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Tyrosinase-Aided Protein Cross-Linking: Effects on Gel Formation of Chicken Breast Myofibrils and Texture and Water-Holding of Chicken Breast Meat Homogenate Gels

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The effects of *Trichoderma reesei* tyrosinase-catalyzed cross-linking of isolated chicken breast myofibril proteins as a simplified model system were studied with special emphasis on the thermal stability and gel formation of myofibrillar proteins. In addition, tyrosinase-catalyzed cross-linking was utilized to modify the firmness, water-holding capacity (WHC), and microstructure of cooked chicken breast meat homogenate gels. According to SDS-PAGE, the myosin heavy chain (MHC) and troponin T were the most sensitive proteins to the action of tyrosinase, whereas actin was not affected to the same extent. Calorimetric enthalpy (ΔH) of the major thermal transition associated with myosin denaturation was reduced and with actin denaturation increased in the presence of tyrosinase. Low-amplitude viscoelastic measurements at constant temperatures of 25 °C and 40 °C showed that tyrosinase substantially increased the storage modulus (G') of the 4% myofibrillar protein suspension in the 0.35 M NaCl concentration. The effect was the most pronounced with high-enzyme dosages and at 40 °C. Without tyrosinase, the G' increase was low. Tyrosinase increased the firmness of the cooked phosphate-free and low-meat chicken breast meat homogenate gels compared to the corresponding controls. Tyrosinase maintained gel firmness at the control level of the low-salt homogenate gel and weakened it when both salt and phosphate levels were low. Tyrosinase improved the WHC of the low-meat and low-salt homogenate gels and maintained it at the level of the corresponding controls of phosphate-free and low-salt/low-phosphate homogenate gels. Microstructural characterization showed that a collagen network was formed in the presence of tyrosinase.

KEYWORDS: Chicken myofibrillar proteins; protein modification; cross-linking; tyrosinase; gelation; thermal stability; texture; water-holding capacity; microstructure

1. INTRODUCTION

The texture of cooked meat products is mainly a consequence of a gel network of myofibrillar proteins of muscle fibers. Textural features are related to the biochemical properties of the muscle fibers and processing conditions. Heat-induced formation of the protein network, a gel, is a complex process involving initial denaturation of the proteins followed by protein−protein aggregation. During heating, myosin, the most abundant of the myofibril proteins, undergoes several conformational changes as a consequence of dissociation of noncovalent bonds in the protein and dissociation of the subunits of the myosin protein and myofilaments (*1*). The degree of denaturation necessary for aggregation depends on the heating conditions as well as on the ionic environment, pH, and intrinsic factors of the protein (*2*, *3*). Actin, the other main protein component of myofibrils, is reported to have only a minor role in gelation (*4*). Obvious reasons for this are, at least, the poorer extractability of actin than that of myosin (*5*) as well as its higher endothermic transition temperature (*4*, *6*). Gelation is mainly related to protein extractability but also to pH. Gel formation of chicken breast myofibril proteins is optimal at pH 6.0 (*7*). The effects of pH, protein concentration, type, and solubility as well as heating conditions on denaturation, aggregation, and gelation of poultry muscle myofibril proteins and further on meat processing have been comprehensively reviewed by Xiong (*8*) and Lesiów and Xiong (*9*, *10*).

In addition to the structure formation, the water-holding properties of meat systems are also pH dependent. Above the average isoelectric point of the muscle proteins (about pH 5.0 and about 4.0 with added NaCl), the water-holding capacity (WHC) is improved because of swelling of the myofibrils along with increasing pH (*11*). In meat with added NaCl, the WHC maximum is reached by about pH 6.0 (*11*). Salt addition has also an impact on the functional properties of meat products. NaCl contributes to flavor, shelf life, and binding properties (*12*), playing also a key role in the extraction of myofibril

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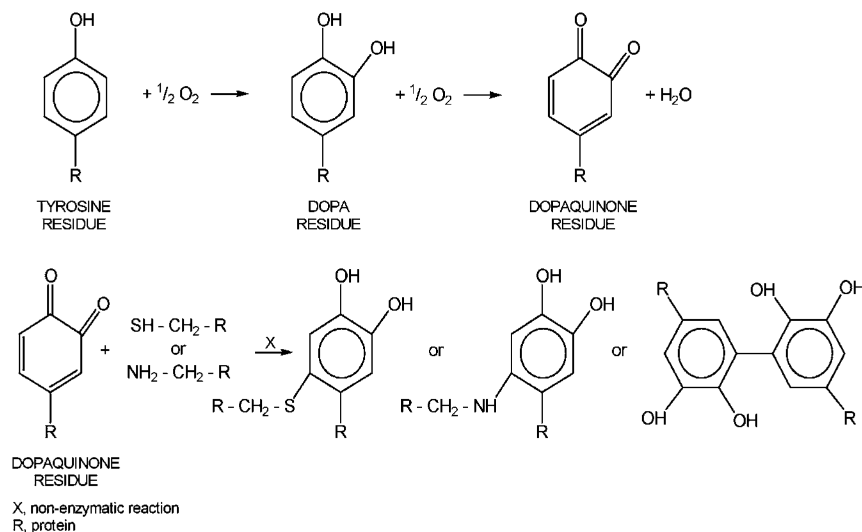

Figure 1. Tyrosinase-catalyzed oxidation of tyrosine and subsequent nonenzymatic reactions of the quinones according to Ito et al. (*26*) and Burzio and Waite (*28*).

proteins and the swelling of myofibrils (*11*, *13*). A review on the effects of NaCl in meat was recently published by Ruusunen and Puolanne (*12*). Besides NaCl, added phosphates have a marked influence on texture and WHC. Phosphates increase pH and ionic strength and affect WHC positively, particularly at NaCl levels around 1.5% (*14*).

