Gideon Oudgenoeg

Peroxidase catalyzed conjugation of peptides, proteins and polysaccharides *via* endogenous and exogenous phenols

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman
in het openbaar te verdedigen
op woensdag 14 april 2004
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G. Oudgenoeg - Peroxidase catalyzed conjugation of peptides, proteins and polysaccharides *via* endogenous and exogenous phenols - 2004

PhD thesis Wageningen University, The Netherlands

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- Tijdens oligomerisatie van tyrosine bevattende peptides geldt welliswaar dat 2+2=4 (Michon et al, Biochemistry, 1997) maar toch vooral dat 1+3=4 (Oudgenoeg et al, JAFC, 2001).

- Mensen die niet hebben doorgeleerd voor het met een factor 1000 opschalen van een reactie kunnen om tijd te winnen beter 1000 maal dezelfde reactie doen dan die ene reactie op 1000-voudige schaal (Dit proefschrift, hoofdstuk 2→3).

- Geen enkel enzym is helemaal geknipt voor digestie van α-lactalbumin (dit proefschrift, niet derhalve).

- Een onderschatte functie van de conformatie van elk monomeer eiwit is de bijkomende ontoegankelijkheid van reactieve aminozuren via welke het zou kunnen polymeriseren.

- De digitalisering van wetenschappelijke literatuur loopt onvermijdelijk vast op de pluriformiteit in voor- en achter-namen van zowel Chinezen als Japanners en hun toenemende dominantie in de wetenschap.

- De populatie verkeersslachtoffers is dusdanig anders samengesteld dan die van tabaksslachtoffers dat bij aankoop van een auto 100% van de lak met waarschuwingen zou moeten zijn bestickerd.

- Het vergoeilijkende 'het is nu eenmaal druk' wanneer een vanzelfsprekende service irritant lang op zich laat wachten dient te worden gehoord als 'we zijn hier slecht georganiseerd, nergens op voorbereid en kunnen absoluut niet anticiperen'.

- Met deelname aan de kabinetten Paars1, Paars2 en Balkenende2 illustreert D66 nog maar eens dat 3 keer naar rechts ook links is.

Stellingen behorende bij het proefschrift

'Peroxidase catalyzed conjugation of peptides, proteins and polysaccharides *via* endogenous and exogenous phenols'

Gideon Oudgenoeg

14 april 2004, Wageningen

Aan mijn moeder

Abstract

Oudgenoeg G.	Peroxidase catalyzed conjugation of peptides, proteins and polysaccharides *via* endogenous and exogenous phenols
Ph.D. thesis	Wageningen University, Wageningen, The Netherlands, 2004
Keywords	Peroxidase, α-lactalbumin, arabinoxylan, ferulic acid, catechol, cross-linking, polymerization, kinetic control, tyrosine, coupling

The research was directed towards peroxidase mediated cross-linking of proteins and polysaccharides. Two approaches were explored, cross-linking by use of ferulic acid (FA) and cross-linking by use of catechol. Within each approach, first model studies were performed with small peptides, of which the findings were applied in subsequent studies with proteins. First, a kinetically controlled incubation that leads to covalently coupled adducts of the tripeptide Gly-Tyr-Gly (GYG) and FA, catalyzed by horseradish peroxidase (HRP) is described. Next, two series of covalent adducts of GYG and FA, comprising dehydrogenatively polymerized FA on the GYG tyrosine were identified. Cross-linking of holo- and apo-α-lactalbumin was subsequently explored. Oligomerization of α-lactalbumin was observed and a direct relation between protein conformation and extent of oligomerization was shown. Application of the findings with GYG, combined with those of α-lactalbumin homo-cross-linking led to the modification of α-lactalbumin with FA. The degree of polymerization of α-lactalbumin was reciprocal to the incident concentration of free FA. Next, cross-linking of FA-containing arabinoxylans with β-casein is described. Maximal formation of protein-arabinoxylan conjugates was observed at high protein to arabinoxylan ratios in combination with a low H_2O_2 concentration and a long reaction time. Finally, the use of catechol in peroxidase catalyzed modification of amino acids and proteins was studied . Covalent attachment of catechol to the side chain of the amino acids tyrosine and histidine was unambiguously proven by tandem MS of the adducts. Cross-linking of globular proteins yielded oligomeric adducts whereas cross-linking of β-casein yielded high molecular weight polymers. Two mechanisms for the cross-linking of catechol with proteins were proposed.

Contents

Chapter I

Introduction

1.1 Oxidative coupling of phenols

Phenols are ubiquitous in nature. Simple phenolics as well as phenolic moieties in large biomolecules compose various tissues in many organisms. Oxidative, covalent coupling of phenols is for that reason an important topic in the biochemistry of plants and animals. Accordingly, it is also of relevance in the industrial processing of raw materials from these sources. Two possible products from oxidative coupling of the simplest phenol, hydroxybenzene, are shown in Figure 1.

