

Raija Lantto

Protein cross-linking with oxidative enzymes and transglutaminase

| Effects in meat protein systems

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Abstract

The oxidative enzymes tyrosinase and laccase, as well as the acyltransferase transglutaminase (TG), are capable of creating covalent cross-links in proteinaceous substrates. These enzymes differ from each other on the basis of their different reaction mechanisms and the amino acid residues with which they react. TG and tyrosinase are well-known protein cross-linkers, and TG is already used industrially in the food sector. The commercial feasibility of tyrosinase is under assessment, whereas the effectiveness of laccase is not known because the reaction mechanism of protein modification by laccase is still poorly understood.

The effects of tyrosinase-, laccase- and TG-catalysed protein modification were studied in different meat protein systems from the myofibrillar proteins of chicken breast muscle to heated ground chicken breast meat systems containing different amounts of meat and salts. The study was focused on the effects of the enzymes on the thermal behaviour and gel formation properties of myofibrils, and on the textural and water-holding properties of the heated meat systems.

The cross-linking efficiency of a novel tyrosinase from the fungus *Trichoderma reesei* was compared to that of the commercial tyrosinase from the mushroom *Agaricus bisporus*. *Trichoderma* tyrosinase was found to be superior compared to the *Agaricus* enzyme in its protein cross-linking efficiency and in the incorporation of a small molecule, dihydroxyphenylalanine, into a complex proteinaceous substrate, wool fibre. *Agaricus* tyrosinase was found to have a propensity towards oxidation of small tyrosine-containing substrates.

All three enzymes, although having different reaction mechanisms, affected the same myofibrillar proteins. Myosin and troponin T were found to be most sensitive to enzymatic modification, whereas actin was clearly more resistant. Tyrosinase, laccase and TG all polymerised myofibrillar proteins, but only

laccase was found to cause protein fragmentation. The fragmentation products originated from myosin and troponin T. In the differential scanning calorimetric (DSC) measurements alteration of the peak temperature of myosin or actin transitions was not observed. However, tyrosinase and TG decreased, whereas laccase increased, the heat of myosin transition. The heat of actin transition was increased with all three enzymes.

Tyrosinase and TG improved the gel formation of a 4% myofibrillar suspension at 0.35 M (2%) NaCl. With both enzymes the gel formation increased along with increasing enzyme dosage, showing a positive connection between covalent cross-link and gel formation. Laccase was able to increase the gel formation slightly only when the NaCl concentration was increased to 0.60 M (3.5%). With an excessive laccase dosage the gel formation declined markedly due to protein fragmentation.

Tyrosinase, laccase and TG had different effects on the texture and water-holding of the heated chicken breast meat homogenates. The homogenates were prepared free of phosphate (75% meat, 2% NaCl), with a low meat content (65% meat, 2% NaCl, 0.34% phosphate), with a low salt content (75% meat, 1% NaCl, 0.34% phosphate) or with low amounts of both NaCl and phosphate (75% meat, 1% NaCl, 0.17% phosphate). Tyrosinase improved the firmness of the homogenate gels free of phosphate and with a low amount of meat. TG improved the firmness of all studied homogenates. Laccase weakened the gel firmness of the low-meat, low-salt and low-salt/phosphate homogenates and maintained the firmness on the control level in the homogenate free of phosphate. Tyrosinase reduced the weight loss in the homogenates containing a low amount of meat and a low amount of NaCl, whereas TG and laccase were not able to decrease the weight loss of any homogenate. TG was the only enzyme that could positively affect the firmness of the homogenate gel containing both low NaCl (1%) and phosphate (0.17%) amounts.

In pilot scale the test products were made of coarsely ground chicken breast fillet with a moderate amount of salt (1.2–1.8% NaCl), a meat amount 70–80% and a TG dosage 0–20 nkat/g protein (0–0.2% of the meat mass). All three factors, meat, salt and TG contents, favoured the development of firmness of the test products. The evaporation loss decreased slightly along with increasing TG and NaCl amounts in the experimental conditions used, indicating a positive interaction between these two factors.

In this work it was shown that tyrosinase, laccase and TG affected the same myofibrillar proteins. However, these enzymes had distinguishable effects on the gel formation of a myofibril system as well as on the textural and water-holding properties of the finely ground meat homogenates, reflecting distinctions at least in the reaction mechanisms and target amino acid availability in the protein substrates for these enzymes.

Academic dissertation

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Preface

The study described in this thesis was carried out at VTT Technical Research Centre of Finland during the years 2004–2006. The research was made in the projects “Controlled modification of carbohydrates and proteins” and “High Performance Industrial Protein Matrices through Bioprocessing”. Tekes – Finnish Funding Agency for Technology and Innovation, European Commission and participating companies are acknowledged for partially funding the research.

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My co-authors Professor Johanna Buchert, Dr. Nisse Kalkkinen, Dr. Kati Katina, Dr. Kristiina Kruus, Dr. Maija-Liisa Mattinen, M.Sc. Markku Niemistö and M.Sc. (Eng.) Emilia Selinheimo are thanked for sharing their knowledge with me, and providing constructive criticism and support during the work. I also thank Michael Bailey for revising the English language and Päivi Vahala for the secretary work. My sincere thanks are due to Heljä Heikkinen, Ritva Heinonen and Eeva Manninen for their skilful technical assistance beyond comparison.

My dearest gratitude belongs to my family, sweet daughters Aino and Eeva, as well as to Matti, their father. Without much complaining they compromised their own comfort and provided me with undisturbed moments to finalise the thesis.

List of publications

This work is based on the following Publications I–IV, which are referred to in the text by their Roman numerals.

- I Mattinen, M.-L., Lantto, R., Selinheimo, E., Kruus, K., and Buchert, J. Oxidation of peptides and proteins by *Trichoderma reesei* and *Agaricus bisporus* tyrosinases. J. Biotechnol. (submitted).
- II Lantto, R., Puolanne, E., Kruus, K., Buchert, J., and Autio, K. (2007) 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. 55: 1248–1255.
- III Lantto, R., Puolanne, E., Kalkkinen, N., Buchert, J., and Autio, K. (2005) Enzyme-aided modification of chicken-breast myofibril proteins: Effect of laccase and transglutaminase on gelation and thermal stability. J. Agric. Food Chem. 53: 9231–9237.
- IV Lantto, R., Puolanne, E., Katina, K., Niemistö, M., Buchert, J., and Autio, K. (2007) Effect of laccase and transglutaminase on the textural and water-binding properties of cooked chicken breast meat gels. Eur. Food Res. Technol. 225: 75–83.

The author of the thesis was responsible for planning the research, biochemical, textural and microscopical analyses and interpretation of the data in all publications, with the following exceptions: the chromatographic and mass spectrometric analyses of the peptides and interpretation of the results was performed by Maija-Liisa Mattinen (Publication I), the mass spectrometric analyses of the proteins and interpretation of the results was carried out by Nisse Kalkkinen (Publication III), experimental work of the pilot scale trial was carried out by Markku Niemistö and design and data analysis by Kati Katina (Publication IV).

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Publications I–IV

*Appendices of this publication are not included in the PDF version.
Please order the printed version to get the complete publication
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Abbreviations

BSA	bovine serum albumin
ΔH	heat of transition
DOPA	dihydroxyphenylalanine
DSC	differential scanning calorimetry
G'	storage modulus
LC	myosin light chain
MALDI-TOF	matrix assisted laser desorption/ionisation – time of flight
MHC	myosin heavy chain
MS	mass spectrometry
MW	molecular weight
nkat	nanokatal
PMS	permonosulphuric acid
PPO	polyphenol oxidase
RP-HPLC	reverse-phase high-performance liquid chromatography
SSP	salt-soluble protein
TG	transglutaminase
UHT	ultra-high temperature
WHC	water-holding capacity
Y	tyrosine

1 Introduction

Foods are multicomponent matrices with complex structures. The macromolecular structure of food affects its mechanical and physical properties, chemical and microbiological stability, diffusion properties, product engineering, sensory properties and nutrition. The textural and water-holding properties of food play a major role in food product quality, as they are recognised by consumers and are factors behind food choice. Proteins along with carbohydrates and fats are the main components affecting the textural and water-holding properties of foods. Cross-linking and aggregation of protein molecules into three-dimensional networks is an essential mechanism for developing food structures with desirable mechanical properties (Dickinson, 1997). In most cases protein cross-linking takes place in a food manufacturing process in which proteins are denaturated, i.e. transformed from biologically active molecules to disordered structures.

The functional properties of a protein network are defined by the type and number of chemical bonds and interactions that hold individual protein molecules together. Hydrophobic and electrostatic interactions, van der Waals forces as well as hydrogen, ionic, and covalent bonds all contribute to the formation of protein gel networks, covalent bonds having clearly the highest binding energy. In proteins covalent cross-links can be formed either within a protein (intramolecular cross-linking) or between proteins (intermolecular cross-linking) (Feeney and Whitaker, 1988).

Cross-links can be introduced to a food protein matrix by chemical, physical and enzymatic means as reviewed by Singh (1991) and Gerrard (2002). Enzymatic cross-linking is an attractive approach due to the high specificity of the enzyme catalysis. Moreover, enzymatic cross-linking can be controlled to a certain degree by changing the temperature or pH. The best-known enzymatically created cross-links are the isopeptide bonds catalysed by transglutaminases (TGs), the bonds in which tyrosine acts as a counterpart catalysed by tyrosinases and peroxidases as well as disulfide bonds catalysed by sulfhydryl oxidases (Matheis and Whitaker, 1984a, b and 1987; Feeney and Whitaker; 1988; Gerrard, 2002). Cross-linking can be a result of a direct enzymatic catalysis as in the case of TGs and sulfhydryl oxidases, or it can occur indirectly by enzymatic

production of reactive cross-linking agents, such as quinones, radicals or hydrogen peroxide, which in turn are able to react non-enzymatically with certain chemical groups in proteins with a subsequent cross-link formation. Tyrosinases, laccases, peroxidases, as well as glucose and hexose oxidases are examples of enzymes performing this type of catalysis. Enzymatically catalysed cross-links in proteins are formed between reactive groups of amino acid residues, such as the primary amino groups of glutamine and lysine, the phenolic group of tyrosine and the thiol group of cysteine. The efficiency of the reactions is dependent on the accessibility of the target amino acids in a protein, the treatment or process conditions used and the type of enzyme. Cross-linking changes the molecular size and/or conformation of a protein, often leading to changes in its functional properties (Gerrard, 2002). Potential cross-linking enzymes for food structure engineering are summarised in Table 1.

Table 1. Potential cross-linking enzymes for food structure engineering.

Enzyme	Reaction	Target amino acid(s) in proteins	Note	Reference
Transglutaminase (EC 2.3.2.13)	Formation of an isopeptide bond	Protein-bound glutamine, lysine		Folk (1980); Kuraishi (2000)
Tyrosinase (EC 1.12.18.1)	Oxidation of the phenolic moiety of tyrosine	Tyrosine		Ito et al. (1984); Matheis and Whitaker (1984a)
Laccase (EC 1.10.3.2)	Radical-generating oxidation of aromatic compounds	Tyrosine	Acts with carbohydrates containing ferulic acid residues	Færgemand et al. (1998a); Figueroa-Espinoza and Rouau (1998); Mattinen et al. (2005)
Peroxidase (EC 1.11.1.7)	Radical-generating oxidation of aromatic compounds	Tyrosine	Acts with carbohydrates containing ferulic acid residues	Matheis and Whitaker (1984b); Hillhorst et al. (1999)
Glucose and hexose oxidases (EC 1.1.3.4 and EC 1.1.3.5)	Formation of H ₂ O ₂ in conjunction with glucose or hexose oxidation		Indirect reaction with proteins due to H ₂ O ₂ formation	van Dijken and Veenhuis (1980); Poulsen and Bak Høstrup (1998)
Sulfhydryl oxidases (EC 1.8.3)	Oxidation of cysteine residues to form disulfide cross-links	Cysteine		Aurbach and Jakoby (1962); Kusakabe et al. (1982)

The effects of cross-linking enzymes are many and they are highly dependent on the applications. In the cereal sector TG has been used to improve the strength of weak and low-quality flour (Köksel et al., 2001; Autio et al., 2005), texture and volume of bread (Wijngaards et al., 1997) and texture of pasta after cooking (Yamazaki and Nishimura, 2002; reviewed in Kuraishi et al., 2001). Cross-linking can also be exploited in milk products such as yoghurts in order to prevent syneresis or to make the soft texture firmer (Lorenzen et al., 2002; reviewed in Sodini et al., 2004). Casein micelles in milk have been reported to be stabilised by TG against e.g. high pressure and heating in the presence of ethanol (Smiddy et al., 2006). The effects of cross-linking enzymes in meat systems, emphasising the role of TG, are reviewed in detail in the following sections.

The high costs of meat production require ways to use the meat raw material in an efficient way. One approach is to restructure or reshape a value-added meat product from lower-value fresh meat parts by binding them together. Improvement of binding between meat pieces with the help of targeted and controlled formation of covalent cross-links by enzymes is an approach already adopted in the meat industry. TG has shown to be an efficient binding agent (Kuraishi et al., 1997). Moreover, many processed meat products such as sausages and hams are today often associated with a negative health image due to their high salt content. As salt contributes to many properties of the meat products, both technological and sensory, reduction of its concentration is not straightforward (Ruusunen and Puolanne, 2005). Improvement of the textural properties of low-salt meat products by TG was recently reported by e.g. Carballo et al. (2006).

The ability of TG to cross-link different meat proteins has been generally known for over two decades. In early studies the TG used was purified from mammalian sources such as human plasma (Kahn and Cohen, 1981), bovine plasma (Kurth, 1983; Kurth and Rogers, 1984) or guinea pig liver (Ikura et al., 1992), all of which are Ca^{2+} -dependant enzymes. Industrial application of TG in meat binding started about a decade ago when microbial TGs were shown to polymerise various food proteins (Nonaka et al., 1989). Microbial TGs are far easier to produce than the mammalian enzymes and are more feasible due to their Ca^{2+} independence.

The following literature review focuses on protein cross-linking enzymes and particularly the exploitation of these enzymes in modification of textural and water-holding properties in meat protein systems. However, it should be noted that these enzymes also have potential in other sectors than food, e.g. the textile and cosmetic industries.

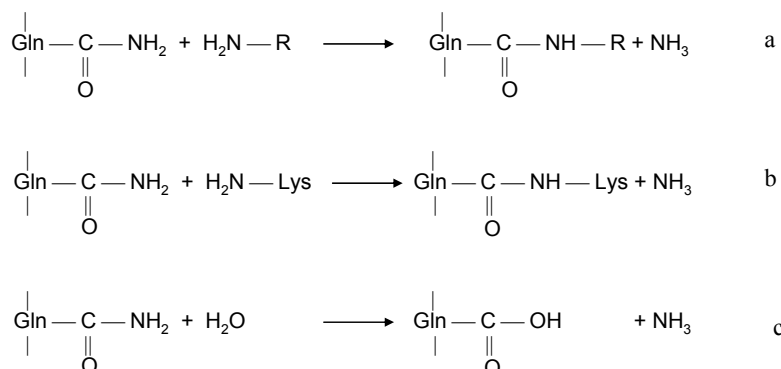
2 Review of the literature

2.1 Enzymatic cross-linking of proteins

A number of enzymes are known to introduce various types of cross-links into proteins. Hitherto only TGs, peroxidases and glucose and hexose oxidases are used as processing aids in the food industry. TG is applied particularly in the meat but also increasingly in the dairy sector (Nielsen, 1995; Jaros et al., 2006). Peroxidases and glucose oxidases are exploited in baking (van Oort, 1996). Recently the potential of tyrosinase (Halaouli et al., 2005), laccase (Yamaguchi, 2000), and sulfhydryl oxidase (Thorpe et al., 2002) as cross-linking agents in food applications has been recognised. TG and the oxidative enzymes all have dissimilar reaction mechanisms but have partially overlapping sets of amino acid residues with which they react. However, the resulting cross-links vary due to the different modes of action of the enzymes. The distribution, physiological roles, reaction mechanisms, biochemical properties and application potential of TG and oxidative enzymes in food systems are reviewed in the following sections.

2.2 Transglutaminases

Transglutaminases (TG, EC 2.3.2.13, γ -glutamyl-peptide, amine- γ -glutamyl transferase) belong to the group of acyltransferases, which catalyze acyl-transfer reactions between a γ -carboxyamine group of a peptide- or protein-bound glutamyl residue and a primary amino group of various substrates including the ϵ -amino group of lysine or lysyl residues in proteins, resulting in polymerisation or amine incorporation (Figures 1a and b). The cross-link formed is called an ϵ -(γ -glutamyl)lysine isopeptide bond (Folk and Finlayson, 1977; Griffin et al., 2002). During the reaction one molecule of ammonia is generated per cross-link. If the amine substrates are not available as acyl acceptors, TG can catalyze deamination of glutamyl residues using water as an acyl acceptor (Figure 1c). In contrast to their limited glutamine (an acyl donor) substrate specificity, TGs possess a wide specificity for the acyl acceptor substrates.



R, protein

Figure 1. TG-catalysed reactions: (a) cross-linking via a lysyl residue, (b) incorporation of an ϵ -amino group of lysine and (c) deamidation (Folk, 1980).

The first TG, cytoplasmic TG 2, was identified in guinea pig liver 50 years ago (Sarkar et al., 1957), and since then TGs have been identified in various mammal tissues and body fluids (reviewed by Ha and Iuchi, 2003), fish (reviewed by An et al., 1996), chicken (Weraarchakul-Boonmark et al., 1992), invertebrates (Singer et al., 1992; Cariello et al., 1997) and plants (Icekson and Apelbaum, 1987; reviewed by Serafini-Fracassini et al., 1995). TG-like activity was found by Lantto et al. (2006) in apple pomace, a co-product of industrial cider production. Biotechnical exploitation of TG in various food and non-food applications was launched over ten years ago after these enzymes had been discovered in bacteria such as *Streptomyces mobaraense* (Ando et al., 1989), *S. ladakanum* (Tsai et al., 1996; Tzeng et al., 2005), *S. cinnamoneum* (Duran et al., 1998), and *Bacillus subtilis* (Kobayashi et al., 1998; Suzuki et al., 2000).

The best known mammalian TG is the extracellular plasma TG, also known as the blood coagulation factor XIIIa. According to its name it plays a role in blood clotting and hence in wound healing (Folk and Finlayson, 1977; Hornyak et al., 1989). Another mammalian TG, the cytoplasmic TG 2 (reviewed by Chen and Mehta, 1999 and Fesus and Piacentini, 2002), has diverse functions ranging from intracellular signalling to apoptosis and pathological conditions such as celiac and Huntington diseases. This enzyme shows general stabilising and protective roles in cells. In its absence conditions such as impaired wound healing, autoimmunity and diabetes have been observed in mice. Mammalian epidermal TG (type 3) has a role in epidermal keratinisation as reviewed by Griffin et al. (2002).

Properties of TGs vary considerably depending on the source. Mammalian blood coagulation factor XIIIa is a tetramer, comprising two catalytic subunits (75 kDa) and two non-catalytic subunits (80 kDa) (reviewed by Ichinose et al., 1990 and Serafini-Fracassini et al., 1995). Human cytoplasmic TG 2 is a 76 kDa protein which occurs as a dimer (Fesus and Piacentini, 2002). *S. mobaraensis* TG is a monomer with a molecular mass of 38 kDa (Ando et al., 1989) and that of *S. ladakanum* has a molecular mass of 30.5 kDa (Tsai et al., 1996). In contrast to the mammalian TGs the *Streptomyces* TGs are independent of a Ca²⁺ cofactor. *S. mobaraense* TG is a secreted protein, which is activated outside the cytoplasm membrane by post-translational processing (Pasternack et al., 1998). The enzyme participates in mycelial growth and has a role in morphological differentiation. The enzyme shows an optimum in the pH range of 5–8, retains full activity at 40°C for 10 min but is rapidly inactivated at 70°C (Ando et al., 1989). Microbial TGs are hitherto the only commercially available food-grade protein cross-linking enzymes.