Enzymes that stabilize protein systems by forming additional covalent bonds between proteins may have the potential to fabricate meat products with better texture in spite of low salt, phosphate, or protein content. Currently, transglutaminases (TG, glutaminylpeptide:amine γ-glutamyltransferase, EC 2.3.2.13) are widely used to improve the texture of various meat products. Positive effects of TG have been obtained in the restructuring of fresh meat (*15*) and also in improving textural properties of various heated meat systems, for example, low-fat chicken meat gels (*16*), phosphate-free restructured pork meat (*17*), low-salt pork batter gels (*18*), and low-salt chicken meatballs (*19*). Improvement of texture by TG has been generally confirmed, but the results reported on the effects of TG on water-holding vary. Tseng et al. (*19*) reported on increasing cooking yields in low-salt chicken meatballs as a function of the TG dosage. A high amount of pork fat (25%) may have contributed to the results. Pietrasik and Li-Chan (*18*) found that TG treatment reduces cooking loss of pork batter gels containing 0.15% sodium tripolyphosphate at all NaCl (0.4−2%) levels. On the other hand, Dimitrakopoulou et al. (*17*) did not observe any effects of TG on cooking loss of cooked pork shoulder regardless of the salt levels used. Negative effects of TG on cooking yields in beef gels containing 2.5% salt were reported by Dondero et al. (*20*) and in pork, chicken, and lamb gels containing 1.5% salt by Carballo et al. (*21*).

Although TG has an established position in the food industry in the processing of many meat products, both fresh and cooked, a new type of cross-linking enzymes, that is, polyphenol oxidases (PPO), may also have great potential. Laccases (EC 1.10.3.2) which use molecular oxygen to oxidize phenols and polyphenols have been found to modify bovine serum albumin (*22*), α-lactalbumin (*23*), and chicken breast myofibril proteins (*24*). Although Mattinen et al. (*25*) showed that laccase cross-links tyrosine-containing short peptides via an isodityrosine bond, at present the mechanism of laccase-catalyzed protein modification is, however, poorly understood. Another PPO, that is, tyrosinase (EC 1.14.18.1), has been known for decades to activate tyrosine residues in proteins to the quinone forms, which react further nonenzymatically with each other or with thiol or primary amino groups, resulting in the formation of covalent tyrosine−tyrosine, tyrosine−cysteine, or tyrosine−lysine cross-links (*26*−*29*; **Figure 1**). Apart from extensive studies on inhibition of endogenic plant tyrosinase (or PPO) activity to control fruit and vegetable browning, only a few reports have focused on the exploitation of tyrosinase in food protein modification (*29*−*33*). Mushroom (*Agaricus bisporus*) tyrosinase has been used in all these studies.

The aim of the present study was to elucidate the potential of a novel tyrosinase isolated from a filamentous fungus *Trichoderma reesei* in modifying chicken breast muscle myofibril proteins. A macrolevel consequence of the tyrosinase-catalyzed protein modification was investigated as the gel formation of chicken breast meat myofibrils. In addition, the effects of tyrosinase on firmness and weight loss of cooked chicken breast meat homogenate gels containing reduced amounts of protein, salt, or phosphate were examined.

2. MATERIALS AND METHODS

2.1. Tyrosinase. Tyrosinase from a filamentous fungus *Trichoderma reesei* was purified to a high degree of purity as reported by Selinheimo et al. (*34*). Tyrosinase activity was assayed using 15 mM L-DOPA (Sigma, St. Louis, United States) as substrate at pH 7 and room temperature according to Robb (*35*). Tyrosinase activity is expressed as nanokatals (nkat). One nkat is defined as the amount of enzyme activity that converts 1 nmol of the substrate used per second under the given assay conditions.

2.2. Myofibrils and Salt-Soluble Myofibrillar Proteins. Commercial chicken breast muscles excised 3−4 days post-mortem were trimmed free of visible fat and connective tissue and were cut into pieces of about 2 cm^3. Myofibrils were isolated according to Xiong and Brekke (*36*). As the isolation buffer 50 mM sodium-phosphate buffer, pH 7.4, containing 0.1 M NaCl was used, 0.1 mM phenylmethylsulfonyl fluoride (Sigma) was added to reduce indigenous protease activity of the myofibril preparation. Isolated myofibrils were used in the differential scanning calorimetric (DSC) and viscoelastic measurements. Salt-soluble proteins (SSPs) for SDS-PAGE were extracted from the isolated myofibrils in 1 M NaCl (*36*). The myofibril and SSP suspensions were stored in liquid nitrogen. The protein concentration was determined according to Lowry et al. (*37*).

For SDS-PAGE analysis, the SSPs were suspended to a concentration of 3 mg/mL in a 50 mM sodium-phosphate buffer, pH 6. NaCl was added to the buffer to obtain a Na^+ concentration of 0.35 M or 0.60

Table 1. Composition (% by Weight) of the Meat Homogenates

meat homogenate	meat, % (w/w)	NaCl, % (w/w)	$Na_3HP_2O_7$, % (w/w)	pH[a]
1. control	75	2	0.34	5.99
2. low meat (LM)	65	2	0.34	5.95
3. no phosphate (NoPP)	75	2		5.86
4. low salt (LS)	75	1	0.34	6.00
5. low salt and phosphate (LSPP)	75	1	0.17	6.13

[a] pH was measured for each meat homogenate at 4 °C before enzyme addition and heating.

