46) Davies, K. J. A.; Delsignore, M. E. Pprotein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. *J. Biol. Chem.* **1987**, *262*, 9908−9913.
(47) Carvajal-Millan, E.; Guigliarelli, B.; Belle, V.; Rouau, X.; Micard, V. Storage stability of laccase induced arabinoxylan gels. *Carbohydr. Polym.* **2005**, *59*, 181−188.
(48) Kütemeyer, C.; Froeck, M.; Werlein, H.-D.; Watkinson, B. M. The influence of salts and temperature on enzymatic activity of microbial transglutaminase. *Food Control* **2005**, *16*, 735−737.
(49) Xiong, Y. L.; Brekke, C. J.; Leung, H. K. Thermal denaturation of muscle proteins from different species and muscle types as studied by differential scanning calorimetry. *Can. Inst. Food Sci. Technol. J.* **1987**, *20*, 357−362.
(50) Stabursvik, E.; Martens, H. Thermal denaturation of proteins in post rigor muscle tissue as studied by differential scanning calorimetry. *J. Sci. Food Agric.* **1980**, *31*, 1034−1042.

Received for review July 6, 2005. Revised manuscript received September 5, 2005. Accepted September 15, 2005. This study was carried out with the financial support of the National Technology Agency of Finland.

JF051602A