TGs have been reported to cross-link e.g. myofibrillar proteins (Kahn and Cohen, 1981), gelatine (Broderick et al., 2004), milk proteins (reviewed by Jaros et al., 2006), soy proteins (Nio et al., 1985; Babiker, 2000), egg yolk and white (Sakamoto et al., 1994; Lim et al., 1998), fish proteins (Joseph et al., 1994) and cereal proteins (Alexandre et al., 1993; Larré et al., 2000; Basman et al., 2002a and b). Some of the TG-induced effects of non-meat food proteins are summarised in Table 2.

Modification of proteins by TG may be exploited in a number of applications in the food industry such as by improving rheological properties, encapsulation of lipids or lipid-soluble materials, improved gel formation and gel properties, modification of protein solubility, foaming properties and water-holding properties (Matheis and Whitaker, 1987; Nielsen, 1995; Seguro et al., 1995; Kuraishi et al., 1997; Motoki and Seguro, 1998; Zhu et al., 1999; Jaros et al., 2006).

TG has also been shown to have potential in the textile industry. Functional molecules have been grafted onto wool fibres by TG (Gembeh et al., 2005). TG has also been shown to remediate the damage caused by chemicals and proteases during wool processing (Cortez et al., 2004) and washing of wool fabrics (Cortez et al., 2005).

Table 2. Effects of TGs on non-meat food proteins.

Protein	Origin of TG	Effect	Reference
Soy proteins	Guinea pig liver	Protein polymerisation	Ikura et al. (1980)
	Bovine plasma	Protein polymerisation	Kurth and Rogers (1984)
	Human placenta	Gel formation	De Backer-Royer et al. (1992)
	<i>Streptomyces</i> sp.	Gel formation	Nonaka et al. (1989)
	Bacterial	Protein polymerisation	Basman et al. (2002a); Ramirez-Suarez and Xiong (2003)
Milk proteins	<i>Streptomyces</i> sp.	Formation of α 1-casein gels	Nonaka et al. (1989)
		Improved strength of Na-caseinate gels	Sakamoto et al. (1994)
		Improved acid-induced gelation of micellar casein	Schorsch et al. (2000a and b)
		Altered Ca-sensitivity, hydrophobicity and viscosity of casein micelles	O'Connell and de Kruijff (2003)
	Guinea pig liver	Polymerisation of α -lactalbumin and β -lactoglobulin	Aboumahmoud and Savello (1990)
	<i>Streptomyces lydicus</i>	Polymerisation of α -lactalbumin and β -lactoglobulin	Færgemand et al. (1997)
	<i>Streptomyces mobaraense</i>	Increased surface shear viscosity of Na-caseinate and casein protein films	Færgemand et al. (1999)
	<i>Streptomyces mobaraense</i>	Improvement of water vapour permeability of a casein-gelatin film	Chambi and Grosso (2006)
		Improved gel formation rate, rheological properties and gel microstructure of acidified caseinate gels	Myllärinen et al. (in press)
		Improved physical and sensory properties of non-fat yogurt	Ozer et al. (2007)
Cross-linking of micellar caseins in the presence of glutathione and without heat pre-treatment		Bönisch et al. (2007)	
Egg white proteins	Bacterial	Protein polymerisation, improved gelation	Lim et al. (1998)
Cereal proteins	<i>Streptomyces</i> sp.	Polymerisation of glutenin subunits, reinforcement of gluten network	Larré et al. (2000)
		Polymerisation of oat globulin, altered textural properties, improved water and fat binding	Siu et al. (2002)
		Improved thermal stability of gluten from damaged flour	Caballero et al. (2005)
	Bacterial	Wheat and barley protein polymerisation	Basman et al. (2002a)

2.3 Oxidative enzymes

Potential oxidative enzymes (oxidases or oxidoreductases) in food processing are phenol oxidases (tyrosinase and laccase), peroxidases, glucose and hexose oxidases and sulfhydryl oxidases (Whitaker et al., 2003). Oxidase is an enzyme which catalyzes the transfer of electrons from one molecule (an oxidant, a hydrogen donor or electron donor) to another (a reductant, a hydrogen acceptor or electron acceptor) (reviewed by Burton, 2003). Except for peroxidases, which use hydrogen peroxide (H_2O_2), the above-mentioned enzymes use molecular oxygen (O_2) as the electron acceptor. Although these enzymes have distinct biological roles in their native host organisms all of them are either emerging (tyrosinase, laccase, sulfhydryl oxidases) or already established (peroxidase, glucose/hexose oxidases) biotechnical tools for modifying protein and carbohydrate polymers and/or heteropolymers in foods.

2.3.1 Tyrosinase

Tyrosinases (EC 1.14.18.1) are widely distributed in nature, and they are mainly involved in the biosynthesis of melanin pigments from tyrosine amino acid as well as from other polyphenolic compounds. Tyrosinases have been characterised from bacteria, fungi, plants, insects and higher animals (Mayer and Harel, 1979; Lerch, 1981; Claus and Decker, 2006). Tyrosinases catalyse two distinct oxidative reactions, namely hydroxylation by monophenolase (cresolase) and oxidation by diphenolase (catecholase) activity (Figure 2). Oxygen is the co-substrate in both of these reactions (Lerch, 1983; Solomon et al., 1996). For the hydroxylation one atom of O_2 is incorporated into the aromatic ring of the monophenolic substrate and the other is reduced to water. As the result of the first oxidation an *o*-diphenol is formed from a monophenol. In the second reaction *o*-diphenol is further oxidised to a corresponding *o*-quinone. Quinones are reactive compounds and can react spontaneously to form high-molecular weight compounds or melanin pigments. The first three-dimensional structure of tyrosinase was published only recently (Matoba et al., 2006). The tyrosinase gene originated from the prokaryote *Streptomyces castaneoglobisporus*.

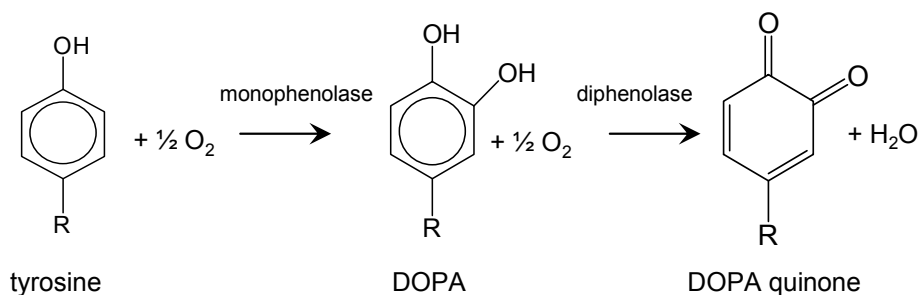


Figure 2. Oxidation of a monophenol (tyrosine) to an o-diphenol (DOPA) and an o-quinone (DOPA quinone) by tyrosinase (modified from Lerch, 1983; Solomon et al., 1996; Claus and Decker, 2006).

Besides their natural substrates i.e. tyrosine and dihydroxyphenylalanine (DOPA) tyrosinases, in principal, are capable of oxidizing any mono- or diphenols having a similar structure to these compounds. Generally, human and animal tyrosinases are relatively specific to L-tyrosine and L-DOPA, whereas a wider set of substrates is known for fungal and plant enzymes (Mayer and Harel, 1979). Tyrosinase has been known for decades to activate tyrosine residues in proteins to their quinone forms, which can further react non-enzymatically with each other or with thiol and/or primary amino groups (Pierpoint, 1969) resulting in the formation of covalent tyrosine-tyrosine, tyrosine-cysteine or tyrosine-lysine cross-links (Ito et al., 1984; Matheis and Whitaker, 1984a; Kato et al., 1986; Takasaki and Kawakishi, 1997; Burzio et al., 2000) as shown in Figure 3.

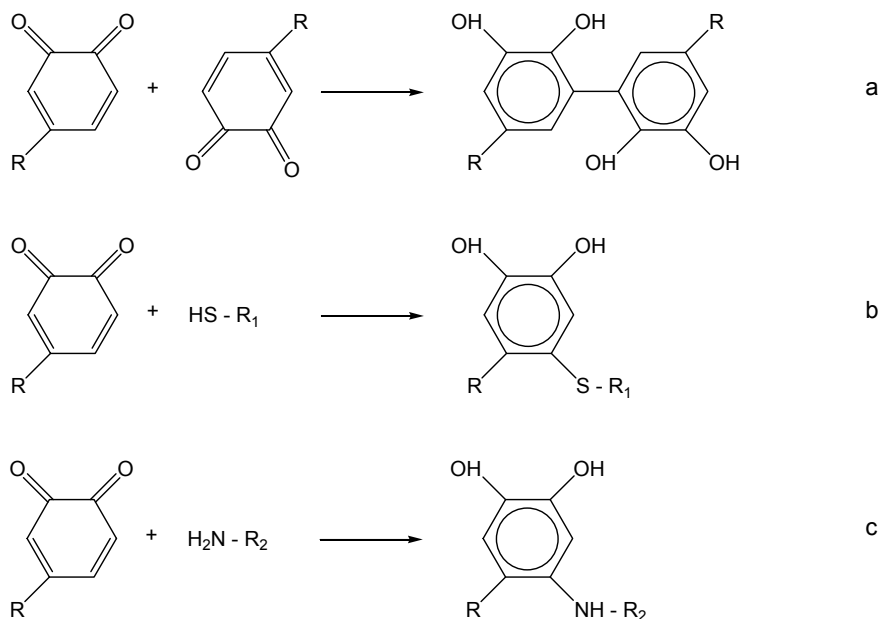


Figure 3. Proposed tyrosinase-activated protein cross-linking: phenol coupling (a) and formation of a cysteinyl-tyrosine (b) and a lysyl-tyrosine (c) cross-link (modified from Ito et al., 1984; Matheis and Whitaker, 1984a; Kato et al., 1986; Takasaki and Kawakishi, 1997; Burzio et al., 2000).

Tyrosinases are often referred to as phenolases, phenol oxidases, catechol oxidases or polyphenol oxidases (PPO) depending on both the origin of the enzyme and the authors who have described them. The name tyrosinase is usually used for the animal and human enzymes and refers to their typical substrate, tyrosine. The biological function of tyrosinases is related to melanin biosynthesis (Sánchez-Ferrer et al., 1995; Claus and Decker, 2006), regardless of the origin of the organism. Melanins are heterogenous insoluble polyphenol biopolymers with a complex structure and colour from yellow to black (Sánchez-Ferrer et al., 1995). Melanins protect bacterial cells and spores against harmful solar radiation, oxidants, heat and antimicrobial compounds and also bind toxic heavy metals (Butler and Day, 1998; Nosanchuk and Casadevall, 2003). In fungi tyrosinases function in differentiation, spore formation and pathogenesis (Seo et al., 2003). The best characterised fungal tyrosinase is that of the common mushroom *Agaricus bisporus*. In plants tyrosinases participate in wound healing, leading to the undesired browning recognised in many edible plants as well as to primary immuno response and regulation of growth (Mayer

and Harel, 1979; Yoruk and Marshall, 2003). In insects, tyrosinases are involved in sclerotization of the cuticle, exoskeletal pigmentation and wound healing and in animals in melanin biosynthesis (Sugumaran, 1991). In humans melanins are mainly synthesised in the retina, skin and hair and have a role, as in bacteria, in protecting cells against solar radiation (Pomerantz and Ances, 1975; Hill, 1992).

Tyrosinases are copper-containing enzymes having a binuclear copper catalytic site (Lerch, 1983; Matoba et al., 2006). The two copper atoms in the catalytic centre shuttle electrons from the substrate to O₂, which is the terminal electron acceptor and is reduced to water during the catalysis. Binding of mono- and diphenols in the catalytic site is regulated by the functional states of the copper atoms. The reaction mechanism of tyrosinase is comprehensively reviewed by e.g. Sánchez-Ferrer et al. (1995) and Rescigno et al. (2002). Unlike most eukaryotic tyrosinases, which are multimeric, the bacterial enzymes appear to be either monomeric (*Streptomyces* species) or dimeric (*Bacillus thuringiensis*) (Liu et al., 2004; Claus and Decker, 2006). The monomeric forms of tyrosinase from *Agaricus bisporus* have molecular masses of 43 kDa and 13.4 kDa (Kim and Uyama, 2005). The molecular mass of the tyrosinase monomer recently isolated from the fungus *Trichoderma reesei* is 43 kDa (Selinheimo et al., 2006). Tyrosinases from the bacterial genus *Streptomyces* have a molecular mass of about 30 kDa (Kim and Uyama, 2005; Claus and Decker, 2006). The largest tyrosinases have been characterised in mammals, the human tyrosinase monomer having a molecular weight of 62–67 kDa (Kwon et al., 1987; Kim and Uyama, 2005). Many microbial and plant tyrosinases have their pH optima in a slightly acidic pH range and often lose their activity within few minutes at temperatures higher than 70°C (Zawistowski et al., 1991).

Tyrosinase has received considerable attention among researchers in food science. Due to undesired tyrosinase-catalysed browning of many plant products, the tyrosinase research has been focused on inhibition of tyrosinase activity (Vamos-Vigyazo, 1981; Rescigno et al., 2002; Kim and Uyama, 2005). Browning occurs in conditions in which oxygen is capable of penetrating the plant tissue and is a result of tyrosinase-initiated oxidation of phenolic compounds in plants to quinones, which eventually polymerise to melanin pigments (Nicolas et al., 1994). These pigments change the colour, flavour, texture and nutritional value of the plant products, causing reduced consumer acceptance and overall value of the plant food products (Vamos-Vigyazo, 1981). In addition to plants, browning

by tyrosinase has been detected in seafood (Rolle et al., 1991). Tyrosinase causes deteriorative post-harvest browning of shells of crustaceans, affecting both quality and consumer acceptance.

Consequently a large number of natural and non-toxic tyrosinase inhibitors mainly for food but also for dermatological use (Lersch et al., 2003; Kim and Uyama, 2005) have been studied. Some of the most recent of them are summarised in Table 3. The mechanism of tyrosinase inhibition as well as the clinical and industrial significance of the inhibition have been reviewed e.g. by Rescigno et al. (2002), Seo et al. (2003) and Kim and Uyama (2005).

Apart from the extensive studies on inhibition of endogenic plant tyrosinase activity to control browning, there are only some studies in the food sector that have been focused on exploitation of tyrosinase in protein modification. Most of the studies have been carried out using *Agaricus bisporus* tyrosinase. Sweet-tasting thaumatins have been stabilised by tyrosinase against proteolytic activities (Ramsohoye and Kozlov, 1991). Tyrosinase-catalysed formation of a 5-S-cysteinyl-3,4-DOPA cross-link was characterised in gluten proteins by Takasaki and Kawakishi (1997) and Takasaki et al. (2001). The whey proteins β -lactoglobulin and α -lactalbumin have been polymerised by tyrosinase (Thalmann and Lötzbeyer, 2002). In the β -lactoglobulin polymerisation a small-molecular co-substrate, caffeic acid, was required, whereas α -lactalbumin was directly cross-linked. A novel tyrosinase from the fungus *Pycnoporus sanguineus* has proved to be an efficient cross-linker of caseins both with and without tested co-substrates L-tyrosine and L-DOPA (Halaouli et al., 2005).

Tyrosinase has been known for a long time to create intra- and intermolecular cross-links via tyrosine residues in tropocollagen (Dabbous, 1966). The firmness of unheated pork homogenate gels have been improved by tyrosinase (Lantto et al., 2006). Except for this preliminary study on pork there are no published reports of the effects of tyrosinase in the meat protein modification.

Table 3. Recently published natural tyrosinase inhibitors.

Inhibitor	Enzyme	Inhibition of	Reference
Gallic acid and gallic acid esters	Mushroom tyrosinase	Diphenolase activity	Kubo et al. (2000, 2003)
3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxy-3-methoxycinnamic acid from <i>Pulsatilla cernua</i> root			Lee (2002)
<i>p</i> -alkoxybenzoic acids	Mushroom tyrosinase	Diphenolase activity	Chen et al. (2005)
Cinnamic acid			Shi et al. (2005)
Glabrene isoliquiritigenin from liquorice root	Mushroom tyrosinase	Mono- and diphenolase activity	Nerya et al. (2003)
Red koji extract			Wu et al. (2003)
Maillard reaction products of onion extract, glycine and glucose	Potato PPO	Diphenolase activity	Lee et al. (2002)
Maillard reaction products of glucose, fructose and cysteine	Apple PPO		Billaud et al. (2004)
Glycine-lysine, glycine-histidine, glycine-asparagine	Mushroom tyrosinase, Apple PPO, Potato PPO	Diphenolase activity of mushroom tyrosinase; browning of apple and potato slices	Girelli et al. (2004)
Combination of kojic acid, L-cysteine, 4-hexylresorcinol	Apple PPO	Enzymatic browning of apple juice	İyidoğan and Bayındırlı (2004)
Ascorbic acid, L-cysteine, 4-hexylresorcinol	Mango PPO	PPO activity in mango puree	Guerrero-Beltrán et al. (2005)

Although the application research of tyrosinase has focused mainly on food-based proteins, certain protein or peptide fractions as well as intact fibres of silk and wool are also reported to be modified by tyrosinase. Chitosan, a linear carbohydrate polymer composed of glucosamine units having various commercial and medical uses, has been efficiently incorporated into silk fibroin by tyrosinase via the amino groups of chitosan (Sampaio et al., 2005; Freddi et

al., 2006). Silk sericin peptides (Anghileri et al., 2007) and intact wool fibres (Lantto et al., 2005) are reported to be oxidised by tyrosinase. In addition, tyrosinase and its ability to form dityrosine and tyrosine-lysine cross-links may be exploited in the production of adhesives from such proteins as the DOPA-rich marine mussel glue protein or various types of extensins (Nelson and Jones, 1998).

2.3.2 Laccase

Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) are a group of enzymes belonging to the blue multicopper oxidases. Laccases are common in nature and occur widely in fungi (Mayer and Harel, 1979), whereas in higher plants their occurrence is more limited (Mayer and Stables, 2002). Laccases or laccase-like proteins have also been identified in insects (Kramer et al., 2001) and bacteria (Claus and Filip, 1997; Claus, 2003; Dalfard et al., 2006).

Oxidation reactions catalysed by laccases take place with the assistance of a cluster of four copper atoms forming the catalytic core of the enzyme. The outcome of the catalytic cycle is the reduction of one molecule of O₂ to two molecules of water and the simultaneous oxidation of the substrate to produce four oxygen-centred free radicals. Basically any substrate similar to *p*-diphenol can be oxidized by laccase. The unstable radicals formed can further convert non-enzymatically to a semi-quinone form. The free radical form and quinone may lead to polymerization of the substrate molecule. Figure 4 shows the laccase-catalysed oxidation of a *p*-diphenol published by Thurston (1994).