M. The protein suspension was tempered to the treatment temperature prior to enzyme addition. Tyrosinase was dosed according to its measured activity per gram of protein. The dosage used was 1000 nkat/g protein. Protein suspensions (with and without tyrosinase) were incubated at 40 °C for up to 24 h. The precipitate formed during the 24-h treatment of the protein suspension containing 0.35 M NaCl was not loaded to SDS-PAGE.

For DSC, isolated myofibrils were suspended to a concentration of 70 mg protein/mL in the 50 mM sodium-phosphate buffer, pH 6. NaCl was added to the buffer to obtain a Na^+ concentration of 0.60 M. Tyrosinase treatments of 1 h at 40 °C were carried out prior to the DSC measurement. The tyrosinase dosages used were 500 and 1000 nkat/g of protein.

For viscoelastic measurements, isolated myofibrils were suspended to a concentration of 40 mg/mL of 50 mM sodium-phosphate buffer, pH 6. NaCl was added to the buffer to obtain a Na^+ concentration of 0.35 M. The myofibril suspension was briefly tempered to 40 °C prior to tyrosinase addition. Tyrosinase was dosed per gram of protein according to the measured activity. The dosages used were 0, 60, 120, and 240 nkat/g of protein.

2.3. Chicken Breast Meat Homogenates. Commercially available chicken breast meat was trimmed free of visible fat and connective tissue, was coarsely ground through a 20-mm plate, and was frozen at −20 °C until further use. The protein content of the meat was analyzed as Kjehldal nitrogen and was found to be 23.3%. Prior to use, a portion of meat was slightly thawed at room temperature and was homogenized in a kitchen blender for 2 min. In addition to water, the ingredients used in the meat homogenate mixtures included NaCl and $Na_3HP_2O_7$ (trisodium pyrophosphate, Chemische Fabrik Budenheim KG, Budenheim, Germany). Water containing the ingredients was oxygenated at 4 °C by bubbling an O_2/N_2 (80%/20%) gas mixture into the solution until the oxygen concentration reached a level of about 50 mg/L. Batches of the meat homogenate (100 g) needed for three replicate experiments were mixed at 4 °C with water (4 °C) containing the ingredients and the enzyme. The composition excluding water of the homogenates is shown in **Table 1**. The composition of the homogenate no. 1 was closest to that used in industry. From the other homogenates (nos. 2−5), phosphate was omitted (NoPP), meat (LM) or salt (LS) amount was reduced, or both salt and phosphate (LSPP) amounts were reduced. To the homogenates nos. 2−5, 20 and 120 nkat tyrosinase/g of protein were added. In addition, every homogenate (nos. 1−5) was treated without tyrosinase as a control. Immediately after the tyrosinase addition, the homogenate samples were stuffed into cylindrical steel tubes (diameter 30 mm, height 45 mm) and were allowed to stand at 4 °C for 1 h after which they were removed to a water bath at 40 °C. After the internal temperature of the samples had reached 40 °C, which took about 20 min, the samples were incubated at 40 °C for 50 min. The samples were moved to a water bath at 77 °C. After 10 min, the internal temperature of the samples had reached 72 °C and the samples were moved to a water bath at 25 °C for 30 min, after which the internal temperature had dropped to 25 °C. The internal temperature was monitored using a thermosensor inserted into the geometrical center of a homogenate sample. After tempering to 25 °C, the sample gels were immediately measured for maximum compression force and weight loss.

2.4. SDS-PAGE. SDS-PAGE analysis was carried out to salt-soluble proteins (SSP) extracted from the isolated myofibrils. Changes in molecular weight and intensity of SSP bands caused by tyrosinase as a function of time at 40 °C were analyzed by SDS-PAGE according to Laemmli (*38*). Ready-made 12% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA) were used. Protein bands were visualized by staining with Coomassie G-250 (GelCode Blue Stain Reagent, Pierce, United States) and were compared with molecular weight markers (Prestained SDS-PAGE standards, broad range, Bio-Rad, United States).

2.5. Differential Scanning Calorimetry. Changes in the thermal stability of the enzymatically cross-linked myofibril proteins of the isolated myofibrils were measured using a Mettler Toledo DSC820 differential scanning calorimeter (Mettler-Toledo GmbH, Greifensee, Switzerland). Pretreated myofibrils (about 16 mg of protein, 70 mg/mL) were weighed into aluminum capsules, were tempered first for 10 min at 40 °C, and then were heated from 40 °C to 100 °C at a scan rate of 10 °C/min. Samples were analyzed in duplicate.

2.6. Small-Amplitude Viscoelastic Measurements. The effect of tyrosinase on the gel-forming ability of the chicken breast 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 the loss modulus (G'') during heating at constant temperatures of 25 °C and 40 °C. The rheometer was equipped with a high-temperature cell and a plate−plate measuring geometry (PP25HT). The gap between the plates was 20 mm, the strain was 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 to the exposed edges of the sample to prevent drying. The samples were equilibrated at the desired temperature for 10 min prior to measuring, and they were sheared in an oscillatory mode at a constant frequency of 1 Hz throughout the measurement.