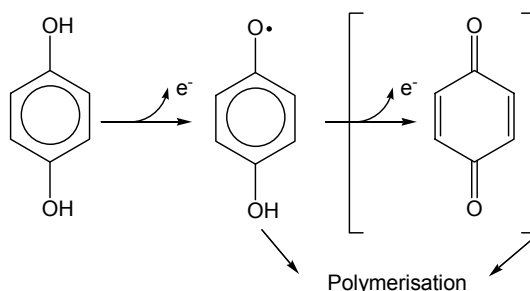
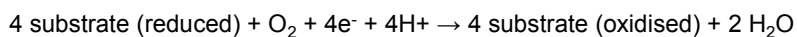


Figure 4. Laccase-catalysed oxidation of a diphenol (modified from Thurston, 1994 and Yaropolov et al., 1994).

Laccases are often grouped together with tyrosinases under the trivial name polyphenoloxidase (PPO). Partially this is due to the fact that these two enzymes are not always easy to classify or to distinguish from each other because of their overlapping substrate specificity. However, laccases can be distinguished from tyrosinases by the fact that they do not catalyse the hydroxylation step, a typical reaction in the tyrosinase catalysis (Figure 2), but are capable of oxidising mono- and diphenols by a radical-generating mechanism.

The physiological functions of laccases are all related to polymerisation or degradation processes. In higher plants laccases are involved in cell wall formation and lignin biosynthesis (Thurston, 1994; Gianfrida et al., 1999) together with peroxidases, whereas fungal laccases are involved in lignin degradation. Delignification is, by definition, the main process in which the laccases of white-rot fungi are involved (Thurston, 1994; Xu, 1996; Mayer and Staples, 2002). In addition, laccases have a role in fungal morphogenesis (Zhao and Kwan, 1999) and can protect fungal pathogens from toxic compounds in host environments, acting as virulence factors in many fungal diseases (Mayer and Staples, 2002). In insects laccases have been reported to be involved in cuticle sclerotization (Kramer et al., 2001; Suderman et al., 2006) and in the bacterial species *Bacillus subtilis* in pigment production of endospores (Martins et al., 2002).

Except for the conserved catalytic site of laccases, there is a great diversity in the rest of the protein structure, molecular size, pH optimum and substrate specificity (Thurston, 1994; Gianfrida et al., 1999; Mayer and Staples, 2002). The molecular mass of laccases varies from 40 to 100 kDa and the enzymes occur as monomers. Although acting on a broad range of substrates, there are substrates that cannot be oxidized directly, either because they are too large to penetrate the active site or because they have too high a redox potential (Xu, 1996). This limitation is overcome by mediators, which are most often small molecules that are readily oxidized by laccase, producing radicals which can then react with the target substrate. Like tyrosinases many of the fungal laccases also have their pH optima in the acidic range (Xu, 1997) and rapidly lose their activity at temperatures above 60°C (Xu, 1996).

Laccases have a surprisingly broad substrate range, being able to oxidize various phenolic compounds, diamines, aromatic amines and benzenethiols (Xu, 1996).

Some laccases can also oxidize monophenols such as cresol (Mayer and Staples, 2002) and tyrosine (Williamsson, 1994; Mattinen et al., 2005), a feature which has traditionally distinguished these enzymes from tyrosinases. In addition to tyrosine oxidation, laccases have also been reported to oxidize thiol-containing compounds and tryptophan (Figueroa-Espinoza et al., 1998; Labat et al., 2000; Tsuchiya et al., 2000; Mattinen et al., 2006). Tyrosine-containing short peptides have been polymerised by *Trametes hirsuta* laccase without a mediator resulting in a covalent ether bond formation between two phenolic rings of tyrosine residues (Mattinen et al., 2005). α -Lactalbumin and reduced β -lactoglobulin have been polymerised by *Polyporus pinsitus* laccase in the presence of chlorogenic acid (Færgemand et al., 1998a). Yamaguchi (2000) reported polymerisation of caseins and bovine serum albumin (BSA) by *Pycnoporus* laccase without a mediator. Mattinen et al. (2006) recently reported *Trametes hirsuta* laccase-catalysed oligomerisation of coactosin without an auxiliary substance but it was concluded that, in general, proteins are poor substrates for laccase. Reported effects of laccases on proteinaceous substrates including peptides and amino acids are summarized in Table 4.

Table 4. Laccase-catalysed modification of proteinaceous substrates.

Substrate(s)	Origin of laccase	Mediator	Effect	Reference
Isolated protoplasts from tobacco leaf mesophyll	<i>Nicotiana tabacum</i>		Polymerisation of tyrosine-rich structural proteins	De Marco and Roubelakis-Angelakis (1997)
Whey proteins	<i>Polysporus pinsitus</i>	Chlorogenic acid	Small and large protein oligomers	Færgemand et al. (1998a)
Gluten dough	<i>Myceliophthora</i> sp.		Oxidation of thiol-groups	Heldt-Hansen (1998)
UHT-treated milk	<i>Myceliophthora thermophila</i>	10-phenothiazine propionic acid; 10-phenoxazine propionic acid	Oxidation of thiol-groups	Tsuchiya et al. (2000)
Wool fibre, tyrosine and cysteine		Violuric acid, 1-hydroxy-benzotriazole (HBT)	Oxidation of wool, tyrosine and cysteine	Lantto et al. (2004)
Cysteine	<i>Pycnoporus cinnabarinus</i>	Ferulic acid	Formation of a disulphide bond	Figueroa-Espinoza et al. (1998); Labat et al. (2000)
Glutathione				Vansteenkiste et al. (2004)
BSA, feruloylated arabinoxylan of wheat			BSA-arabinoxylan co-polymer formation	Yamaguchi (2000)
BSA, casein, pork	<i>Pycnoporus coccineus</i>		Cross-linking of BSA and caseins; improved gel strength of pork sausage	Yamaguchi (2000)
Gelatine, fish	<i>Cortolius versicolor</i>		Cross-linking of gelatine; improved elasticity of kamaboko gels	Yamaguchi (2000)
Gelatine and catechin	not given		Formation of a gelatine-catechin conjugate	Chung et al. (2003)
Tyrosine and tyrosine-containing short peptides	<i>Trametes hirsuta</i>	Ferulic acid	Dityrosine cross-link formation in the presence and absence of ferulic acid	Mattinen et al. (2005)
Coactosin, Gly-Leu-Tyr, cysteine, tyrosine and tryptophan			Oligomerisation of coactosin and tripeptide, oxidation of Cys, Tyr and Trp	Mattinen et al. (2006)
β -lactoglobulin	<i>Trametes versicolor</i>	Ferulic acid or a phenolic mono- or oligosaccharide	Oligomerisation of β -lactoglobulin	Boumans et al. (2006)
Recombinant insect cuticular proteins	<i>Pyricularia oryzae</i>	N-acetyl catecholamines	Small and large protein oligomers	Suderman et al. (2006)

Laccases are already exploited industrially in non-protein applications and their application potential is well recognized in several fields of the food sector. Bioremediation of process wastewaters by precipitation of phenolic compounds in the brewery, distillery and olive oil manufacture sectors, removal of polyphenols from musts and wine to stabilise the colour and flavour of wines (Minussi et al., 2002) and removal of polyphenols from wort to minimise the formation of haze and thus to stabilise beer (Mathiasen, 1995) are applications in which laccase has already shown its usefulness. Significant improvement of gel formation (Norsker et al., 2000) and rheological properties (Kuuva et al., 2003) has been detected in sugarbeet pectin gels treated with laccase, indicating cross-linking of the pectin molecules via dimerisation of the ferulic acid residues of this pectin type. Laccases are also of interest in the baking sector due to their ability to cross-link arabinoxylans (Figueroa-Espinoza and Rouau, 1998; Figueroa-Espinoza et al., 1998) via formation of diferulic acid.

2.3.3 Other oxidative enzymes

Peroxidases (EC 1.11.1.7) are a diverse group of oxidative enzymes occurring in plants, animals and microbes, the substrate specificity of which varies with the origin. Most peroxidases are heme-proteins with a ferric protoheme group, whereas others have magnesium, vanadium or selenium or the flavin group at their active site (Smith and Veitch, 1998). The biological roles of plant peroxidases are in cell wall lignin biosynthesis and biodegradation, providing defence against pathogens, and in regulation of growth (Yuan and Jiang, 2003). Many biological roles have been assigned to horseradish peroxidase, which is perhaps the most intensively studied of all peroxidases. In addition to the biological roles mentioned, horseradish peroxidase is involved in the indole-3-acetic acid metabolism (Krylov and Dunford, 1996) having a role in tumor development (Veitch, 2004). The structure, reaction mechanism and applications of peroxidases were comprehensively reviewed by Veitch (2004).

Peroxidases utilize H_2O_2 as an electron acceptor to oxidise a variety of organic and inorganic substrates such as phenols (Yuang and Jiang, 2003), aromatic phenols, phenolic acids, indoles, amines, sulphonates (Veitch, 2004), thiols (Obinger et al., 1996), halides (Ekstrand and Björck, 1986) and amino acids (Gross and Sizer, 1959; Malencik and Anderson, 1996). As a result of oxidation a

radical is formed that can react further with other substrates. Most reactions catalysed by peroxidases can be expressed by Equation (1)

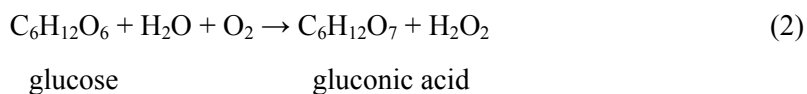


in which AH_2 represents a substrate and $\text{AH}\cdot$ its free radical product. Concomitantly H_2O_2 is converted to water. Stahmann et al. (1977) showed that horseradish peroxidase is capable of cross-linking proteins in the presence of H_2O_2 and low a molecular weight phenolic hydrogen donor. It has also been shown that when a diamine is present as a hydrogen donor, peroxidases can catalyse complex protein cross-linking reactions resulting in covalent bond formation between tyrosine, cysteine and lysine residues as reviewed by Matheis and Whitaker (1984a and b); Matheis and Whitaker (1987) and Feeney and Whitaker (1988).

Apart from the feasibility of peroxidases in cancer therapy (Wardman, 2002), organic synthesis (Lim and Yoo, 2000), analytical purposes (Krig and Halbhuber, 2003) and treatment of wastewaters (Wagner and Nicell, 2002; Duran and Sakurai, 2003), the potential of peroxidases in food protein cross-linking has been evaluated using soy proteins (Matheis and Whitaker, 1984b), gelatine and casein (Matheis and Whitaker, 1984b; Yamaguchi 2000), β -lactoglobulin (Stahman et al., 1977; Færgemand et al., 1998a), ovalbumin (Stahman et al., 1977) and wheat gliadin (Michon et al., 1999) as substrates.

Due to unavoidable addition of H_2O_2 to the system or alternatively its generation *in situ*, peroxidases as protein cross-linkers are of interest mainly in the baking sector. In baking, oxidants are frequently added to flour to improve the baking performance. Substituting chemical oxidants with a peroxidase (or with a peroxidase/glucose [hexose] oxidase system) is an option that has attracted considerable interest among researchers in the field. A dityrosine bond is formed in wheat gliadins with horseradish and soybean peroxidases and a manganese-dependent peroxidase from the fungus *Phanerochaete chrysosporium* (Michon et al., 1999). It has also been hypothesised, although not demonstrated, that peroxidases affect the gluten network by cross-linking the gluten proteins, or alternatively by attaching arabinoxylans to gluten via the ferulic acid residues of arabinoxylans to tyrosine or cysteine residues of gluten (Hillhorst et al., 1999; Dunnewind et al., 2002).

Glucose oxidases (EC 1.1.3.4) are predominantly produced by fungi such as *Aspergillus* (Zetelaki and Vas, 1968; Kona et al., 2001) and *Penicillium* (van Dijken and Veenhuis, 1980). The biological role of glucose oxidase is not well understood but it is thought to give the fungus an ecological advantage in the soil (Vroeman, 2003). Glucose oxidase is induced in the presence of glucose and it catalyses oxidation of glucose to form first glucono δ -lactone and eventually gluconic acid and H₂O₂ as expressed in the following summary Equation (2) by Vroeman (2003):

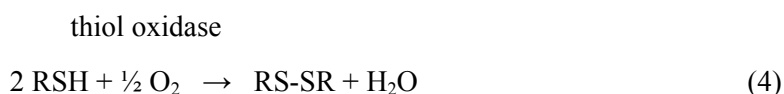
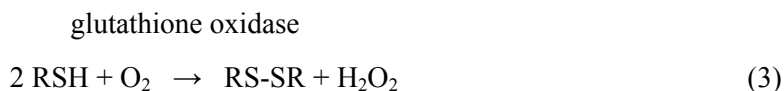


In dough systems, H₂O₂ generated due to the action of glucose oxidase has been suggested to oxidize cysteine residues of dough proteins to form disulphide bonds (Vermulapalli et al., 1998; Rasiah et al., 2005). H₂O₂ produced in the glucose oxidase-catalysed reaction is proposed to act as an electron acceptor for endogenous peroxidase in the wheat flour. In the presence of H₂O₂ this peroxidase is capable of catalysing the tyrosyl cross-link formation in gluten (Miller and Hosney, 1999; Ameille et al., 2000; Tilley et al., 2001). The glucose oxidase/peroxidase system has potential especially in the baking sector to improve the baking performance.

Hexose oxidases (EC 1.1.3.5) have many similarities to glucose oxidase but their substrate specificity range is broader. Hexose oxidases are capable of oxidising many mono- and oligosaccharides to the corresponding lactones with a concomitant formation of H₂O₂. Hexose oxidases have been found e.g. in the marine algal species *Chondrus crispus* (Sullivan and Ikawa, 1973), *Iridophycus falaccidum* (Bean and Hassid, 1956) and a bacterial species *Malleomyces pseudomallei* (Dowling and Levine, 1956). *C. crispus* hexose oxidase has the highest affinity for D-glucose, D-galactose, cellobiose and maltose (Poulsen and Bak Høstrup, 1998) and in baking this hexose oxidase was capable of increasing the dough strength and bread volume more efficiently than glucose oxidase.

Sulfhydryl oxidases (EC 1.8.3) including both thiol oxidases (EC 1.8.3.2) and glutathione oxidases (EC 1.8.3.3), exclusively generate a disulphide bridge between two thiols. During the reaction glutathione oxidase reduces O₂ to H₂O₂

(Kusakabe et al., 1982; Sliwkowski et al., 1984) as shown in Equation (3), whereas thiol oxidase reduces O₂ to water (Aurbach and Jakoby, 1962) as shown in Equation (4)



The nomenclature of sulfhydryl oxidases is still somewhat confusing, and classification of sulfhydryl oxidases to the two above-mentioned classes is by no means straightforward.

Glutathione and thiol oxidases differ from each other not only in their reaction product, H₂O₂ or water, but also in their protein characteristics and substrate specificity. Glutathione oxidases are flavin-dependent proteins catalyzing oxidation of small thiol compounds such as glutathione, cysteine or dithiotreitol and have been isolated from rat seminal vesicles (Ostrowski and Kistler, 1980), fungi, e.g. *Penicillium* (Kusakabe et al., 1982), *Aspergillus niger* (de la Motte and Wagner, 1987), and *Saccharomyces* (Gerber et al., 2001) as well as from chicken egg white (Hoover et al., 1996). On the other hand, metal-containing thiol oxidases (sulfhydryl oxidase) have broader substrate specificity, are not flavin-dependent and have been found in bovine milk (Janolino and Swaisgood, 1975) and other mammalian tissues (Clare et al., 1984) but not hitherto from e.g. fungal sources. Bovine milk sulfhydryl oxidase catalyses the oxidation of thiol groups of cysteine as well as cysteine residues in peptides and proteins to the corresponding disulphides, leading to protein polymerization. However, it cannot oxidize a small thiol compound dithiotreitol (Janolino and Swaisgood, 1975 and 1992). At present, research on potential exploitation of sulfhydryl oxidases in food protein modification is rather limited. For the time being sulfhydryl oxidases are not available commercially for industrial use. In addition, the substrate specificity of the fungal enzymes isolated and characterized so far suggests oxidation of small thiol compounds rather than cysteine residues in proteins. Even so, a clear synergistic effect of glutathione

oxidase with glucose oxidase to improve baking results has been claimed (Haarasilta and Pullinen, 1992). Furthermore, the bovine milk sulfhydryl oxidase has been successfully exploited in elimination of 'cooked' flavour in UHT-treated milk. This invention was already patented a long time ago by Swaisgood (1977).

2.4 Structure of meat

The dominant feature of muscle is its fibrous structure that makes the function of a muscle in contraction possible. Due to its structure muscle is capable of providing tensile strength and transmitting the force needed in contraction. Hence the eating quality of muscle of a freshly slaughtered animal is not usually accepted by consumers because of its toughness. Conversion of muscle to meat is prerequisite for the development of desired eating qualities. Muscle begins to convert to meat when an animal is slaughtered (Lawrie, 1998, p. 96; Ouali et al., 2006). At the death of an animal a complex set of biochemical and biophysical changes is initiated in muscle, which is far from being well understood. Degradation of muscle proteins by the indigenous protease system leads to softening of myofibrillar structure (Ouali, 1992; Sentandreu et al., 2002) and ultimately to meat tenderisation. However, the fibrous structure is maintained, which gives meat its characteristic texture recognised by consumers.

A skeletal muscle is an assembly of bundles of muscle fibres. Muscle fibres are narrow, long (commonly several centimetres long with a diameter of 10–100 μm), and multinucleate cells (Walls, 1960). They are the basic cellular units of living muscle and meat, and are surrounded by a connective tissue sheath called endomysium consisting mainly of collagen. Muscle fibres in turn consist of bundles of myofibrils, composed of a number of parallel myofilaments, thick and thin. The functional (contractile) unit of a myofibril, a sarcomere, is an organised assemblage of thin and thick filaments interacting with each other during contraction. The thick filament is composed of about 200 rod-shaped myosin molecules (Huxley, 1963) with a molecular size of about 500 kDa (Gershman et al., 1969). Each thick filament is surrounded by six thin filaments composed of actin, with a molecular size of about 42 kDa (Elzinga et al., 1973). The arrangement of globular actin molecules in the thin filament is like two strings of pearls twisted around each other. Myosin and actin are the proteins

responsible for the contractile action in a living muscle. In meat these proteins affect such functional properties as texture and water-holding (Macfarlane et al., 1977). In addition to myosin and actin, myofibrils also contain such proteins as troponins (21–35 kDa, reviewed in Asghar et al., 1983) and tropomyosin (65–70 kDa, Cummins and Perry, 1974), having a regulatory role during contractions of muscle, as well as e.g. titin and nebulin, which appear in smaller quantities and form the cytoskeleton of a muscle (Locker, 1987; Wang and Wright, 1988; Lawrie, 1998; Belitz and Grosch, 1999). The assembly of muscle is depicted in Figure 5.