2.7. Gel Firmness. The firmness of enzyme-treated and control meat homogenate samples (diameter 30 mm, height 40 mm) was measured by a compression test using a TA-XT2 Texture Analyzer (Stable Micro Systems, Godalming, United Kingdom). The samples were pressed with a cylinder probe P50 (diameter of the pressing surface 50 mm) at ambient room temperature. The trigger force, compression distance, rate of compression, and measurement time were 10 g, 60%, 1.5 mm s^{-1}, and 7 s, respectively. Each result is an average of three replicate measurements.

2.8. Weight Loss. Weight loss of the homogenate samples was determined as fat and water loss after heating to 72 °C and subsequent cooling to 25 °C, according to Hermansson and Lucisano (*39*). The homogenate samples were centrifuged at 20 °C for 10 min at 490*g* (Biofuge Stratos, rotor no. 3047, Heraeus Instruments, United States). The amount of released liquid was determined by weighing after centrifugation. Weight loss was determined from three replicate samples and calculated from the formula

$$\text{weight loss (\%)} = (\text{weight of liquid phase/weight of sample}) \times 100\%$$

2.9. Microscopy. For microscopy, cooked chicken breast meat homogenate samples (75% meat/1% salt and 65% meat/2% salt) were prepared in a similar manner to the samples for the gel hardness and weight loss measurements. Tyrosinase treatments were carried out using the enzyme dosage of 120 nkat/g protein. Sections (10-μm thick) from the frozen gel samples of 5 × 5 × 5 mm^3 were cut in a cryostat (Leica CM 3050 S, Leica Microsystems Nussloch GmbH, Nussloch, Germany), were fixed with Bouin's solution (Sigma-Aldrich Co., St. Louis, United States), and were stained with Aniline Blue (Aldrich Chemical Co. Inc., Milwaukee, United States) and Orange G (Fluka, Steinheim, Germany) according to Ofstad et al. (*40*). After staining, the samples were examined under an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan) and were photographed with a SensiCam PCO CCD camera (PCO Computer Optics GmbH, Kelheim, Germany).

2.10. Statistical Analysis. One-way ANOVA and Tukey's b test were used to compare means and to identify significant differences among the DSC data of the myofibril treatments and the gel firmness and weight loss data of the meat homogenate treatments. Values were considered to be significantly different at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Cross-Linking of Myofibrillar Proteins. The major modifications of the salt-soluble myofibril proteins caused by

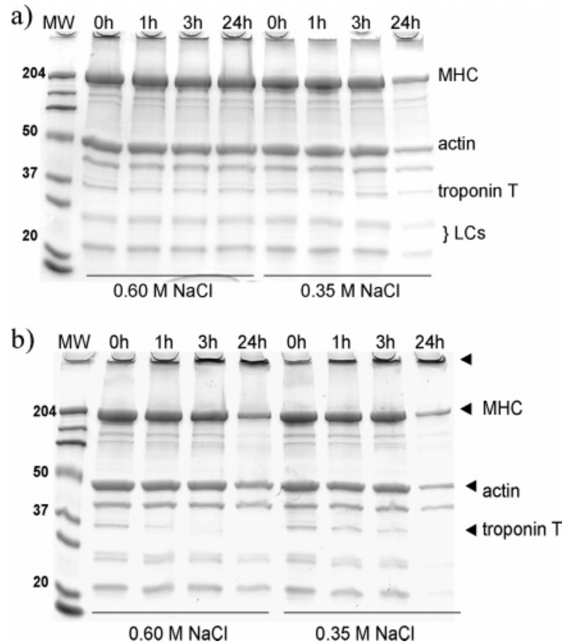

Figure 2. SDS-PAGE of SSPs treated (**a**) without tyrosinase and (**b**) with 1000 nkat tyrosinase/g of protein. Samples were drawn in the beginning and after 1, 3, and 24 h of treatment. SSPs were treated in 0.60 and 0.35 M NaCl. Treatment conditions: 50 mM sodium-phosphate buffer, pH 6, protein concentration 3 mg/mL, 40 °C. Protein amount per lane was approximately 20 μg except in the sample treated for 24 h in 0.35 M NaCl in which the amount of protein was about 10 μg.

tyrosinase (**Figure 2b**) were 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 the enzyme addition (**Figure 2a**) when the NaCl concentration was either 0.35 M or 0.60 M. Six distinct bands, for example, myosin heavy chain (MHC), actin, troponin T, and myosin light chains (LCs), were visible on SDS-PAGE when the proteins were incubated without added tyrosinase (**Figure 2a**). At 0.60 M NaCl, myofibril proteins are practically solubilized, and even a prolonged incubation time (24 h) did not alter the protein pattern. When the NaCl concentration was reduced to 0.35 M, proteins had a tendency to precipitate out of the solution over time. Precipitation indicating the decreased solubility of proteins at the low ionic strength was observed only after 24 h of incubation and can be seen as fading of the protein bands on the gel (**Figure 2a** and **b**).

Molecular changes caused by tyrosinase are shown in **Figure 2b**. Tyrosinase catalyzed similar changes in protein mobility in both NaCl concentrations. Protein polymerization could be observed after 3 h of treatment as an accumulation of high molecular weight protein unable to penetrate the gel. Accumulation was not observed in the control samples. Of the proteins, the myosin heavy chain (MHC, about 200 kDa) was clearly the most susceptible protein to the tyrosinase action although the MHC band began to disappear only when the treatment was prolonged to 24 h. Troponin T (about 30 kDa), on the other hand, was found to start to disappear after 1 h of the treatment in 0.60 M NaCl and after 3 h of the treatment in 0.35 M NaCl. Actin seemed not to be affected by tyrosinase. No conclusion regarding the effect of tyrosinase on actin can be drawn from the 24-h tyrosinase treatment in 0.35 M NaCl as precipitation of proteins occurred also in the control sample.

The three-dimensional shape of myosin and the better accessibility of the target amino acid residues (tyrosine, lysine, and cysteine) may explain the observed higher susceptibility of myosin to cross-linking than that of the less-soluble actin. Interestingly, a similar difference in reactivity between MHC, troponin T, and actin was observed also with TG and laccase (*24*). Tyrosinase (*26*), laccase (*41*), and TG (*42*) have distinct reaction mechanisms and they primarily react with different amino acids. Tyrosinase is known to catalyze a covalent cross-link formation between two phenolic rings of tyrosine residues and at least between tyrosine and an amino group (lysine residues in proteins) and between tyrosine and a thiol group (cysteine residue in proteins) (*26, 28*; **Figure 1**). Myosin and actin contain different amounts of tyrosine, lysine, and cysteine (*5*, *43*). In myosin and actin, the average amount of tyrosine is 3 and 6 g/16 g N, respectively (*5*). The amount of lysine in myosin and actin is 12 and 7 g/16 g N, and the amount of cystine/cysteine is 1 and 1 g/16 g N, respectively (*5*). Chicken breast muscle myosin is reported to contain about 43 thiol groups, 42 of which are located on the outer parts of the molecule (*43*) and thus are readily available for intermolecular bonding. Actin contains only five thiol groups (*44*). Thus, the amount of tyrosine does not explain why myosin is polymerized by tyrosinase while actin is quite resistant to polymerization. In addition to the differences in size, shape, and solubility, myosin contains higher amounts of amino and thiol groups compared to actin, which may explain, at least partially, the observed better susceptibility of myosin to the action of tyrosinase.

Table 2. Heat Transition (ΔH) and Peak Temperature (T_{max}) of the Tyrosinase-Pretreated Myofibrils[a]

tyrosinase dosage (nkat/g protein)	first transition peak (myosin)		second transition peak (actin)	
	ΔH (J/g)	T_{max} (°C)	ΔH (J/g)	T_{max} (°C)
0	6.79 a (0.30)	59.6 a (0.11)	0.22 a (0.09)	66.7 a (0.47)
500	4.71 b (0.20)	59.4 a (0.08)	0.54 ab (0.06)	68.1 ab (0.56)
1000	4.43 b (0.60)	59.2 a (0.44)	0.75 b (0.13)	67.9 ab (0.03)

[a] Pretreatment conditions: 70 mg protein/mL of 50 mM Na–phosphate buffer, pH 6, 0.60 M NaCl, 40 °C, 1 h. Myofibrils were heated from 40 to 100 °C at 10 °C/min. Means with letters in the same column are significantly different at $p < 0.05$. Values in parentheses are standard deviations of the means.

3.2. Thermal Properties of Myofibrils. Depending on the tyrosinase dosage, the myofibril samples showed different thermal behavior (**Table 2**, **Figure 3**). Two major endothermic transitions were observed with peak temperatures (T_{max}) at about 59 °C and 67 °C. These transitions were presumably due to thermal denaturation of myosin and actin, respectively (*6*). The calorimetric enthalpy (ΔH) and peak temperature (T_{max}) of the heat transitions after 1 h pretreatment at 40 °C without tyrosinase were 6.79 J/g and 59.6 °C (first transition peak, myosin) and 0.22 J/g and 66.7 °C (second transition peak, actin). When tyrosinase was added to the system, the ΔH of the myosin peak decreased ($p < 0.05$) with both enzyme dosages, which may be due to tyrosinase-catalyzed polymerization of myosin to larger (**Figure 2**) but less heat energy requiring structures. A similar reduction in thermostability has been detected in TG-treated chicken myofibrils (*24*) and ground beef (*45*). Tyrosinase treatment did not affect the T_{max} of the myosin peak. Treating myofibrils with tyrosinase caused an increase ($p < 0.05$) in the ΔH ($p < 0.05$) and T_{max} ($p > 0.05$) of the second transition, indicating possible conformational changes in the protein requiring more heat energy for denaturation. Actin showed a marked resistance to tyrosinase-catalyzed modification (**Figure 2**) probably because of its globular shape and lack of the target

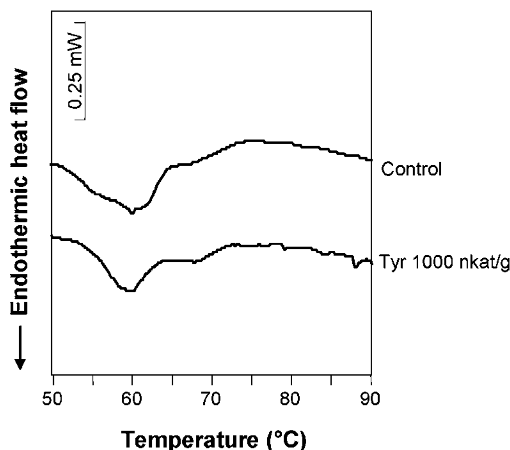

Figure 3. DSC thermogram of chicken breast myofibril suspension (70 mg protein/mL) pretreated with 1000 nkat of tyrosinase/g protein in 50 mM Na phosphate buffer, 0.6 M NaCl, pH 6 for 1 h at 40 °C and heated from 40 to 100 °C at 10 °C/min.

amino acids in the protein accessible to the enzyme. Lantto et al. (*24*) observed a similar increase in the ΔH of the actin transition after treating chicken breast meat myofibrils with TG. Furthermore, in this study actin was found to be resistant to polymerization. TG is reported to form an intramolecular isopeptide bond in rabbit actin (*46*). It is also reported that additional intramolecular bonds in a protein may lead to more stable structures (*47*).