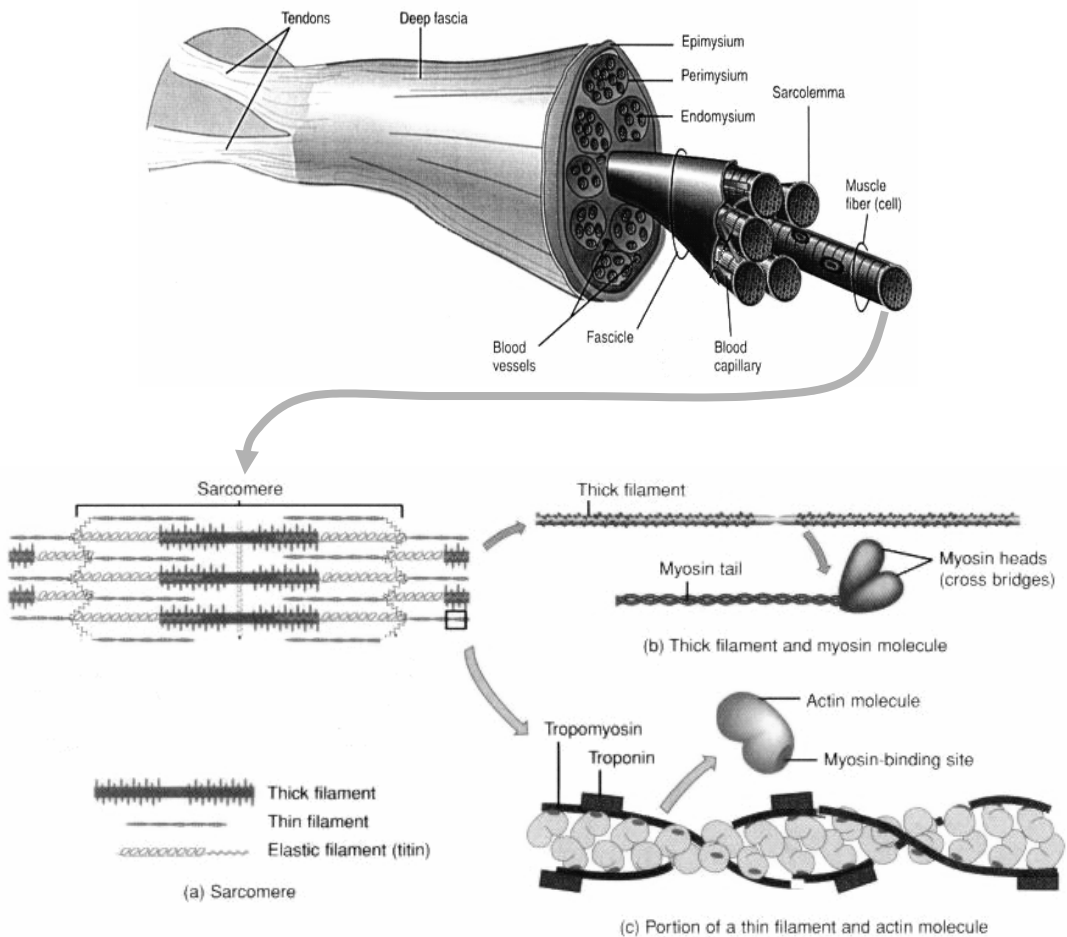


Figure 5. Assembly of muscle. Modified from the images at <http://faculty.etsu.edu/currie/images/>.

2.5 Gel formation and water-holding in meat systems

Protein functionality is a term which defines the physico-chemical behaviour of a food protein affecting the quality characteristics of the final product. In meat processing protein functionality means rheological behaviour such as gel formation, but also textural and binding properties. Heat-induced gelation of muscle proteins is a crucial event in meat processing, which determines the textural characteristics of the meat products. It involves denaturation (unfolding), aggregation and formation of a three-dimensional network, a gel, and is affected by the type and amount of protein, intrinsic characteristics of proteins as well as pH, ionic environment, temperature, rate of heating and protein oxidation (Lesiów and Xiong, 2001a and b). Myosin, the most abundant of the structural proteins in muscle and meat, plays the most important role in gelation. Heat-induced gelation starts with partial unfolding of the protein molecules, exposing binding sites for protein-protein aggregation.

Water-holding capacity (WHC) describes the ability of meat or a meat product to retain its natural or added water (Hamm, 1960; Huff-Lonergan and Lonergan, 2005). WHC is a substantial economic and technological factor, influencing not only process yields but also the sensory properties of the product such as juiciness, texture and flavour. WHC is essentially related to the microstructure of meat. Muscle contains approximately 75% water, most of which (up to 90%) is immobilised in the spaces between the thick and thin filaments of the myofibrils (Offer and Knight, 1988a, p. 79). In addition, a substantial amount (0–10%) of the water is located in the extracellular space, i.e. spaces between muscle fibres and muscle fibre bundles (Offer and Trinick, 1983; Huff-Lonergan and Lonergan, 2005). The rest of the water is associated with the muscle protein surfaces via hydrogen bonds and remains so under most conditions (Hamm, 1960; Fennema, 1985; Huff-Lonergan and Lonergan, 2005).

WHC is affected by NaCl added to the meat system because it makes the myofibrils swell. Swelling leads to enlarged cavities in the myofilament lattice, which are capable of retaining more water (Offer and Trinick, 1983; Offer and Knight, 1988a, p. 82). In addition to the change of volume of the myofibrils due to NaCl, this salt also solubilises myofibrillar proteins. WHC is also greatly dependent on pH values. When a meat system does not contain added salt, WHC is at its minimum at pH 5, which is the average isoelectric point of the

myofibrillar proteins (Hamm, 1972). WHC increases when pH is changed towards either acidic or basic pH-values from pH 5. Adding NaCl to the system shifts the minimum WHC (and the average isoelectric point of meat proteins) towards pH 4 due to selective binding of Cl⁻ ions to the proteins (Hamm, 1960). The maximum swelling of the myofibrils with NaCl occurs at pH 6 (Hamm, 1972). According to Hamm (1972), NaCl affects meat and its WHC most probably due to stronger binding of the Cl⁻ than Na⁺ ions to the meat proteins. As a result the negative charge of the proteins is increased, which generates repulsion between myofibrillar proteins and finally leads to swelling and disintegration of the myofibrillar lattice, but also to partial solubilisation of the myofibrillar proteins.

Offer and Knight (1988a, pp. 119–126) speculated that the negative net charge of the myofilaments caused by the stronger binding of Cl⁻ ions into protein attracts Na⁺ ions close to the filament surfaces, thus forming an Na⁺ ion cloud around the myofilaments. As myofibrillar proteins cannot move freely in meat, a negative and uneven distribution of ions in the water phase is inevitably created. This leads to increased osmotic pressure in the filament network, attracting water molecules into the system. According to Offer and Trinick (1983), increasing the NaCl concentration up to 1 M increases swelling, but myofibrils do not swell indefinitely as proposed by Millman and Nickel (1980) due to structural resistance of the system such as transverse van der Waals forces and covalent cross-links in the myofilament system. A comprehensive review of the effects of NaCl in meat was presented by Ruusunen and Puolanne (2005).

In practice, many meat products such as sausages are manufactured by chopping meat in the presence of water and salts to a fine meat homogenate, in which the muscle structure is partially destroyed, and are finally heated. Increasing temperature alters the native microstructure of muscle. As reviewed by Tornberg (2005), myofibrillar proteins in solution start to unfold at 30–32°C and aggregate at 30–40°C, after which gelation occurs at 45–50°C. As reviewed by Lesiów and Xiong (2001b), poultry myofibrillar proteins unfold at 30–39°C, aggregate at 36–46°C, and form gels at 45–51°C. At temperatures around 65°C the collagen covering muscle fibres is denatured (Bailey and Light, 1989). Hermansson (1986) proposed that it is the pores in these protein gels, rather than an arranged myofilament network that maintain the capillary force and thus determine the WHC of processed emulsion-type meat products. For example during heating

the cooking loss of sausages (finely minced) is much less compared to hamburger-type products (more coarsely ground) or whole meat. According to Tornberg (2005) this is probably due to the shrinkage of meat fibres that is prevalent during heating in meat systems consisting of intact muscle structure, whereas in sausages in which the muscle structure is largely destroyed due to chopping and homogenation, a higher proportion of myofibrillar proteins is extracted, allowing the formation of denser gels holding more water.

2.6 The role of cross-linking enzymes in meat systems

2.6.1 Meat protein modification

In addition to meat tenderisation by proteolytic enzymes, meat proteins are modified by creating new covalent bonds in the system rather than by degrading them. Hitherto TG has been the main enzyme studied and applied in meat protein modification. Although the most obvious source of TG for the meat industry would be blood, microbial enzymes have superseded the mammalian ones due to their Ca^{2+} -independence, feasible pH and temperature profiles and commercial availability for industrial use.

TG was examined in covalent cross-link formation of myofibrillar proteins already more than four decades ago when Derrick and Laki (1966) used the enzyme to label actin and tropomyosin with ^{14}C -putrescine. TGs of different origins that have been studied ever since in myofibrillar protein modification have differed in their cross-linking specificity. Ca^{2+} -dependent mammalian TGs, i.e. bovine plasma TG (Kahn and Cohen, 1981), human placental factor XIII (De Backer-Royer et al., 1992) or pig plasma TG (Tseng et al., 2002), are capable of modifying myosin and actin, whereas microbial enzymes originating from *Streptomyces* (Nonaka et al., 1989) have shown limited activity on actin. Although actin has not exhibited major changes in the presence of microbial TG, an intramolecular cross-link catalysed by this enzyme has been identified in rabbit actin (Eli-Berchoer et al., 2000). It has been suggested that TGs from different origins recognize different peptide sequences in the same proteins (Matsumura et al., 2000), thus leading to differences in cross-linking efficiency. Studies on the effects of TGs in myofibrillar protein modification are summarised in Table 5.

Table 5. TG-catalysed modification of myofibrillar proteins.

Myofibrillar protein substrate	Origin of the protein	Origin of TG	Modification	Reference
Myosin	Beef	Bovine plasma	Polymerisation	Kurth and Rogers (1984)
		Human placental factor XIIIa		De Backer-Royer et al. (1992)
Actomyosin	Turkey	Guinea pig liver	Polymerisation	Akamittath and Ball (1992)
	Beef	Guinea pig liver		Kim et al. (1993)
	Tilapia	Tilapia (<i>Oreochromis niloticus</i>) muscle		Worratao and Yongsawatdigul (2003)
Actin and myosin	Rabbit	Human plasma factor XIII	Coupling of actin to myosin	Kahn and Cohen (1981)
Actin	Rabbit	<i>Streptomyces mobaraense</i>	Intramolecular isopeptide bond	Eli-Berchoer et al. (2000)
	Beef	Human placental factor XIIIa	Partial polymerisation of actin	De Backer-Royer et al. (1992)
Myofibrillar proteins	Mackerel	<i>Streptomyces ladakanum</i>	Polymerisation of MHC	Tsai et al. (1996)
	Beef heart	Microbial	Polymerisation of myosin, possible intramolecular bond in MHC	Ramirez-Suarez et al. (2001)
				Ramirez-Suarez and Xiong (2002a)
	Chicken breast		Intramolecular cross-links in MHC and actin in salt-free conditions. Suggested proteolytic fragmentation.	
	Spent hen breast	Pig plasma	Polymerisation of MHC and actin	Tseng et al. (2002)
Beef	<i>Streptomyces mobaraense</i>	Polymerisation of myosin	Aktaş and Kiliç (2005)	

In addition to myofibrillar proteins, TG is capable of cross-linking collagen, the main protein component of the connective tissue. Although no detailed reports on how TG acts with intramuscular collagen or about the subsequent consequences on meat quality have been published, it is known that TG cross-links at least porcine (Chen et al., 2005) and bovine (Chau et al., 2005) skin type

I collagen, resulting in greater resistance to proteolysis, increased tensile strength and shift of thermal denaturation towards higher temperatures compared with the native collagen.

Although meat protein cross-linking has hitherto been carried out almost solely by TG, mushroom tyrosinase is also known to be capable of cross-linking collagen (Dabbous, 1966; Chau et al., 2005) and increasing the firmness of gels of unheated pork (Lantto et al., 2006). In addition, laccase has been claimed to cross-link BSA and gelatin and to improve the textural properties of pork sausage and kamaboko gels (Yamaguchi, 2000).

2.6.2 Effects on meat protein gels

By adding a cross-linking enzyme to a meat protein system the gel formation, texture and water-holding properties of a gel can be modified. Due to the efficient cross-linking of myosin TG is generally capable of positively affecting gelation and the texture of meat protein gels. As well as by enzymatic protein modification, gelation is affected by the protein type and quantity, protein solubility, pH, temperature and the amount and types of salts in the system. Gelation of solubilised myofibrillar proteins is a functional property that is necessary for restructuring of fresh meat, but particularly in processing meat products such as sausages or hams.

TG has been used in meat systems to improve the binding of fresh meat pieces together (Nielsen et al., 1995; Kuraishi et al., 1997), low-temperature gelation of fish (Lee et al., 1997), heat-induced gelation of meat and fish (Joseph et al., 1994; Ramirez-Suarez and Xiong, 2002a and b, 2003; Ramirez-Suarez et al., 2005) and to strengthen meat gels (Dondero et al., 2006; Lantto et al., 2006). Ramirez-Suarez et al. (2001) reported about 2 to 3-fold increase in storage modulus (G') in the presence of TG for heated gels containing 3% or 5% of beef heart protein with poor binding and water binding characteristics. Improvement of G' was dependent on TG but independent on the TG-pretreatment time (0.5–15 h) and temperature (5 and 15°C) within the given range of these variables. TG-aided myosin cross-linking was observed in these pretreatment conditions whereas actin did not show major modifications. The result indicated that myosin polymerisation occurring during the TG-pretreatment was sufficient to

cause clear improvement in heat-induced gelation. Tsai et al. (1996) obtained 3.9-fold improvement in gel strength when fish surimi was set at 25°C for 80 min in the presence of TG and then heated to 90°C. Lantto et al. (2006) recently reported a 2-fold increase in firmness of unheated pork gels by TG and 52% increase by mushroom tyrosinase. The enzymatic treatment was carried out at 4°C for 22 h. In this study gel formation during heating was improved by TG but not by tyrosinase.

Enzyme-catalysed formation of additional covalent bonds in structural proteins leads, by definition, to firmer gel structures as has been clearly shown in many studies (Table 6) and is often desired in various meat product applications. However, excessive increase in gel firmness due to additional transverse bonds in the protein network may cause constraint in protein mobility and flexibility of myofibrils, leading to undesired decrease in water-holding (Carballo et al., 2006).

Decreased water-holding is mostly an unwanted phenomenon and has been observed to take place particularly in heated comminuted meat systems when salt concentrations are low (Jiménez Colmenero et al., 2005; Ruusunen and Puolanne, 2005; Carballo et al., 2006). TG has not been shown unequivocally to be an adequate salt replacer, but in high enough salt levels by ensuring sufficient protein solubility it is capable of improving water-holding properties.

When salt levels are low (< 2% NaCl), introduction of strong bonds in proteins by TG has not generally been found to improve water-holding of heated meat or fish systems, although textural properties have been improved (Hammer, 1998; Ramírez et al., 2002; Uresti et al., 2004; Dimitrakopoulou et al., 2005; Jiménez-Colmenero et al., 2005; Carballo et al., 2006). The reason is most probably the increased protein-protein and reduced protein-water interaction due to TG-catalysed covalent bonds and subsequent limited protein solubility and swelling ability resulting in reduced WHC, as e.g. Carballo et al. (2006) concluded in their study. The authors postulated that the combined effect of TG and low amount of salt (1.5%) in a comminuted and heated meat system leads to increased cooking loss because of the reduced WHC of solubilised meat proteins cross-linked with TG. This conclusion is very much in line with what has been known for years about water-holding in unheated and heated meat systems (reviewed in Offer and Knight, 1988a and b; Ruusunen and Puolanne, 2005;

Tornberg, 2005). Extra cross-links between myofibrillar proteins may prohibit the swelling of the myofibrils, which is a requirement for good water-holding.

Table 6. Effects of TGs on texture and water-holding properties of processed meat gels with a low NaCl content.

Raw materials	Origin of TG	Processing conditions	Effect on texture	Effect on water-holding	Reference
Ground chicken breast, 25% pork fat, 1% salt, tripolyphosphate	Pig plasma	Cooking for 30 min at 85°C	Improved gel strength	Improved cooking yield	Tseng et al. (2000)
Beef and pork meat chopped with TG, 26% fat, 18% ice, 1.65% nitrite curing salt, diphosphate	<i>Streptomyces mobaraense</i>	Cooking to a core temperature of 70°C	Improved breaking strength	No effect on cooking loss	Hammer (1998)
Pork batter (10% protein), 0.4–2% NaCl, tripolyphosphate		TG-pretreatment, cooking to a core temperature of 70°C at various heating temperatures	Increased gel hardness and chewiness at all NaCl levels	Decreased cooking loss at all NaCl levels	Pietrasik and Li-Chan (2002)
Homogenised chicken thigh, 30% water, 10% egg yolk and 10% white, 1% NaCl, tripolyphosphates		TG-pretreatment, discontinuous pressure at 500 MPa for 30 min at 20, 40 or 75°C	Improved gel hardness at high pressure	Decreased expressible moisture	Tres-palacios and Pla (2005)
Pork meat pieces massaged with TG, 13% water, 1 or 2% NaCl		Cooking for 65 min at 72°C and for 65 min at 78°C	Slightly improved firmness	No effect on cooking loss or juiciness	Dimitrakopoulou et al. (2005)
Pork batter, 15% water, no NaCl, 1% KCl, 20% fibre, 2% caseinate		Heating for 15 min at 40°C, then cooking to the final temperature of 70°C	Decreased gel hardness with TG>TG/fibre >TG/KCl > TG/caseinate	Increased cooking loss with TG>TG/KCl> TG/fibre >TG/caseinate	Jiménez Colmenero et al. (2005)
Pork, chicken or lamb batter, 13% water, 1.5% NaCl, tripolyphosphate		Cooking for 30 min at 70°C	Improved gel hardness	Increased cooking loss	Carballo et al. (2006)

However, contrasting results indicating improved water-holding caused by TG in low salt concentrations have also been reported at least by Tseng et al. (2000), Pietrasik and Li-Chan (2002) and Trespalacios and Pla (2005). Tseng et al. (2000) were able to increase cooking yield as a function of increasing TG dosage in chicken meatballs containing 1% salt, 0.2% tripolyphosphate and 25% pork fat. The high fat amount, phosphate and no added water might have contributed to the result. Pietrasik and Li-Chan (2002) studied the effects of TG and various salt levels (0–2%) on pork batter gel properties. The authors found that low salt levels, as expected, caused decreased gel firmness and cooking yield, but TG was able to increase both properties in the low-salt batters but not to the same level as with the normal salt amount (2%). Trespalacios and Pla (2005) reported significant reduction of the expressible moisture of low-salt chicken batters treated with TG. However, in their study ground chicken meat was mixed with fresh egg yolk and dehydrated egg white, both at 10% of the meat mass. Although the reported effects of egg proteins on hydration properties of meat gels are said to be somewhat contradictory (discussed in Trespalacios and Pla, 2005), they may have played a role in water-holding in the studied chicken meat batters.

High quality but moderate prices of meat products demanded by consumers have been the driving forces to develop methods to restructure low-value cuts and muscles of poorer quality to improve their market value by making them palatable steaks resembling intact muscle and to maximise the efficiency of carcass utilisation. Traditionally salt and phosphates in conjunction with heat treatment have been used to bind meat pieces together. Unheated comminuted products are usually frozen to enhance meat binding. Nowadays when consumers demand fresh, unfrozen meat as well as lower salt contents, technologies have been developed to eliminate the need for freezing and to enable the use of less salt. One of these technologies is TG-aided restructuring (Kuraishi et al., 1997; Uresti et al., 2004), which has been adopted in commercial scale production already some time ago and is still the main TG application in the meat sector. TG has been found to improve the strength of restructured meat protein gels with or without low levels of added salt and phosphates (Wijngaards and Paardekooper, 1988; Nielsen et al., 1995; Kuraishi et al., 1997). Kuraishi et al. (1997) reported that restructured meat products that are traditionally prepared using salt and phosphates to promote extraction of proteins can be prepared without added salt using TG, and that binding strength

can be boosted with caseinate added to the system as an extender. Caseinate is an excellent substrate for microbial TG (Færgemand et al., 1998b). It polymerises during the enzymatic reaction, it becomes viscous and thus acts like a glue binding the meat pieces together. The TG/caseinate technology has also been tested in fish products (Uresti et al., 2004). Textural parameters of fish gels, i.e. firmness and springiness were best when TG and caseinate were present. In addition, all the measured textural parameters increased along with the increasing salt concentration (0, 1 and 2%). The amount of expressible water was drastically increased (indicating reduced WHC) by TG in all tested salt concentrations, particularly in non- and low-salt gels. Adding caseinate into the system reduced expressible water to some extent but not to the level of the normal salt control.

As already stated, WHC is a feature that is affected by many technological factors, but also by many intrinsic features of meat proteins themselves. Due to the complexity of WHC *per se* and the inconsistent findings about the effects of TG in the development of WHC reviewed in this section, no generalisations concerning the role of TG in the water-holding of meat systems can be made. Despite this, the positive effect of TG on the textural properties is beyond dispute.

2.7 Aims of the study

Oxidative enzymes are promising tools for various industrial applications, both food-based and others, in which protein-containing materials are modified by protein polymerisation or incorporation of functional molecules to the proteins to achieve better feasibility or novel characteristics in the system. The aim of the present work was to evaluate the potential of oxidative enzymes, tyrosinase and laccase, and TG in the modification of meat protein matrices. More specific aims were:

1. to compare the oxidation efficiency of a novel tyrosinase from the fungus *Trichoderma reesei* on tyrosine, peptide, protein and protein fibre substrates to that of the well-known *Agaricus bisporus* mushroom tyrosinase (Publication I)

2. to study the effects of tyrosinase-, laccase- and TG-catalysed reactions in myofibrillar protein model systems, particularly on gel-forming and thermal properties (Publications II, III)
3. to study the effects of tyrosinase, laccase and TG on the textural and water-holding properties in model meat systems (Publications II, III, IV)
4. to evaluate the feasibility of TG in improving texture and water-holding in the manufacture of a cooked low-salt chicken meat product in pilot scale (Publication IV).

3 Materials and methods

The materials and methods used in this study are described in detail in the original publications I–IV. A brief summary of the methodology is given below.

3.1 Raw materials

Tyrosine (Y), DOPA, the tyrosine-containing peptides glycine-tyrosine-glycine (GYG) and glutamic acid-glycine-valine-tyrosine-valine-histidine-proline-valine (EGVYVHPV), β -casein, BSA and wool fibres were used in Publication I as model compounds to compare the action of the two tyrosinases, i.e. from the fungus *Trichoderma reesei* and the mushroom *Agaricus bisporus*. Throughout the rest of the study (Publications II–IV), commercially produced chicken breast meat as well as myofibrils and salt-soluble myofibrillar proteins (SSPs) prepared from chicken breast muscle were used as substrates for the enzymes. Substrates, their concentrations and analyses, in which the substrates were used, are listed in Table 7.

Table 7. Substrates used in the study.

Substrate	Concentration	Used in analysis	Used in Publication
Y DOPA GYG	1 mM	O ₂ consumption	I
EGVYVHPV	1 mM	O ₂ consumption RP-HPLC Maldi-TOF MS	I
β -casein BSA	1 mM	SDS-PAGE	I
Wool fibres	24 mg/ml	Microscopy	
SSPs	3 mg/ml	SDS-PAGE	II, III, IV
Myofibrils	3 mg/ml	O ₂ consumption	IV
	40 mg/ml	Gel formation	II, III
	70 mg/ml	DSC	II, III

For myofibril preparation commercial chicken breast muscles were trimmed of visible fat and connective tissue and cut into pieces. Myofibrils and SSPs were isolated according to Xiong and Brekke (1989) and stored in liquid nitrogen until use. Protein concentration was determined according to Lowry et al. (1951). Prior to use myofibrils and SSPs were suspended in 50 mM sodium phosphate buffer, pH 6, containing either 0.35 M or 0.60 M NaCl.

Commercial chicken breast muscles were coarsely ground and stored as portions in a freezer at -20°C in airtight plastic bags until use. For the laboratory scale experiments of gel firmness and water-holding as well as for light microscopy the frozen ground meat was slightly thawed and homogenised in a kitchen blender. The homogenate was mixed with water supplemented with NaCl and trisodium pyrophosphate as shown in Table 8 and enzymes, and stuffed into the steel cylinders for treatments. Protein concentration of the meat was analysed as Kjeldahl nitrogen and was found to be 23.3%. For pilot scale processing the meat coarsely ground through a 20 mm plate was thawed and mixed with water supplemented with the ingredients shown in Table 9 and stuffed into fibrous casings prior to the cooking process.

Table 8. Composition (wt-%) of the chicken breast meat homogenates prepared for the laboratory scale experiments (Publications II and IV).

Name of the homogenate	Meat content (%)	NaCl content (%)	Phosphate content (%)
Phosphate-free	75	2	–
Low-meat	65	2	0.34
Low-salt	75	1	0.34
Low-salt and -phosphate	75	1	0.17

Table 9. Composition^a (wt-%) and pH of the chicken breast meat test products prepared in the pilot scale (Publication IV).

Meat content (%)	NaCl content (%)
80	1.2
80	1.8
75	1.5
70	1.2
70	1.8

^a 0.3% phosphate, 0.06% ascorbic acid, 0.1% glucose and 0.012% nitrite were added as ingredients. TG was suspended in the brine.

3.2 Enzymes and enzyme activity assays

Tyrosine, DOPA, tyrosine-containing peptides, β -casein, BSA and wool fibres were treated with the tyrosinase purified from the filamentous fungus *Trichoderma reesei* (Selinheimo et al., 2006) and with a commercial mushroom tyrosinase preparation (Publication I). Chicken SSPs, myofibrils and meat homogenate samples were treated with *T. reesei* tyrosinase, laccase purified from *T. hirsuta*, a laccase preparation NovoSample 51003 or *S. mobaraense* TG purified free of maltodextrin (Publications II–III). Coarsely ground chicken breast meat used in the pilot scale study was treated with a commercial TG preparation (Publication IV). A summary of the enzymes used in this study is presented in Table 10.

Table 10. Enzymes used in the study.

Enzyme	Origin	Remarks	Used in Publication	Reference
Tyrosinase	<i>Trichoderma reesei</i>	Purified	I, II	Selinheimo et al. (2006)
	<i>Agaricus bisporus</i>	Tyrosinase from mushroom, Fluka, Switzerland	I	
Laccase	<i>Trametes hirsuta</i>	Purified	III	Rittstieg et al. (2002)
	<i>Myceliophthora thermophila</i>	NovoSample 51003, Novozymes A/S, Denmark	IV	
TG	<i>Streptomyces mobaraense</i>	Partially purified	III, IV	III
		Activa®WM, Ajinomoto Inc., Japan	IV	

Tyrosinase activity was assayed according to Robb (1984) using 2 mM L-tyrosine or 15 mM L-DOPA as substrate. Laccase activity was assayed according to Leonowicz and Grzywnowics (1981) using 0.06 mM syringaldazine as substrate. TG activity was assayed according to Folk (1970) using 0.03 M N-carbobenzoxy-L-glutaminyglycine as substrate. Endoprotease activity was assayed using azurine cross-linked casein as substrate (Megazyme International Ireland Ltd.). All enzyme activities are expressed as nanokatal (nkat). One nanokatal is defined as the amount of enzyme activity that converts one nmol of substrate per second in the assay conditions.

Reactivity of the tyrosinases with tyrosine and tyrosine-containing peptides as well as that of *Myceliophthora thermophila* laccase with the myofibrillar proteins was also evaluated as oxygen consumption monitored with an oxygen meter (Fibox 3, Precision Sensing GmbH, Germany) in closed vessels (Publications I, IV).

3.3 Biochemical and chemical methods

Changes in molecular weight and intensity of the enzymatically treated proteins were analysed with SDS-PAGE (Publications I–IV). SDS-PAGE is a simple method to detect polymerisation by covalent bonding but also fragmentation of proteins. Prior to running a protein sample into a polyacrylamide gel the protein is dissolved, reduced and denaturated.

Mass-spectroscopic (MALDI-TOF or MALDI-TOF/TOF MS) methods were carried out for analysis of the polymerisation products of the EGVYVHPV-peptide by tyrosinases using Autoflex MALDI-TOF MS (Bruker-Daltonik GmbH, Germany) (Publication I) and the fragmentation products of SSPs by laccase by using an Ultraflex MALDI TOF/TOF MS (Bruker-Daltonik GmbH, Germany) (Publication III).

Chromatographic analyses to the reaction products formed during the tyrosinase-catalysis of the EGVYVHPV peptide were performed by Waters 600 E chromatographic system (Waters Inc., USA) (Publication I).

Conformational changes and thermal stability of the enzymatically treated myofibrils were monitored by DSC820 differential scanning calorimetry (Mettler Toledo, Switzerland) (Publications II, III).

The commercial TG product Activa™ WM (Ajinomoto Inc., Japan) was purified free of maltodextrin, the content of which was 99% of the product. The one-step purification was carried out by cation-exchange chromatography using a CM Sepharose FF (Pharmacia, Sweden) column and 30 mM to 500 mM Na-acetate, pH 5.5, as an elution buffer (Publication III).

3.4 Enzyme treatments and process conditions

The enzyme treatments of tyrosine, DOPA, the tyrosine-containing peptides, β -casein, BSA and wool fibres are described in Publication I. The enzyme treatments of the SSPs, myofibrils and laboratory-scale chicken breast meat homogenate samples are described in Publications II–IV. The processing

conditions for the preparation of the chicken breast test products are presented in Publication IV.

3.5 Viscoelastic measurements

The effects of enzymes on gel-forming of the myofibril suspensions were measured using a Bohlin VOR rheometer (Bohlin Reologi AB, Sweden) in an oscillatory mode to determine the storage modulus (G') during heating of the myofibril suspensions at a constant temperature (Publications II, III).

3.6 Gel firmness

The effect of enzymes on the firmness of the cooked meat homogenate gel samples was measured in laboratory scale as a compression test in a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd.,UK) (Publications II, IV). The samples were pressed with a cylinder probe with a pressing surface diameter of 50 mm at room temperature. The trigger force, compression distance and measurement time were 10 g, 60%, 1.5 mm/s and 7 s, respectively. The effect of the commercial TG Activa®WM on the firmness of the pilot scale test products was measured using a texture analyzer (Instron Ltd., UK) (Publication IV). Test samples of the products were pressed by a probe with a pressing surface diameter of 45 mm at room temperature. The force required to compress the samples 5 mm was measured. The speed of the probe was 50 mm/min.

3.7 WHC analyses

The effect of enzymes on the WHC of the cooked meat homogenate gel samples was determined as weight loss by a low speed centrifugation method according to Hermansson and Lucisano (1982) after heating the samples to 72°C and subsequent cooling to 25°C (Publications II, IV). The effect of the commercial TG Activa®WM on the evaporation loss of the pilot scale test products was measured by weighing the products in casings before and after the cooking process (Publication IV).

3.8 Microscopical techniques

Incorporation of tyrosine and DOPA derivatives into wool fibres by tyrosinases was detected using Olympus BX-50 microscope (Olympus Corp., Japan) (Publication I). Ten fields per treatment were photographed and analysed for fluorescence caused by the attachment of the derivatives of tyrosine or DOPA to the wool fibres by tyrosinase.

The microstructure of heated meat homogenate gels was studied using light microscopy (Publication II). Both tyrosinase-treated and control gels were studied in order to examine the role of tyrosinase in the collagen matrix modification in two NaCl and meat concentrations. Cryosections of the cooked gel samples were stained with Aniline Blue (a stain specific for hydroxyproline in collagen) and Orange G (a protein stain) according to Ofstad et al. (1993). After staining, ten fields per sample were examined under an Olympus BX-50 microscope and photographed.

3.9 Statistical analysis of the experimental data

The experimental data were analysed using standard statistical procedures as described in Publications II–IV. One-way analysis of variance or multivariate analysis together with Tukey's b test was used to compare the mean values and to determine the significances of differences between them. Values were considered to be significantly different at $p < 0.05$.

3.10 Experimental design and modelling of the pilot scale production of test products

To study the effects of the TG, meat and NaCl contents on the compression force and cooking loss of the pilot scale chicken breast meat test products, a statistical design of the experiment and a regression analysis of the experimental data were applied (Publication IV). A two-level factorial design with 12 experiments was chosen to screen for the effects caused by the factors on the compression force and cooking loss. The experimental data was analysed by the PLS (partial least squares) multiple linear regression method. Regression analysis was carried out

and the response surfaces were plotted with the MODDE 6.0 (Umetrics AB, Sweden) software. The reliability of the models was evaluated by calculating R^2 , which explains the extent to which the variance of the modelled variable can be explained by the model. The models were also validated by calculating Q^2 , which explains how well the model will predict the responses in new experimental conditions.

4 Results and discussion

4.1 Comparison of *Trichoderma* and *Agaricus* tyrosinases (Publication I)

The capability of the novel tyrosinase from *Trichoderma reesei* to catalyse the oxidation of various tyrosine-containing substrates, from simple amino acids L-tyrosine and L-DOPA to a complex proteinaceous system of wool fibres, was compared to that of a well-characterised *Agaricus bisporus* tyrosinase.

The oxidation capacity of *Trichoderma* and *Agaricus* tyrosinases was examined in a buffer solution containing tyrosine, GYG or EGVYVHPV peptides. It was found that with *Trichoderma* tyrosinase the oxidation capacity was best with the peptide EGVYVHPV (Figure 6a), whereas *Agaricus* tyrosinase could efficiently oxidise tyrosine and GYG while oxidation of EGVYVHPV was much slower (Figure 6b). According to the results obtained by RP-HPLC and Maldi-ToF MS analyses, *Trichoderma* tyrosinase could form a number of reaction products from the peptide EGVYVHPV, including peptide oligomers and their oxidation products (Figures 2 and 3, Publication I). On the other hand, in the presence of *Agaricus* tyrosinase the number of reaction products from EGVYVHPV was smaller and only a dimer of the peptide was detected.

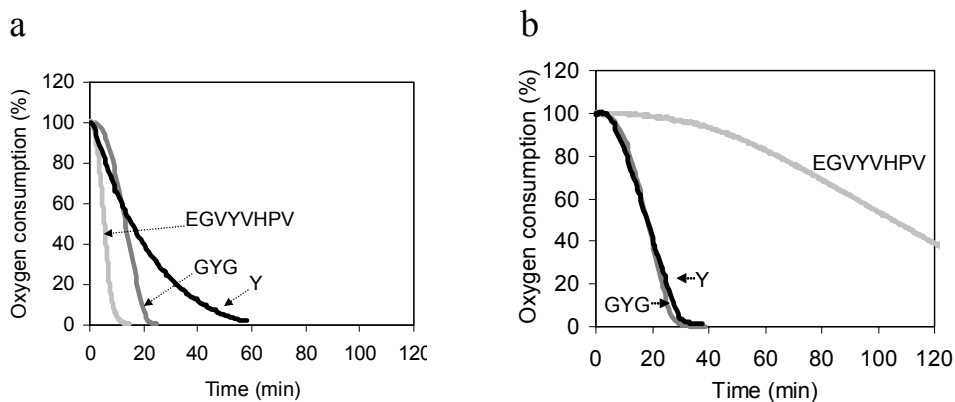


Figure 6. Oxygen consumption of tyrosine (Y), GYG- and EGVYVHPV-peptides with (a) *Trichoderma* and (b) *Agaricus* tyrosinase. Reaction conditions: enzyme dosage 1000 nkat/g, 50 mM Na-phosphate buffer pH 6.0, substrate concentration 1 mM, room temperature.

Cross-linking efficiency of the tyrosinases was studied with two model proteins, a random coil β -casein and globular BSA (Figure 4, Publication I). *Trichoderma* tyrosinase-catalysed cross-linking of β -casein was observed on SDS-PAGE, whereas *Agaricus* tyrosinase was not able to cross-link this protein. Neither of the tyrosinases could cross-link BSA, indicating that large size and/or bulky shape of this protein prevented entry of the protein substrate to the catalytic site of the enzymes.

The efficiency of the tyrosinases to activate tyrosine residues to their quinone forms in a complex proteinaceous substrate was studied with PMS (permonosulphuric acid)-pretreated wool fibres in buffer. Oxidative PMS opens up the extensive sulphhydryl bridging of the fibre cuticle, resulting in conversion of the cuticular disulphides to cysteic acid residues (Lewis, 1992). This pretreatment is assumed to facilitate penetration of the enzymes into the fibre. Tyrosinase-catalysed activation of the tyrosine residues in wool fibres was analysed utilizing the phenol coupling reaction initiated by tyrosinase (Figures 2 and 3). The study was based on the theory according to which tyrosinase oxidises both the tyrosine residues in the fibre and the free tyrosine or DOPA molecules in the surrounding solution to the corresponding diquinones (Figure 2). Subsequently the free quinones formed from tyrosine or DOPA can couple non-enzymatically with the quinones in the fibre or be bound to cysteine or lysine residues in the fibre, resulting in the formation of covalent bonds as depicted in Figure 3. The efficiency of *Trichoderma* and *Agaricus* tyrosinases in the incorporation of activated tyrosine and DOPA derivatives to the wool fibres was analysed by monitoring the increase of fluorescence caused by the attachment of the tyrosine or DOPA derivatives to the fibres under a microscope. The structures of DOPA and tyrosine are depicted in Figure 2. The fluorescence increase in wool fibres due to the incorporation of the tyrosinase-activated DOPA is presented in Figure 7.

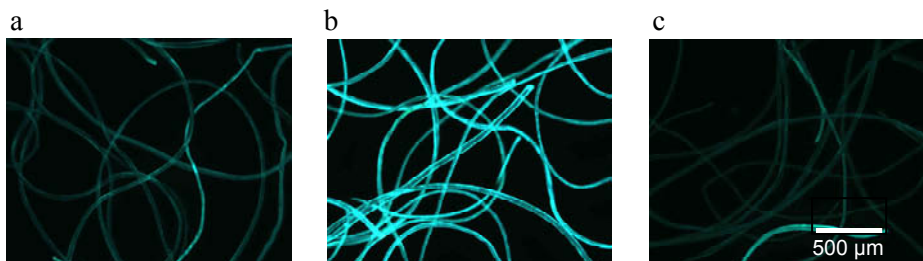


Figure 7. Micro images of the fluorescent wool fibres: (a) buffer-treated fibres, (b) *Trichoderma* and (c) *Agaricus* tyrosinase-treated fibres in the presence of DOPA.

As shown in Figure 7a, wool fibres in buffer without an enzyme or a graftable molecule autofluoresced only very slightly. The percentage of the highly fluorescent fibres of the total area of the fibres was about 4%. The heterogeneous nature of the fibres is shown as uneven fluorescence. When DOPA was added to the system, fluorescence of the fibres increased markedly in the presence of *Trichoderma* tyrosinase (Figure 7b). The percentage of the highly fluorescent fibres after the *Trichoderma* tyrosinase treatment was about 70%, whereas the fluorescence was remained the buffer-treated fibre level when *Agaricus* tyrosinase was used (Figure 7c). When DOPA but no enzyme was added to the wool fibres the detected fluorescence was about the same as that of the buffer-treated fibres (image not shown). The results show that *Trichoderma* tyrosinase had a markedly better capacity than *Agaricus* tyrosinase to activate tyrosine residues in the wool fibres and consequently to incorporate DOPA quinone derivatives either by phenol coupling or via the amino group of the molecules onto the wool fibres. Using tyrosine instead of DOPA did not lead to increased fluorescence (Table 11). The reason for this is that tyrosinase must first convert the tyrosine residues to DOPA before the reactive quinones can be formed. It has also been shown that L-tyrosine is converted to L-DOPA more slowly than L-DOPA to L-DOPA quinone (Espin et al., 2000). The fluorescence intensities of the tyrosinase-treated wool fibres are summarised in Table 11.