3.3. Gel Formation of Myofibrils. The effect of tyrosinase on gel formation of a 4% chicken myofibril suspension was monitored by measuring the G' and G'' of the sample suspensions at constant temperatures of 25 °C and 40 °C for 3 h at 0.35 M NaCl. Without tyrosinase, the G' increase of the myofibril gel was very low at both temperatures, 168 Pa at 25 °C and 118 Pa at 40 °C (**Figure 4**). The G' values were low because of the limited solubility of the myofibril proteins at the ionic concentration used. Tyrosinase addition to the system enhanced the gel formation. A dosage of 60 nkat tyrosinase/g protein increased the G' by 66 Pa at 25 °C and by 146 Pa at 40 °C. When the tyrosinase dosage was doubled to 120 nkat/g protein, the G' increase compared to the control without the added enzyme was 93 Pa at 25 °C and 277 Pa at 40 °C. As expected, the tyrosinase dosage of 240 nkat/g of protein increased the G' even further to 195 Pa at 25 °C and 469 Pa at 40 °C compared to the control. As shown, tyrosinase was capable of cross-linking MHC and troponin T according to SDS-PAGE (**Figure 2b**). Increasing the G' values by increasing the tyrosinase dosages showed that tyrosinase had catalyzed cross-links among these proteins, causing polymerization. The increase in gel formation when the temperature was raised from 25 °C to 40 °C was due to the enhancement of tyrosinase activity as a function of raising the temperature.

3.4. Firmness and Weight Loss of the Cooked Chicken Breast Meat Homogenate Gels. The effect of tyrosinase treatment on firmness and weight loss was studied by carrying out the treatments in closed cylinders without mixing of the meat homogenates. The oxygen supply was assured by oxygenating the brine before adding it to the meat homogenate. Determining the influence of oxidation per se, which is known to be detrimental to some extent to the meat product quality (*2*), was not, however, an object in this study. To demonstrate the effects of tyrosinase on textural and water-holding properties, meat homogenate samples containing reduced amounts of meat (LM), salt (LS), or phosphate (NoPP, LSPP) (**Table 1**) were treated under the temperature conditions described in section 2.3 in the presence of tyrosinase (0, 20, and 120 nkat/g protein) and were analyzed for maximum compression force and weight loss. The values of the maximum compression force and weight loss of the no-enzyme homogenates (control, NoPP, LM, LS, LSPP) and the tyrosinase-treated homogenates (NoPP, LM, LS, LSPP) are presented in **Table 3**.

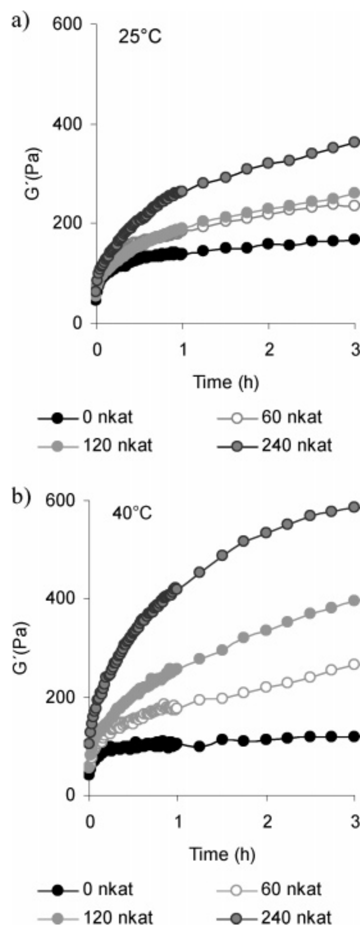

Figure 4. Storage modulus (G') of chicken breast myofibril suspension treated with 0, 60, 120, and 240 nkat of tyrosinase/g of protein. G' was measured at (**a**) 25 °C and (**b**) 40 °C for 3 h. Reaction mixture: 40 mg protein/mL, 50 mM sodium phosphate buffer, 0.35 M NaCl, pH 6.

Table 3. Maximum Compression Force and Weight Loss of the Cooked Control Homogenate and Tyrosinase-Treated Homogenates Containing Lowered Amounts of Phosphate, Meat, or Salt

tyrosinase dosage (nkat/g)	control	NoPP[a]	LM	LS	LSPP
		Maximum Compression Force (g)			
0	13 384 (148)	10 228 a (262)	6551 a (181)	6601 a (748)	6162 a (358)
20	nd	11 922 b (375)	7171 a (255)	6575 a (268)	4575 b (604)
120	nd	11 993 b (950)	9554 b (459)	6509 a (382)	4428 b (446)
		Weight Loss (%)			
0	5.8 (0.20)	6.9 a (0.12)	9.0 a (0.72)	11.0 a (0.51)	12.4 ab (1.06)
20	nd	6.9 a (0.23)	7.0 b (0.65)	8.0 b (0.85)	12.7 ab (1.48)
120	nd	6.2 a (0.12)	6.3 b (0.74)	9.3 ab (0.47)	13.2 b (0.64)

[a] NoPP, phosphate-free; LM, low-meat; LS, low-salt; LSPP, low-salt/low-phosphate (see composition of the homogenates in Table 1). Treatment conditions: 1 h at 4 °C, 1 h at 40 °C, heating until the core temperature of the samples reached 72 °C. Means with different letters in the same column are significantly different at $p < 0.05$. Values in parentheses are standard deviations of the means. nd, not determined.

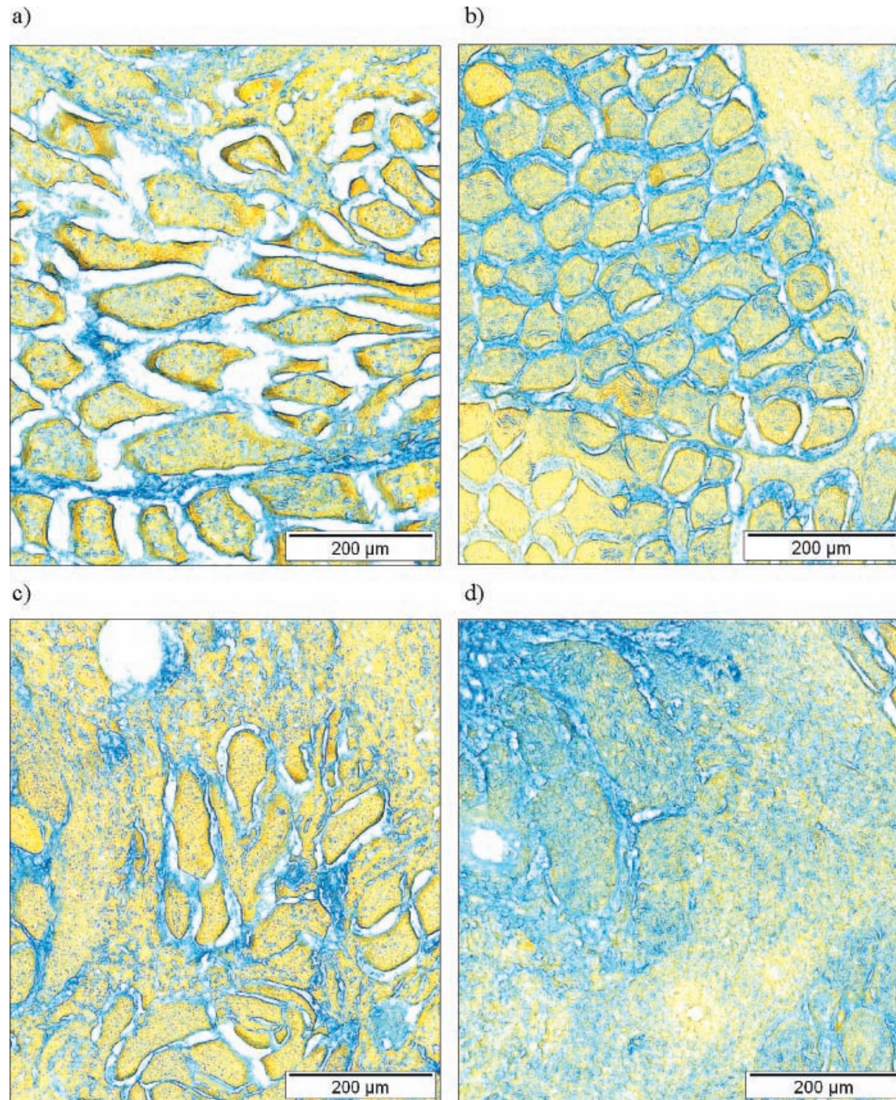

Figure 5. Micrographs showing cooked chicken breast meat homogenates. LS homogenates containing 75% meat and 1% NaCl treated (**a**) in the absence and (**b**) in the presence of tyrosinase. LM homogenates containing 65% meat and 2% NaCl treated (**c**) in the absence and (**d**) in the presence of tyrosinase.

3.4.1. Effect of Tyrosinase on Firmness and Water-Holding of Cooked Chicken Breast Meat Homogenate Gels. A meat homogenate containing 75% meat, 2% salt, and 0.34% phosphate (no. 1 in **Table 1**) was used as a benchmark and was not treated with tyrosinase. As expected, the marked impairment of texture and water-holding occurred when the meat (LM) or salt (LS) content was reduced in the system (**Table 3**). Omitting phosphate from the system (NoPP) led to softer texture although water-holding was not affected. The potential of tyrosinase to improve either texture or water-holding of the NoPP, LM, LS, and LSPP (low salt and phosphate content) systems was studied. Comparison of the tyrosinase-treated homogenates to the corresponding no-enzyme control of each homogenate type (nos. 2−5 in **Table 1**) as well as to the benchmark control (no. 1 in **Table 1**) is shown in **Table 3**.

A higher tyrosinase dosage improved the firmness of the phosphate-free (NoPP) homogenate from 10 228 g to 11 993 g ($p < 0.05$), which is not significantly ($p > 0.05$) lower than the firmness of the benchmark control. Regardless of tyrosinase, weight loss was maintained on the level of the NoPP control and was not significantly ($p > 0.05$) higher than that of the benchmark control.