Table 11. Fluorescence intensity (%) of the wool fibres treated with *Trichoderma* and *Agaricus* tyrosinases in the presence of tyrosine or DOPA.

Origin of tyrosinase	Incorporated molecule	Amount of the highly fluorescent fibres (%)
–	–	4.3 + 3.2
<i>Trichoderma</i>	–	3.7 + 2.4
<i>Trichoderma</i>	Tyrosine	2.5 + 1.6
<i>Agaricus</i>	Tyrosine	4.7 + 2.6
<i>Trichoderma</i>	DOPA	70 + 6.7
<i>Agaricus</i>	DOPA	1.0 + 1.5

4.2 Enzymatic modification of myofibrillar proteins (Publications II, III)

The modifications of the SSPs caused by *Trichoderma* tyrosinase (Publication II), *Trametes* laccase and TG (Publication III) were determined by comparing the relative mobilities of the enzyme-treated proteins on SDS-PAGE to those treated otherwise similarly but without enzyme addition (Figure 8). SDS-PAGE has been widely used to monitor the cross-linking efficiency of TG and mushroom tyrosinase in various protein systems, both food-based and other. Covalent cross-links except for the disulphide bridges are not opened during the samples preparation for SDS-PAGE. Thus modification of the molecular size of a protein by the enzymatic cross-linking can be detected on SDS-PAGE. Myosin heavy chain (MHC \approx 205 kDa, Starr and Offer, 1971), actin (\approx 42 kDa, Elzinga et al., 1973), troponin T (\approx 30 kDa, Pearlstone et al., 1976) and myosin light chains (LCs 16–22 kDa, Starr and Offer, 1971), were visible on SDS-PAGE (Figure 8a). The SSPs as such did not show polymerisation on SDS-PAGE.

With enzymes the SSPs gave rise to changes in the electrophoretic pattern when compared with the enzyme-free control. Changes in the protein pattern caused by the enzymes (Figures 8b–d) were found to occur in the same proteins regardless of the enzyme. Myosin was clearly the most susceptible protein. To a lesser degree troponin T, low molecular weight proteins consisting of LCs, and

actin were also found to be modified. To compare the effects of the enzymes on the molecular sizes the proteins were enzyme-treated in 0.6 M NaCl.

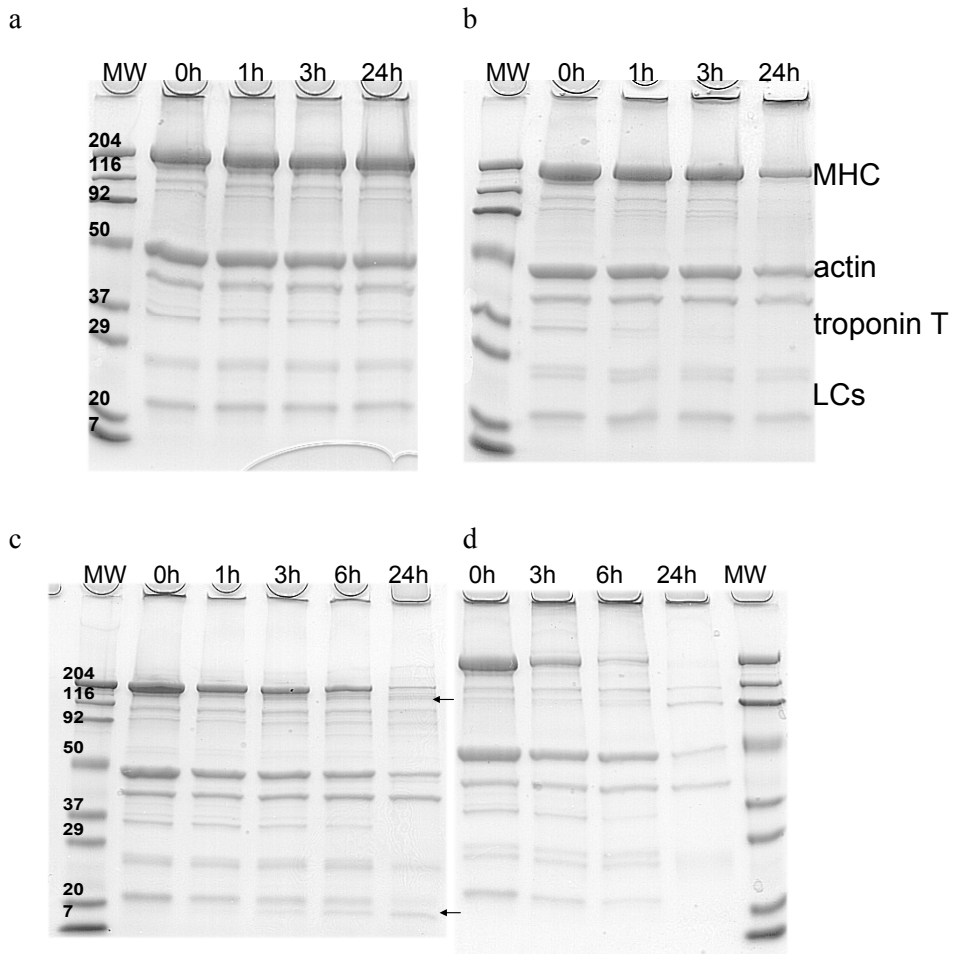


Figure 8. SDS-PAGE pattern of the SSPs treated in buffer (a), and with 1000 nkat tyrosinase/g protein (b), 500 nkat laccase/g protein (c) and 100 nkat TG/g protein (d). Samples were drawn from different time points of the treatments. The concentration of NaCl in the treatments was 0.6 M. Arrows show the laccase-catalysed fragmentation products. (Publications II, III.)

Of the three studied enzymes tyrosinase had the most limited effect on the electrophoretic pattern of the SSPs (Figure 8b). Tyrosinase polymerised troponin T, myosin (MHC) and actin as a function of time. Tyrosinase action could first be

visualised as fading of the troponin T, whereafter the MHC band faded. The actin band started to fade only during the overnight incubation. LCs were not found to be affected by tyrosinase.

With TG the electrophoretic pattern changed drastically (Figure 8d). Myosin was the most susceptible of the proteins. During the prolonged incubation the other myofibrillar proteins including actin were also efficiently cross-linked with TG. This can be seen as total fading of the protein bands on SDS-PAGE after 24 h incubation. Concomitantly, insoluble protein material was formed.

The action of laccase differed from that of the other two enzymes (Figure 8c). In the presence of laccase the MHC, actin and troponin T bands, respectively, started to fade within the incubation time as with tyrosinase and TG. However, a small band (7–20 kDa) and extra bands under the MHC band (indicated with arrows in Figure 8c) started to emerge after 1 h incubation. A peptide mapping and subsequent mass spectrometric analysis of the emerging new bands revealed that the small band (7–20 kDa) contained fragments of troponin T and the larger bands under the MHC band contained fragments of myosin. This indicates that simultaneously with protein polymerisation detected as precipitation of insoluble protein material during the laccase incubation, this enzyme also causes partial fragmentation of the same proteins. As the preparation contained no protease activity, which was determined before the experiment, the protein fragmentation was evidently caused by the laccase activity. These results are in good agreement with those obtained by Martinaud et al. (1997) using chemical radical generating systems leading to myofibrillar protein polymerisation and fragmentation during oxidation. In this study, the most sensitive of the proteins to the oxidative treatments were myosin and troponin T, of which both polymerisation and fragmentation products were detected on SDS-PAGE.

The different amounts of the target amino acid residues for TG, tyrosinase and laccase (Table 12) of the main myofibrillar proteins, myosin and actin, may partly explain the differences of these proteins in their susceptibility to the cross-linking with TG. The amounts of glutamine/glutamic acid and lysine are higher in myosin than in actin. In addition the amounts of glutamine/glutamic acid and lysine are higher than the amounts of tyrosine and cysteine/cystine used by tyrosinase or laccase in the cross-link formation. This may partially explain why TG seemed to be a more efficient myosin cross-linker in the experimental

conditions compared to tyrosinase or laccase. Nevertheless, the exact location of the target amino acids in the native proteins is not known. It can be assumed that the rod-like three-dimensional structure, large size of myosin and apparently better accessibility of the target amino acid residues when the protein is dissolved may render myosin more susceptible to the action of the enzymes than actin, which is a less soluble, smaller and globular protein.

Table 12. The amounts of the target amino acid residues of myosin and actin (Belitz and Grosch, 1999) for the action of TG, tyrosinase and laccase.

Amino acid	The amount of amino acid (g / 16 g N)	
	Myosin	Actin
Tyrosine	3	6
Glutamine/glutamic acid	22	14
Cysteine/cystine	1	1
Lysine	12	7

As the three enzymes have different reaction mechanisms, and particularly because the action of laccase in protein systems is poorly understood, no general conclusion about the relative efficiencies of these enzymes as protein cross-linkers can be made. However, on the basis of the results obtained it can be said that under the chosen experimental conditions tyrosinase was not nearly as efficient as TG in the myofibrillar protein cross-linking. The feasibility of laccase is open to doubt due to its tendency to fragment meat protein. Modifications of the myofibrillar proteins caused by the enzymes and detected by SDS-PAGE are summarised in Table 13.

Table 13. Enzymatic modifications of the myofibrillar proteins detected by SDS-PAGE.

Myofibrillar protein	Tyrosinase	Laccase	TG
Myosin heavy chain (MHC)	+	++ ^a	+++
Actin	+	++	+++
Troponin	++	+ ^a	+++
Myosin light chains (LCs)	–	+	++

^a Fragmentation products were detected by Maldi TOF/TOF MS analysis.

4.3 Gel formation of myofibrils (Publications II, III)

Protein gels are formed by intermolecular interactions resulting in a three-dimensional network of protein fibres that promote structural rigidity (Fennema, 1976). The most important heat-initiated process in a meat system is the thermal denaturation of the muscle proteins, which ultimately leads to gelation. Myofibrillar proteins play an essential role in this process. Of the main myofibrillar proteins, myosin alone is capable of efficient gel formation, while the role of actin is less significant (Yasui et al., 1980).

In this study gel formation was followed by measuring the storage modulus (G') of the 4% myofibril suspensions at pH 6 in the presence of the enzymes and at a constant temperature of 40°C (Figure 9). The efficiency of tyrosinase and laccase in gel formation was compared to that of TG.

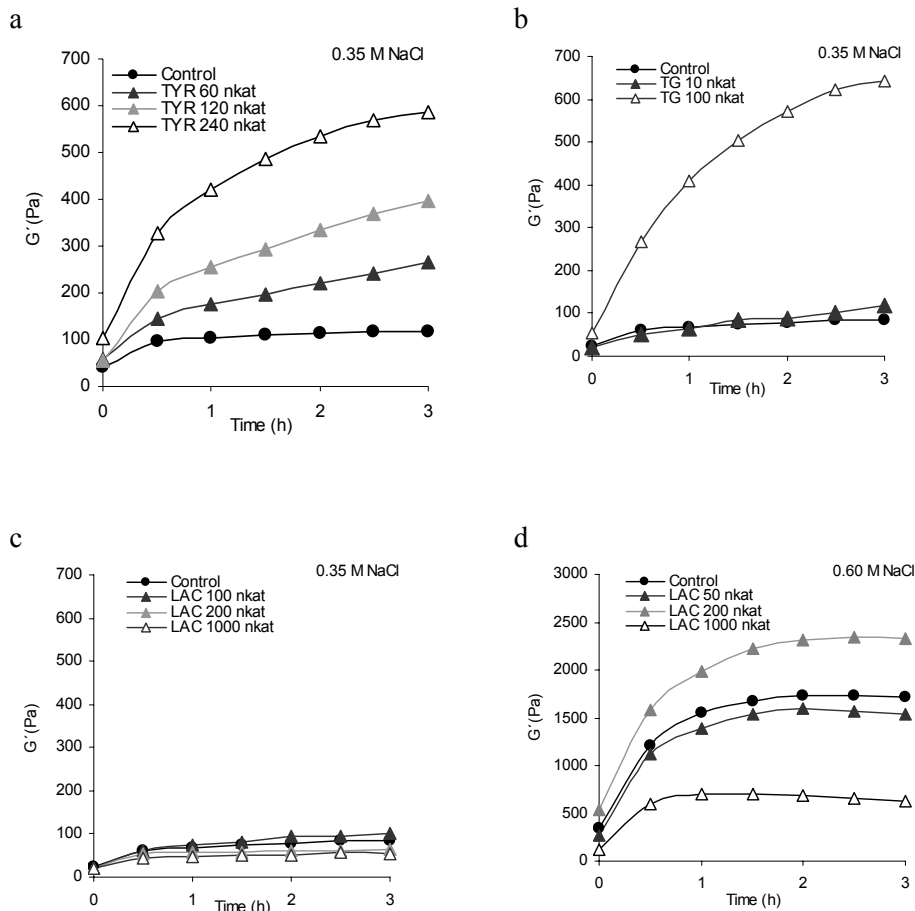


Figure 9. Storage modulus (G') of 4% myofibril suspension treated with a) tyrosinase, b) TG and c) laccase at 0.35 M NaCl and d) laccase at 0.60 M NaCl. G' was measured at 40°C for 3 h. Reaction mixture: 40 mg protein/ml, 50 mM sodium phosphate buffer, pH 6. Enzymes were dosed as nkat/g protein.

At the lower NaCl concentration of 0.35 M, gel formation without enzyme addition was slow (Figures 9a–c). G' increased to the level of 85–118 Pa. The main reason for the poor gel formation was the limited solubility of the myofibrillar proteins at 0.35 M NaCl. However, both tyrosinase and TG were able to enhance the gel formation at this salt concentration (Figures 9a–b). G' increased along with the increasing tyrosinase dosage, indicating a clear connection between gel and cross-link formation. In less than one hour of treatment with 240 nkat tyrosinase/g the increase of G' began to slow down,

approaching a plateau level at about 590 Pa indicating saturation in the cross-link formation due to the possible extinction of accessible cross-link sites in the proteins. With the lower enzyme dosages the plateau phase was not reached, although the rate of gel formation began to decrease just as with the high dosage after one hour of treatment.

The dose of 10 nkat TG/g was too low to enhance gel formation. With this dosage G' remained at the control level of 85 Pa. With 100 nkat TG/g the G' increase was pronounced and rather similar to that obtained with 240 nkat tyrosinase/g. A plateau at a G' level of 570–590 Pa was reached in 2.5 h of the treatment indicating saturation in the cross-link formation as in the case of 240 nkat tyrosinase/g. Gel formation with TG is shown in Figure 9b.

When laccase was added to the myofibril suspension, the gelation process differed markedly as compared to that obtained with tyrosinase or TG. At the low NaCl concentration laccase was not capable of enhancing the gel formation (Figure 9c). Apparently the limited solubility of the myofibrillar proteins at this salt concentration combined with the poor protein cross-linking ability of laccase as proposed by Mattinen et al. (2006) were the reasons. When the NaCl concentration was increased to 0.60 M the gel formation was substantially increased in the enzyme-free control suspension as compared to the gel formation at 0.35 M NaCl (Figure 9d). G' of the control remained on the level of 85 Pa in 0.35 M NaCl throughout the 3 h measurement, whereas in 0.60 M NaCl G' reached a level of 1735 Pa. Adding 50 nkat laccase/g had practically no impact on G' , which reached a level of 1600 Pa. 200 nkat laccase/g protein increased G' slightly compared to the control. The gel formation started instantaneously, as in the case of tyrosinase and TG. The plateau level in the G' development was reached at 2350 Pa after about 1 h of treatment. Increasing the laccase dosage to 1000 nkat/g protein resulted in slower development of G' compared to the control. The plateau phase of G' was reached at about 700 Pa during the first hour of the treatment.

Protein polymerisation due to covalent cross-linking by the enzymes was detected as enhanced G' . On the other hand, impaired gel formation apparently caused by the protein fragmentation was observed, when an extensive laccase dosage was used. Protein fragmentation was detected also on SDS-PAGE of the SSPs treated with laccase (Figure 8c).

Radicals formed in the laccase reaction are proposed to be the reason for fragmentation of the most susceptible myofibrillar proteins. As discussed earlier, fragmentation products of myosin and troponin T were detected after a laccase treatment. Oxidation reactions catalysed by iron or copper have also been shown to impair the gel formation ability of myofibrils, detected as weakening of gels (Xiong and Decker, 1995). Oxygen radicals generated by e.g. ionizing radiation are known to cause various chemical changes in proteins, e.g. aggregation, polymerisation and fragmentation (Garrison, 1987; Davies, 1987; Davies and Delsignore, 1987). There is no definite reason to believe that radicals formed during the laccase catalysis would not affect protein in a similar way as the free radicals formed due to metal ions, ionic irradiation, or other non-enzymatic radical generating sources. It has been reported by Carvajal-Milan et al. (2005) that arabinoxylan gels induced by laccase have poor stability properties. Furthermore, softening of dough due to a high dosage of laccase has been reported by Selinheimo et al. (2005).

TG has been shown to boost gel formation of various protein suspensions such as gluten (Larré et al., 2000), micellar casein, (Schorsch et al., 2000a and b), oat globulin (Siu et al., 2002), bovine cardiac myofibrillar proteins (Ramirez-Suarez et al., 2001) and mixtures of soy, whey and gluten with myofibrillar proteins (Ramirez-Suarez and Xiong, 2002a, b, 2003; Ramirez-Suarez et al., 2005). In all these cases the improvement of gel formation is presumed to be due to the covalent bond formation between proteins by TG. In the present study it was shown that tyrosinase, but not laccase, can be used to enhance gel formation in a myofibrillar protein system due to covalent protein cross-linking.

4.4 Thermal properties of myofibrils (Publications II, III)

The heat stability of proteins influences their functional properties (Montejano et al., 1984). The most crucial heat-induced process in a meat system is the thermal denaturation of muscle proteins, contributing to gelation. Differential scanning calorimetry (DSC) permits evaluation of the conformational changes in proteins during thermal denaturation at high concentrations, thus allowing study of the proteins in their natural environment (Findley et al., 1986). When a whole muscle sample is heated in DSC, three major endotherms are usually observed (Stabursvik and Martens, 1980; Findley et al., 1986; Xiong et al., 1987; Amako

and Xiong, 2001). However, the endotherms with their distinguishable peaks and interpretation of the peaks vary considerably. The first transition peak is observed at 54–60°C and is attributed to myosin, the second peak occurs at 65–67°C is attributed to connective tissue proteins such as collagen and sarcoplasmic proteins and finally the third peak assigned to actin is found at 78–83°C (Wright et al., 1977; Stabursvik and Martens, 1980; Findley et al., 1986; Xiong et al., 1987).

It should be emphasised that although intact muscle samples usually show three distinct transition phases in DSC, in isolated myofibril systems devoid of connective tissue and sarcoplasmic proteins three or only two transition phases are observed. Stabursvik and Martens (1980) observed two transition phases at pH 6.10 with peaks at about 60°C and 77°C, ascribing them to the denaturation of myosin and actin, respectively. Xiong et al. (1987) reported two transition peaks in isolated myofibrils of chicken, lamb, pork and beef, at approximately 60°C and 70°C, which they cautiously ascribed to myosin and actin, respectively. Recently, Aktaş and Kiliç (2005) reported two transition peaks in the thermal scanning of beef myofibrils, at about 62°C and 71°C, ascribing them to myosin and actin, respectively. The precise peak temperatures, which reflect unfolding and aggregation, are dependent on pH, ionic environment and species and are also sensitive to heating rate (Findley et al., 1986; Xiong et al., 1987; Foegeding, 1988). Consequently, straightforward comparison between different studies should be avoided.

In this study thermal analysis using DSC was performed with the isolated chicken breast muscle myofibrils treated with tyrosinase (Figure 10a and Table 14), laccase and TG at pH 6.0 (Figure 10b and Table 15) at a protein concentration of 70 mg/ml. A heating rate of 10°C/min was used to scan the samples over the temperature range of 40–100°C. The heat of transition (ΔH) associated with the heat-induced conformational changes of the myofibrillar proteins during denaturation was estimated. The peak temperatures (T) of the transitions, providing information about unfolding and aggregation and thus reflecting thermal stability were also recorded.

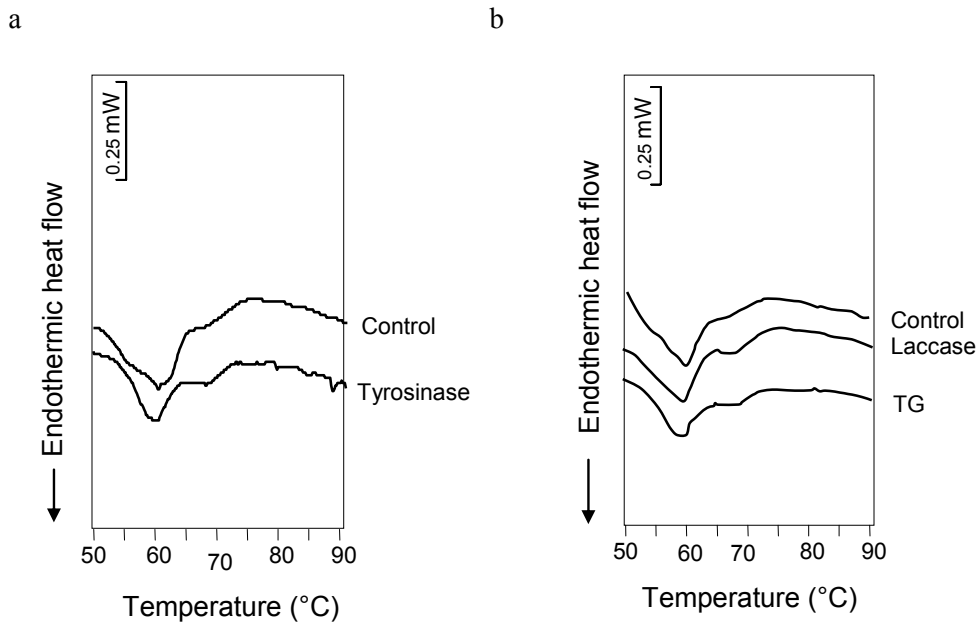


Figure 10. DSC curves of the myofibrils treated with tyrosinase (a) and laccase and TG (b). Pretreatment conditions: tyrosinase 500 nkat/g, laccase 200 nkat/g and TG 100 nkat/g protein, 40°C, 1 h, 50 mM sodium phosphate buffer, pH 6, 0.60 M NaCl, 70 mg protein/ml.

Table 14. Heat of transition (ΔH) and peak temperature (T) of the tyrosinase-treated myofibrils.

Pretreatment	1 st transition		2 nd transition	
	ΔH (J/g)	T (°C)	ΔH (J/g)	T (°C)
Control	6.8 a (0.3)	59.6 a (0.1)	0.2 a (0.1)	66.7 a (0.5)
Tyrosinase	4.7 b (0.2)	59.4 a (0.1)	0.5 ab (0.1)	68.1 ab (0.6)

Means with different letters in the same column are significantly different at $p < 0.05$. Values in parentheses are standard deviations of the means.

Table 15. Heat of transition (ΔH) and peak temperature (T) of the laccase- and TG-treated myofibrils.

Pretreatment	1 st transition		2 nd transition	
	ΔH (J/g)	T (°C)	ΔH (J/g)	T(°C)
Control	5.9 ab (0.3)	59.4 a (0.0)	0.2 a (0.1)	66.3 a (0.8)
Laccase	6.5 b (0.7)	59.1 a (0.6)	0.5 b (0.0)	67.0 a (0.6)
TG	4.3 a (0.0)	58.5 a (0.1)	0.4 b (0.0)	66.7 a (0.5)

Means with different letters in the same column are significantly different at $p < 0.05$. Values in parentheses are standard deviations of the means.

DSC curves of the isolated myofibrils (Figures 10a–b) with or without enzymes showed two transition peaks at about 59–60°C and 66–67°C, indicating apparently thermal transitions of myosin and actin, respectively (Xiong et al., 1987; Aktaş and Kiliç, 2005). When tyrosinase was added to the system the ΔH of the myosin transition decreased. With TG the ΔH decreased and with laccase increased, but not significantly. Decreased ΔH of myosin by tyrosinase and TG may indicate conformational changes of the protein because of polymerisation (Arnfield and Murray, 1981) needed less energy for denaturation. On SDS-PAGE both tyrosinase and TG were found to polymerise myosin. It is noteworthy that after 3 h of tyrosinase treatment visible changes in MHC were not observed on SDS-PAGE, whereas modification of MHC by TG was clear (Figures 8b–d). On the other hand, in DSC the conformational change determined as ΔH decrease was detected with tyrosinase already after 1 h enzyme treatment, whereas the ΔH decrease by TG was detected but the change was not significant. The reason why the ΔH of myosin increased slightly as a result of laccase treatment is not clear but may be due to the type of reaction of myosin with laccase, leading to both protein polymerisation and fragmentation. Modification of MHC by *Trametes hirsuta* laccase was visible on SDS-PAGE after 1 h of treatment (Figure 8c).

All three enzymes increased the ΔH of the actin transition, indicating conformational changes in the protein structure requiring more heat energy for denaturation. On SDS-PAGE the actin band showed less modification by the enzymes than MHC (Figure 8), reflecting either changes which were not detectable on SDS-PAGE or better resistance of actin to the enzyme-catalysed

polymerisation. Probably due to its globular shape and the limited number of accessible target amino acid residues in the protein, actin was less prone to polymerisation. Although nothing is known about the intramolecular cross-linking of actin by tyrosinase or laccase, TG is reported to form an intramolecular isopeptide bond in rabbit actin, perturbing the structure of the actin monomer (G-actin) but improving the thermostability of F-actin (Eli-Berchoer et al., 2000). However, in this study the conformational changes or thermostability of the cross-linked actin were not evaluated by DSC. Thermostability was studied by the ability of the cross-linked actin to activate S1-ATPase activity at 60°C.

TG has been shown to affect the thermal properties of myofibrillar proteins. Ramirez-Suarez et al. (2005) reported the thermal property changes of isolated myofibrils of chicken breast meat. TG promoted disappearance of the 1st transition peak at 61.5°C and increase of the ΔH of the 2nd peak at 68°C, whereas the peak temperature of the 2nd peak was not affected. TG was found by Aktaş and Kılıç (2005) to decrease the denaturation enthalpy of myosin in ground beef meat, but did not affect that of actin. Changes in the heat of transition are thought to be related to the conformational changes caused by intra- and/or intermolecular cross-linking catalysed by TG whereas changes in the peak temperature are regarded as being due to the changes in thermal stability (Ramirez-Suarez and Xiong, 2003).

In our DSC measurements the peak temperature of myosin and actin did not change with any of the enzymes used, indicating that thermal stability of the proteins was not affected by the enzymes in the treatment conditions (Tables 14 and 15). Changes in ΔH may reflect some conformational changes in the proteins due to the intermolecular and/or intramolecular cross-linking by tyrosinase and TG and cross-linking and fragmentation by laccase.

4.5 Texture and water-holding of chicken breast meat homogenate gels (Publications II, IV)

Texture and water-holding are features that significantly influence palatability and consumer acceptance of foods. Replacement or reduction of different ingredients in foods usually leads to changes in these features of a food gel.

Changes in structure are perceived by altered texture. From the perception point of view firmness of a gel is an essential attribute. Textural characteristics of gels are often analysed, as in this study, by compressing a gel using a constant pressure and measuring the resistant force of the gel. The capability of a gel to hold water can be measured in a number of ways. In this study WHC was evaluated by measuring the weight loss of the meat homogenate gel samples after cooking, cooling and centrifuging the samples.

The effects of *Trichoderma* tyrosinase, *Myceliophthora* laccase and TG on texture and WHC of the meat homogenates were evaluated by carrying out the enzyme treatments of the meat homogenates in closed cylinders without mixing. The meat homogenates (see compositions in Table 8, Section 3.1) treated with the enzymes contained decreased amounts of meat (65%), salt (1% corresponding to 0.13 M Na⁺) or phosphate (0% or 0.17%). The fat content of the meat used was less than 1%. After the enzyme treatments the core temperature of the gels was increased to 72°C. Firmness of the gels was analysed as maximum compression force and WHC as weight loss after cooling the gels to room temperature and centrifugation. The figures for maximum compression force of the enzymatically treated homogenate gels are given in Table 16 and for weightloss in Table 17.

The results show that tyrosinase increased the maximum compression force of the meat homogenates free of phosphate and with a low meat content, both containing 2% NaCl. Tyrosinase did not affect the maximum compression force of the homogenate with a low salt content (1%). When NaCl and phosphate contents both were low, 1% and 0.17%, respectively, the decrease of the maximum compression force was significant. It is worthy of note that tyrosinase did not significantly increase the weight loss of any of the studied meat homogenate systems. The lower dosage of tyrosinase (20 nkat/g protein) was capable of decreasing the weightloss of the homogenates containing either a low NaCl content (1%) or a low meat content (65%).

Surprisingly, laccase had a tendency to decrease the maximum compression force and increase the weight loss of all other homogenates except that free of phosphate. The laccase preparation used did not contain detectable protease activity and no fragmentation of the myofibrillar proteins was detected on SDS-PAGE (Publication IV, Figure 1) was observed with this laccase preparation. It

is emphasised that the laccase preparation used in this study (Publication IV) was different from that used to treat the myofibril proteins in Publication III, in which myofibril protein fragmentation due to laccase was reported.

The higher dose of TG increased the maximum compression force of all the homogenates, but on the other hand also increased the weight loss of them. The lower dose of TG increased the maximum compression force of the low-salt homogenate and the weight loss of the low meat and low salt/phosphate homogenates. The higher doses of laccase and TG seemed both to improve the compression force of this homogenate. However, with the lower dosages of these enzymes the effect was the opposite reflecting unreliability of the results and/or instability of this homogenate system in the presence of laccase and TG. The weight loss of this homogenate increased markedly with both laccase and TG regardless of the enzyme dosage.

Table 16. The maximum compression force of the homogenate gels affected by tyrosinase, laccase and TG after cooking and cooling of the samples.

Enzyme and dosage (nkat/g)	Phosphate-free	Low-meat	Low-salt	Low salt and phosphate
	Maximum compression force (g)			
Control	10 200 a (260)	6 600 a (180)	6 600 a (750)	6 200 a (358)
Tyrosinase 20	11 900 b (380)	7 200 a (260)	6 600 a (270)	4 600 b (600)
Tyrosinase 120	12 000 b (950)	9 600 b (460)	6 500 a (380)	4 400 b (450)
Control	12 300 bc (300)	9 100 bc (230)	8 700 b (90)	5 400 b (20)
Laccase 50	11 800 ab (240)	5 900 a (700)	6 200 a (80)	4 400 a (6)
Laccase 200	11 600 ab (450)	5 800 a (610)	6 800 a (480)	5 800 c (8)
TG 20	13 000 c (50)	8 700 b (560)	11 100 c (420)	5 000 b (70)
TG 200	15 800 d (590)	11 900 d (1000)	11 000 c (1100)	5 900 c (300)

Homogenate compositions are given in Table 8 (Section 3.1). The enzymes were dosed as nkat/g of protein. Means with different letters in the same column are significantly different at $p < 0.05$. The experiments with tyrosinase and the corresponding control were carried out separately from the laccase and TG experiments which were carried out concurrently. Values in parentheses are standard deviations of the means.

Table 17. Weight loss of the homogenate gels affected by tyrosinase, laccase and TG. Weight loss was measured after cooking, cooling and centrifugation of the gel samples.

Enzyme and dosage (nkat/g)	Phosphate-free	Low-meat	Low-salt	Low salt and phosphate
	Weight loss after centrifugation (%)			
Control	6.9 a (0.1)	9.0 a (0.7)	11.0 a (0.5)	12.4 ab (1.1)
Tyrosinase 20	6.9 a (0.2)	7.0 b (0.7)	8.0 b (0.9)	12.7 ab (1.5)
Tyrosinase 120	6.2 a (0.1)	6.3 b (0.7)	9.3 ab (0.5)	13.2 b (0.6)
Control	7.2 ab (0.1)	6.1 a (0.1)	7.4 a (1.6)	13.2 a (0.1)
Laccase 50	8.5 ab (0.7)	10.8 c (0.6)	11.6 b (1.0)	15.8 b (0.5)
Laccase 200	9.5 bc (0.7)	11.6 c (0.2)	12.1 b (0.9)	16.5 b (0.1)
TG 20	8.0 ab (1.4)	9.0 b (0.2)	9.3 ab (0.4)	17.4 b (0.9)
TG 200	11.5 c (0.7)	11.3 c (0.1)	12.2 b (0.9)	21.4 c (0.6)

Homogenate compositions are given in Table 7 (Section 3.1). The enzymes were dosed as nkat/g of protein. Means with different letters in the same column are significantly different at $p < 0.05$. The experiments with tyrosinase and the corresponding control were carried out separately from the laccase and TG experiments which were carried out concurrently. Values in parentheses are standard deviations of the means.

With tyrosinase the experiments were carried out several months later than those with laccase and TG, which were carried out concurrently. Thus the freeze-storage time at -20°C of meat used in the tyrosinase experiments was considerably longer. Compression force of the gels was generally weaker in the tyrosinase-free controls compared to the laccase/TG-free controls (Table 16). In addition, the weight loss of the tyrosinase-free low-meat and low-salt controls was higher than that of the corresponding laccase/TG-free controls (Table 17). In several studies it has been found that increasing duration of frozen storage has adverse effects on meat functionality (Awad et al., 1968; Farouk et al., 2004), water distribution causing reduced cooking yield (Bertram et al., 2007) and WHC of pork sausage raw materials (Puolanne and Turkki, 1985). Hitherto, the changes in functionality of meat systems during frozen storage and subsequent thawing are by no means fully understood. In addition to the difference in the duration of frozen storage of the raw material between the tyrosinase experiments and laccase/TG experiments, the added water was oxygenated to ensure a sufficient oxygen supply in the tyrosinase experiments.

Because of the different frozen storage time of the meat raw material and water oxygenation in the tyrosinase experiments, the results obtained with tyrosinase should be compared with those obtained with laccase or TG only with caution. Although replicate measurements showed good similarity within a single experiment, the values of the maximum compression force and weight loss of the low-meat and low-salt enzyme-free controls of the two separate experimental sets differed markedly from each other at least partly because of the reasons discussed above. However, the following general conclusions can be drawn from the results.

TG was an efficient texture improver when the enzyme was used sufficient amounts. This was expected, as the efficiency of TG in texture improvement has been recognised in a number of studies (Table 6). TG has been reported to increase yield or WHC in low-salt meat products (Tseng et al., 2000; Pietrasik and Li-Chan, 2002), but it is also agreed that cross-linking catalyzed by TG may negatively affect development of the meat protein matrix during heating, resulting in reduced WHC in the system. This view was supported by e.g. Carballo et al. (2006). Although tyrosinase seemed inferior to TG in texture improvement, it did not cause decline in water-holding like TG.

Myofibrillar proteins affect the textural and water-holding properties most strongly. When meat rather than isolated myofibrillar proteins is treated with cross-linking enzymes collagen should also be regarded as a target substrate. Collagen is known to act as a substrate for TG (Chen et al., 2005) and tyrosinase (Dabbous, 1966). In the myofibrillar proteins the amount of tyrosine is much lower than that of glutamine or lysine (Table 11). In collagen, the amount of tyrosine is < 0.5%, whereas the amount of glutamine/glutamic acid is 7–11% (Bailey and Light, 1989). The low tyrosine amount compared to glutamine, both in myofibrillar proteins and in collagen, apparently results in a limited effect of tyrosinase. However, limited action may be an advantage as too extensive formation of covalent cross-bridges may well lead to a too restricted swelling of myofibrils, therefore causing a decline in WHC.

Processing such grinding enhances oxidative reactions in meat by introducing O₂ to the system. Oxidative conditions cause protein polymerisation or fragmentation, conformational changes, amino acid derivatisation and formation of protein and other radicals, leading possibly to alterations in protein

functionality such as gel formation and protein-water interactions such as water-holding and viscosity (reviewed by Xiong and Decker, 1995). Tyrosinase and laccase are oxidative enzymes both requiring O₂ for their action, thus exhausting it from the system. However, these enzymes have different reaction mechanisms. Whereas laccase generates free radicals, in tyrosinase catalysis free radicals are not formed. The laccase-catalysed free radicals were most probably the reason for the decline in protein functionality detected as decreased compression force and increased weight loss of the homogenate gels, although myofibrillar protein fragmentation was not observed in SDS-PAGE with the used laccase.

Tyrosinase caused different effects compared to laccase. Tyrosinase utilises more O₂ per cross-link formed than laccase resulting in more efficient O₂ exhaustion from the system which is an advantage in meat processing. Tyrosinase caused protein polymerisation, not fragmentation (Figure 8a). Protein polymerisation was also observed as enhanced gelation in the presence of tyrosinase (Figure 9a). In a meat homogenate system tyrosinase was capable of increasing the compression force of the gels to a certain degree. Possible lower efficiency compared to TG may have been due, in addition to the limited amount and accessibility of the target amino acid residues in the substrate proteins, to O₂ depletion in the system leading to cessation of the tyrosinase action during the treatment. O₂ exhaustion by tyrosinase may also be regarded as a controlling factor, with which over-efficient tyrosinase action could be restricted in order to prevent reduction of WHC in a meat system. Furthermore and unlike laccase or TG, in the treatment conditions used tyrosinase did not cause decline in water-holding but could improve it. Restricted protein cross-linking efficiency of tyrosinase may have led to the limited protein network formation, enabling swelling of the myofibrils which is crucial for water-holding. This may explain the positive effects of tyrosinase on the development of WHC. Unimpaired weight loss indicates that the action of tyrosinase did not cause reduction of protein functionality.

The role of tyrosinase in the collagen network formation in the meat homogenates may also have affected the WHC by entrapping water in the collagen network created by tyrosinase. It is known that a substantial amount of water is located in the extracellular spaces in meat (Offer and Trinick, 1983; Huff-Lonergan and Lonergan, 2005).

4.5.1 Microstructure of the gels – effect of tyrosinase on collagen (Publication II)

In order to study the role of tyrosinase in the collagen modification, meat homogenate samples treated with tyrosinase were examined under a light microscope. Heated chicken breast muscle as well as breast meat homogenate gels were stained with Aniline Blue and Orange G to visualise collagen in the samples. Figure 11a shows the structure of a heated muscle. Cross-sections of the muscle fibres (MF) can clearly be seen. Collagen (C) is stained blue and the muscle cells yellow. Intramuscular connective tissue (endomysium, E) containing collagen is located around the muscle cells. Broad extracellular spaces (ES) can be seen between the cells and are most probably due to growth of ice crystals during freezing (Ofstad et al., 1993) and/or shrinkage of myofibrils during heating.