When the meat content was reduced from 75% to 65% (LM), gel firmness reduced drastically (from 13 384 g to 6551 g). However, when using the higher tyrosinase dosage, gel firmness was significantly ($p > 0.05$) improved compared to the LM control (from 6551 g to 9554 g), but it did not reach the gel firmness level of the benchmark control (no. 1, 13 384 g). However, tyrosinase was capable of improving water-holding to the level of the benchmark control. The weight loss decreased from 9.0% to 6.3% ($p < 0.05$) with the higher tyrosinase dosage. When the salt amount of the homogenate was reduced from 2% to 1% (LS), tyrosinase treatment maintained the texture of the homogenate at the LS control level but could not improve it. However, tyrosinase decreased weight loss in the system. In the LS homogenate, weight loss was reduced from 11.0% to 8.0% ($p < 0.05$) and 9.3% ($p > 0.05$) with a lower and higher tyrosinase dosage, respectively. The lower enzyme dosage led to better water-holding than the higher. The result can be regarded as a consequence of the variation in the experimental setup. In conclusion, tyrosinase was capable of a slight improvement of the weight loss in the LS system, but the improvement was not significant compared to the weight loss of the benchmark control having a higher salt content. In the

low-salt and low-phosphate system (LSPP), tyrosinase decreased gel firmness. Both tyrosinase dosages resulted in significant ($p < 0.05$) reduction of the gel firmness, that is, from 6162 g to 4575 g with the higher dosage and to 4428 g with the lower. Weight loss was, however, maintained at the level of the LSPP control with both tyrosinase dosages.

On the basis of the results obtained, it can be concluded that tyrosinase did not significantly increase the weight loss in any of the studied meat homogenate systems and it improved the texture of the meat systems free of phosphate (NoPP) and with low meat amount (LM). The obtained results are promising when comparing them to those obtained in meat systems treated with TG. Although TG has been reported to increase yield or WHC in low-salt meat products (*18*, *19*), it is also agreed that cross-linking catalyzed by TG may negatively affect development of the meat protein matrix during heating, resulting in reduced water holding in the system. This view is supported by, for example, Carballo et al. (*21*).

In the tyrosinase-catalyzed cross-links, a tyrosine residue is always the other counterpart of the covalent bond. In TG-catalyzed cross-links, the essential amino acid is glutamine. When isolated myofibril proteins are treated, myosin is supposed to be the main substrate for the enzymes. In myosin, the amount of tyrosine residues is about 3% and glutamine/glutamic acid is about 22% (*5*). When meat is treated with these enzymes, collagen should also be regarded as the target protein as it is known to act as a substrate for TG (*48*) and tyrosinase (*49*). In collagen, the amount of tyrosine is <0.5% and glutamine/glutamic acid is 7−11% (*50*). It is possible that because of the much lower amount of tyrosine compared to glutamine, both in myosin and collagen, the action of tyrosinase is more limited than that of TG. This may be an advantage as too extensive a formation of covalent bonds may well lead to a too-restricted swelling of myofibrils therefore causing decline in WHC. A reduction of WHC because of tyrosinase-aided cross-linking was not observed in the used chicken meat homogenate systems with low meat, salt, and phosphate levels. Being the first reported study in which tyrosinase was used in the modification of texture and WHC of a cooked meat system, generalization of the action of tyrosinase in meat matrixes cannot, however, be made and further research to confirm the reported results is definitely needed.

3.4.2. Effect of Tyrosinase on the Microstructure of Cooked Chicken Breast Meat Homogenate Gels. To study the effect of tyrosinase on collagen, the cooked LS and LM homogenate gels were stained with Aniline Blue, a collagen-specific stain, and Orange G. The control homogenates, LS (**Figure 5a**) and LM (**Figure 5c**), showed a more granulated collagen structure than the corresponding homogenates treated with tyrosinase (**Figure 5b** and **d**). In the tyrosinase-treated LS homogenate (**Figure 5b**), collagen appeared to form a network rather than granular or fibrous structures as in the LS control (**Figure 5a**). In the LM system, the difference in the collagen state between the tyrosinase-treated (**Figure 5d**) and the control homogenates (**Figure 5c**) was even more apparent. Collagen covered the other proteins with a dense network. During homogenation, the muscle structure was not completely destroyed and muscle fibers covered by traces of the endomycium sheath could be observed (**Figure 5a** and **c**). Because of limited solubility of the myofibrillar proteins in the LS system, the muscle fibers may have maintained their structure to a certain degree and thus may have prevented the proteins from acting as efficient substrates for tyrosinase, whereas collagen of the endomycium had apparently been exposed to the action of tyrosinase resulting in cross-linking between collagen molecules and subsequent network formation as shown in **Figure 5b**. The higher amount of salt in the LM homogenates increased the solubility of the myofibrillar proteins, possibly also causing disruption of the fiber structure and finally enabling formation of the more even collagen network shown in **Figure 5d**. Apparent tyrosinase-catalyzed cross-linking of collagen and the subsequent network formation may have had a role in the increase of the gel firmness and decrease of weight loss detected in the cooked LM homogenate gels and also to a lesser degree and limited to the reduction of weight loss, in the cooked LS homogenates (**Table 3**).

In conclusion, these results show, for the first time, the potential of using a cross-linking enzyme tyrosinase, particularly that from the filamentous fungus *Trichoderma reesei*, to catalyze cross-links between myofibrillar proteins and to exploit the cross-link formation to modify thermal properties, gel forming, and gel properties of meat protein systems. Although TG-aided cross-linking is well-adopted and well-working means for texture modification of many meat products, reduced WHC observed often as a consequence of TG treatment limits its exploitation. In the future when tyrosinase is available for industrial use, it may well offer an interesting alternative to TG in various meat applications, as reduced WHC did not turn out to be a problem in the tyrosinase-treated low-salt or low-meat systems studied in this work.

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