The structure of a meat homogenate containing 75% meat and 1% salt is presented in Figure 11b. After homogenisation the fibre structure of meat was disintegrated and collagen was observed as granular material (indicated with an arrow in Figure 11b). Ofstad et al. (1993) observed the emergence of granular material in the intercellular space of ground cod and salmon samples during heating and proposed it to be a mixture of gelatine and sarcoplasmic proteins.

Two homogenised meat systems containing 75% meat and 1% salt or 65% meat and 2% salt were prepared, the rest being water in both cases. 0.34% phosphate was added to the homogenates. Tyrosinase was added and the homogenates were pretreated at 4°C for 1 h and at 40°C for 1 h before heating to 72°C in a water bath. The tyrosinase-treated homogenates were compared to the enzyme-free controls. The form of collagen changed from localised granular forms towards a more network-like structure in the presence of tyrosinase in both homogenates (Figures 11c and d). This was observed as spreading and levelling of the blue colour in the micro images.

In the tyrosinase-treated homogenate containing 65% meat and 2% salt the formation of a collagen network was more apparent (Figure 11d) than in the homogenate containing 75% meat and 1% salt (Figure 11c).

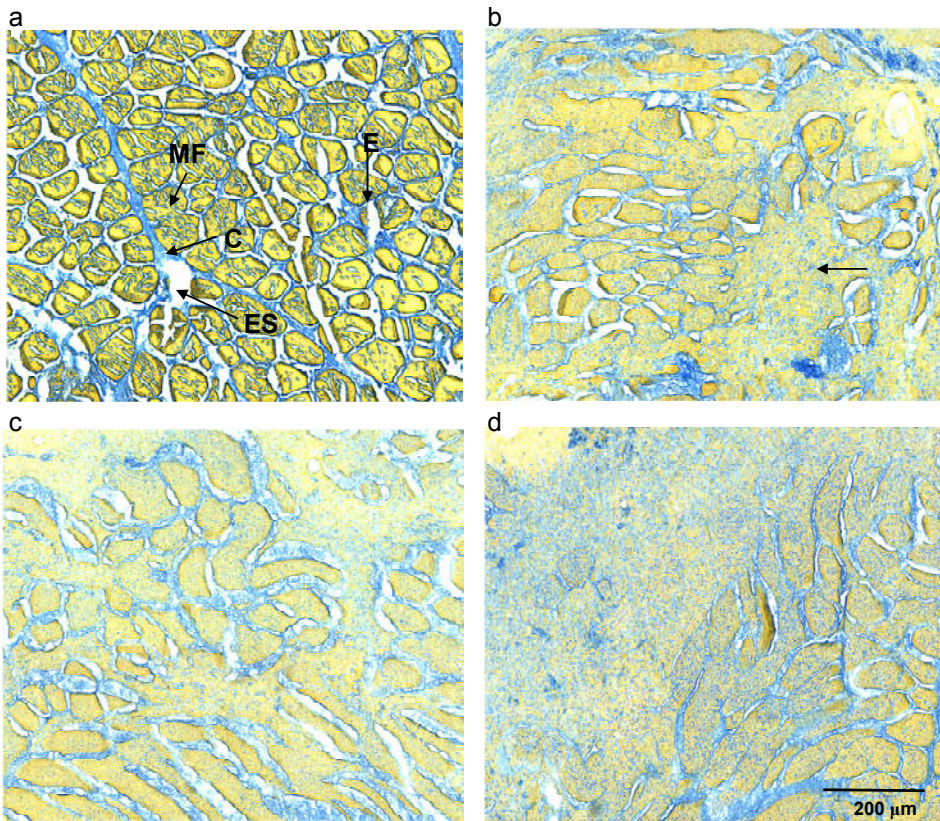


Figure 11. Heated breast muscle and (a) homogenated chicken breast meat containing 75% meat and 1% salt (b). Tyrosinase-treated and heated homogenate containing (c) 75% meat and 1% NaCl and (d) 65% meat and 2% NaCl (d).

Apparently the amount of salt rather than the amount of meat affected the observed result. Due to the limited solubility of the myofibrillar proteins in the low-salt system the muscle fibres may have maintained their structure to a certain degree, thus preventing myofibrillar proteins from acting efficiently as substrates for tyrosinase. Consequently collagen of the endomysium located around the myofibres may have been exposed to the action of tyrosinase, resulting in cross-linking between collagen molecules and leading to network formation as seen in the micrographs. In this respect it is difficult to interpret the observed enhanced network formation of collagen in the higher salt content. One explanation could be that the higher amount of salt in the low-meat homogenate increased the solubility of the myofibrillar proteins, possibly leading to more

pronounced disruption of the muscle fibre structure and hence enabling tyrosinase to act more efficiently with collagen.

4.6 Manufacture of chicken breast meat products (Publication IV)

A pilot-scale trial was carried out to study whether the effects on texture and water-holding obtained with TG in a simplified laboratory scale system could also be accomplished in a semi-industrial and commercially feasible production process. The laboratory-scale experiments confirmed that laccase neither improved texture nor WHC. Therefore laccase was not applied in the pilot trial. Although the laboratory scale results with tyrosinase were promising, particularly regarding WHC, the laboratory capacity to produce tyrosinase, which is presently a research enzyme and not produced commercially, did not allow a pilot scale experiment.

A set of test products was prepared of coarsely ground chicken breast meat containing 70–80% meat, 1.2–1.8% salt and 0–20 nkat TG/g protein (compositions of the products are given in Table 9 in Section 3.1). The salt amount 1.8% is normally used in industrial processes and was chosen as the highest salt level. The TG dosage of 10 nkat/g protein (0.1% the enzyme product of the meat mass) was chosen according to the recommendation of the importer of the TG product for the chicken meat product type used in this study. The amount of meat, i.e. 75%, is usually used in industrially processed ground meat products in Finland. The analysis data for the firmness and the cooking loss determined as evaporation loss of the test products are presented in Table 2 in Publication IV. Two-dimensional response surfaces for the firmness and the evaporation loss of the test products are shown in Figure 12.

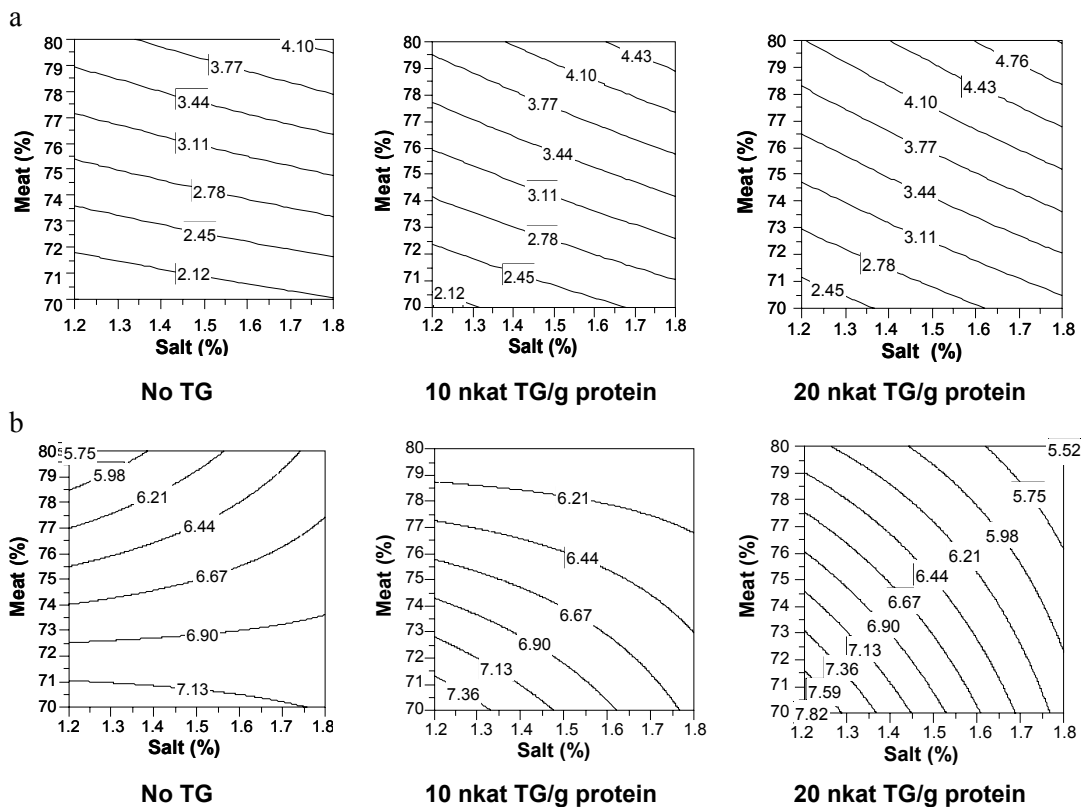


Figure 12. Response surfaces for firmness (a) and weight loss (b) of the test products at different meat, salt and TG contents. Isobars show the firmness values (kp) in (a) and evaporation loss values (%) in (b).

As expected, increase of meat, salt and TG contents resulted in increased firmness of the products. All three factors had a significant effect. No interaction between salt and TG was found. Texture improvement obtained by TG followed the general understanding about the textural effects of TG in various meat systems. The results are well in line with those reported e.g. with finely comminuted pork beef sausages by Hammer (1998), pork and chicken breast meat sausages with 2% salt by Mugumura et al. (1999), low-salt (1%) chicken meat balls by Tseng et al. (2000), low-salt (0.4–2%) pork patties by Pietrasik and Li-Chan (2002), and beef paste (2.5% NaCl) by Dondero et al. (2006). Tseng et al. (2000) used crude pig plasma TG, whereas in the other studies microbial TG was applied.

Increasing the meat content reduced the evaporation loss, also as expected. Surprisingly, in the absence of TG, a decrease of the evaporation loss along with the increasing salt content was not observed. When the meat content is high, e.g. 80%, the water-holding remains on an acceptable level although the salt content is low. However, when the meat content is low, salt is normally required to control the water-holding. An explanation to this unexpected result cannot be given. In the presence of TG the effect of salt was clearly observed and became stronger at the higher dosage of TG.

TG alone did not affect the evaporation loss, but there was a clear positive synergy between salt and TG. Pietrasik and Li-Chan (2002) obtained similar results, although the interaction between salt and TG was not statistically significant. The minimum cooking loss was obtained with high meat, salt and TG amounts. This result is in agreement with that reported by Pietrasik and Li-Chan (2002), who found that TG affected favourably cooking loss in high-salted (2% NaCl) pork products but not to the same extent when the salt content was low. On the other hand, evaporation loss was also at its maximum in the presence of the high dosage of TG when the meat and salt mounts were low. This result is consistent with the findings of Carballo et al. (2006), who reported a significant increase in weight loss due to TG in cooked chicken, pork and lamb meat containing 1.5% salt and 0.5% tripolyphosphate. Salt reduction has an adverse effect on water-holding by increasing cooking loss (Hamm, 1972). The negative impact of TG on WHC in the low-salt conditions may have been a result of the limited protein solubility due to a low salt amount, fortified by the protein cross-linking by TG, inhibiting uniform development of the protein network and consequently causing insufficient entrapping of water as discussed by Carballo et al. (2006).

In the meat systems used the increase of the salt amount alone from 1.2% to 1.8% favoured significantly the development of texture, whereas a positive synergistic effect of salt and TG in water-holding was observed. Salt enhances solubility of the myofibrillar proteins and thus presumably also improves the accessibility of the target amino acid residues, glutamine and lysine, of these proteins for the cross-linking action of TG. Protein solubility and the reported activity and thermostability increase of TG in the presence of salt (Kütemayer et al., 2005) together may explain the positive interaction observed between salt and TG.

5 Summary and conclusions

Cross-linking enzymes, which catalyse the formation of covalent bonds in or between proteins, are highly interesting tools to modify textural properties of foods, but their potential is also recognised in other industrial sectors.

This work focused on studying the effects of the protein cross-linking enzymes, tyrosinase, laccase and TG, starting from simplified amino acid, peptide or protein systems in laboratory scale and ending up in a pilot trial, in which coarsely ground chicken meat products were manufactured. The potential of the oxidative enzymes, tyrosinase and laccase, to modify meat proteins and subsequently affect various properties in meat systems were compared to those of the well-known protein cross-linker, TG. In addition, the work describes how a novel *Trichoderma reesei* tyrosinase differs from that of *Agaricus bisporus* mushroom in its efficiency to modify various proteinaceous substrates.

It was shown that *Trichoderma* tyrosinase had a tendency to oxidise proteins more efficiently than *Agaricus* tyrosinase, whereas *Agaricus* tyrosinase was found to favour small tyrosine-containing substrates rather than proteins. The superiority of *Trichoderma* tyrosinase compared to the *Agaricus* enzyme in functionalising a complex insoluble proteinaceous substrate i.e. wool was clearly shown. The result justified the use of *Trichoderma* tyrosinase in meat protein modification. *Trichoderma* tyrosinase will also be an interesting new tool to modify other proteins than those of meat in various food and cosmetic applications, not forgetting the textile sector.

It was discovered that tyrosinase, laccase and TG affected the same salt-soluble myofibrillar proteins, of which myosin and troponin T were the most susceptible. Both these proteins have been found in other studies to be the most sensitive to oxidation. Susceptibility to oxidation has been explained by the accessibility of the oxidation sites in these proteins. Of the oxidation sites cysteine, tyrosine and lysine take part in the enzyme-aided cross-link formation. Hence it could be reasoned that in the proteins in which these amino acid residues are accessible for oxidation they may also be accessible at least to a certain degree to enzymatic modification. This view is also valid in the case of actin. Actin has been found to be more resistant to oxidation than myosin. In this study actin was observed to be more resistant to the enzymatic modification than myosin.

Tyrosinase and TG polymerised protein but did not cause myofibrillar protein fragmentation. Laccase was shown to do both. In meat systems laccase acted similarly to several non-enzymatic radical generating systems studied elsewhere. It was shown that tyrosinase and TG increased gel formation of the myofibril suspension. Increased gel formation was due to the enzyme-catalysed formation of covalent cross-links between proteins. The impact of laccase in gel formation differed from that of the other two enzymes. At a certain dosage level laccase was capable of enhancing gel formation to some degree, but a higher laccase dosage caused decline in gel formation obviously due to protein fragmentation. Because of the myofibrillar protein fragmentation by laccase, textural effects caused by this enzyme in meat systems may be difficult to predict and control.

According to the DSC measurements, tyrosinase, laccase and TG appeared to induce conformational changes in myosin and actin. These thermal changes can reflect intermolecular and/or intramolecular cross-linking in the proteins by all three enzymes and protein fragmentation by laccase.

Surprisingly, in the experimental conditions used tyrosinase, laccase and TG had different effects on the texture and water-holding of the heated meat homogenates containing reduced amounts of meat, salt or phosphate. Laccase had a tendency to weaken both texture and water-holding, which was most probably due to laccase-catalysed free radicals reducing protein functionality. If texture and/or water-holding are to be improved in heated ground meat products, laccase is hardly a feasible alternative. Tyrosinase was capable of improving the texture only of those homogenates which contained 2% of salt. However, water-holding was improved or maintained on the control level by tyrosinase even in the lower-salt (1%) conditions. TG improved the texture in low and higher salt concentrations, but contrary to tyrosinase, weakened water-holding in both cases. TG capable of efficient cross-link formation both in low and higher salt concentrations caused severe restriction in swelling of the myofibrils, leading to decreased water-holding. On the other hand, lower cross-link formation by tyrosinase compared to TG enabled adequate swelling of the myofilament network. As a result, reduction of water-holding was not observed after the tyrosinase treatments. Why tyrosinase increased water-holding both in low and higher salt concentrations, but improved the texture only in the high salt concentration is an interesting question to which this work does not provide an

answer and consequently remains to be resolved in the future. Nevertheless, tyrosinase is a promising new tool for improvement of water-holding and texture of processed meat products.

Addition of TG to the coarsely ground meat together with moderate amounts of salt favoured texture development in pilot scale. Texture improvement was also obtained in the low-meat systems. These results are in line with that obtained in laboratory scale and with a number of studies published elsewhere. However, TG did not significantly reduce the water-holding. This result is contradictory to that obtained in laboratory scale in this study but in agreement with several studies published earlier. The amounts of TG, salt and meat were such that together they favoured the texture improvement without significant reduction of water-holding, reflecting a delicate balance between the two properties both affected by TG.

This work provided information on tyrosinase, laccase and TG in protein modification, especially that of meat. All these enzymes have been known for a long time and are well-characterised, but the effects of tyrosinase and laccase compared to that of TG in food protein matrices have not been efficiently studied. In this work it was shown that a novel *Trichoderma reesei* tyrosinase was a more efficient protein modifier than that from the mushroom *Agaricus bisporus*. It was also shown that although tyrosinase, laccase and TG affected the same structural proteins of muscle, their effects on gel formation, thermal and textural properties as well as on water-holding were different. Tyrosinase was found to be an interesting new alternative to TG in meat protein modification due partly to its capability to improve textural properties but particularly because its positive effect on water-holding in the studied heated meat systems. The ability of tyrosinase to act as a water-holding and texture improver remains to be evaluated in suitable meat applications, e.g. ham and sausage, and in real production process conditions. Laccase is unlikely to be useful as a protein modifier in meat applications.

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Author(s) Lantto, Raija		
Title Protein cross-linking with oxidative enzymes and transglutaminase Effects in meat protein systems		
Abstract <p>The effects of tyrosinase, laccase and TG were studied in different meat protein systems. The study was focused on the effects of the enzymes on the thermal behaviour and gel formation properties of myofibrils, and on the textural and water-holding properties of the heated meat systems.</p> <p>The cross-linking efficiency of a novel <i>Trichoderma reesei</i> tyrosinase was compared to that of the commercial <i>Agaricus bisporus</i> tyrosinase. <i>Trichoderma</i> tyrosinase was found to be superior compared to the <i>Agaricus</i> enzyme in its protein cross-linking efficiency and in the incorporation of a small molecule into a complex proteinaceous substrate.</p> <p>Tyrosinase, laccase and TG all polymerised myofibrillar proteins, but laccase was also found to cause protein fragmentation. A positive connection between covalent cross-link and gel formation was observed with tyrosinase and TG. Laccase was able to increase the gel formation only slightly. With an excessive laccase dosage the gel formation declined due to protein fragmentation.</p> <p>Tyrosinase, laccase and TG had different effects on the texture and water-holding of the heated chicken breast meat homogenates. Tyrosinase improved the firmness of the homogenate gels free of phosphate and with a low amount of meat. TG improved the firmness of all studied homogenates. Laccase weakened the gel firmness of the low-meat, low-salt and low-salt/phosphate homogenates and maintained the firmness on the control level in the homogenate free of phosphate. Tyrosinase was the only enzyme capable of reducing the weight loss in the homogenates containing a low amount of meat and a low amount of NaCl. TG was the only enzyme that could positively affect the firmness of the homogenate gel containing both low NaCl and phosphate amounts.</p> <p>In pilot scale the test products were made of coarsely ground chicken breast fillet with a moderate amount of salt. Increasing the amount of meat, salt and TG contents favoured the development of firmness of the test products. The evaporation loss decreased slightly along with increasing TG and NaCl amounts in the experimental conditions used, indicating a positive interaction between these two factors.</p> <p>In this work it was shown that tyrosinase, laccase and TG affected the same myofibrillar proteins. However, these enzymes had distinguishable effects on the gel formation of a myofibril system as well as on the textural and water-holding properties of the finely ground meat homogenates, reflecting distinctions at least in the reaction mechanisms and target amino acid availability in the protein substrates for these enzymes.</p>		
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