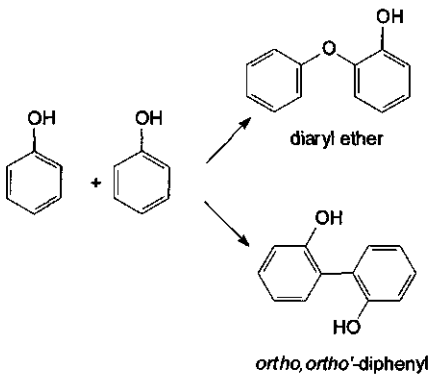

Figure 1: Two possible products of oxidative coupling of two phenols

Three abundant biopolymers, protein, polysaccharide and lignin, can contain phenolic moieties. In proteins, the phenol tyrosine (Figure 2) is one of the constituent amino acids, and evidently widespread throughout all organisms. In plants, cell wall polysaccharides can be esterified with cinnamic acid derivatives, phenols that are substituted with a propenoic acid group at the *para* position of the aromatic ring. A cinnamic acid derivative that has often been found esterified to polysaccharides is ferulic acid, (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid) (Figure 2).

Both tyrosine and ferulic acid have been shown to be oxidatively cross-linked by dehydrogenation. A shared feature in the history of tyrosine and ferulic acid coupling is the first indication in *in vitro* gelling experiments, their subsequent discovery in biological tissues finally resulting in targeted *in vitro* experiments.

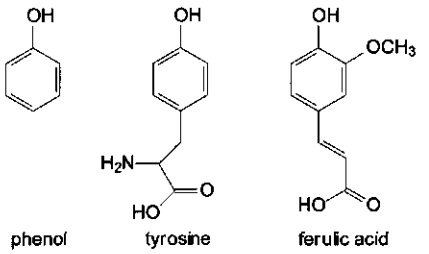

Figure 2 : Three phenols; hydroxybenzene, tyrosine and ferulic acid

In the second half of the twentieth century oxidatively coupled tyrosines were identified both in plant and animal tissue, while oxidatively coupled ferulic acids were found in tissue of plant origin. In unison, the biosynthetic systems responsible for the coupling reactions were clarified and mimicked *in vitro*. The molecular complexes resulting from the cross-linking contribute to physiological function or cause physiological damage, depending on the temporal and spatial incidence.

1.2 **Covalent coupling of tyrosine**

The first indication for a role of tyrosine in protein cross-linking arose from the markedly altered polymerization and fibrogenesis of tropocollagen after removal of the terminal and near-terminal ends of the protein (*1*). The peptides that were removed by pepsin appeared to be extremely rich in tyrosine and were, therefore, deduced to be responsible for the interprotein bonding. The nature of this linkage was yet unclear but in the same and subsequent year two decisive steps towards the unraveling of this interprotein cross-linking were made. First, in some elastic ligaments of the rubber-like resilin protein in arthropods two 'fluorescent amino acids' were discovered. These 'fluorescent amino acids' were concluded to be dityrosine and trityrosine after comparison with synthetic dityrosine (*2, 3*). This was the first identification of dityrosine *in vivo* and Anderson, correctly,

attributed the cross-linking of these proteins to post-translational maturation of resilin protein to a rubber-like structural tissue.

That these dityrosines really stem from cross-linked proteins, and not from free tyrosine linked to protein tyrosine, was shortly after this elegantly demonstrated by ^{14}C-pulse-labeling studies that showed a continuous formation of dityrosine from tyrosine residues in preformed elastin (*4*) Elastin, the vertebrate homologue of resilin and abductin, found in invertebrates such as mollusks, belongs to a class of proteins, which form highly elastic biological structures. The lack of secondary structure of these proteins allows the formation of di- and tri-tyrosine inter-protein cross-linkages, therewith adding elasticity to tissue composed of these proteins. Dityrosine was subsequently identified in other native, structural proteins, with similar functionality but in recent years also in men (Table 1).

organism	tissue	year	ref
arthropod	elastic ligaments	1963	(*2, 3*)
chick embryo	aorta	1967	(*5*)
rat	collagen	1968	(*6*)
cow	ligamentum nuchae proteins	1969	(*7, 8*)
tussah silk moth	silk fibroin and keratin	1971	(*9*)
clamp		1973	(*10*)
dragonfly	egg envelopes	1974	(*11*)
sea urchin	fertilization membrane	1977	(*12*)
sea mussel	adhesive discs	1977	(*13*)
man	cataractous lens	1978	(*14, 15*)
rabbit	aorta	1979	(*16*)
cow	dental enamel matrix	1982	(*17*)
merino	wool	1984	(*18, 19*)
Tomato	Cell wall	1984	(*20*)
yeast	ascospore wall	1986	(*21*)
sea urchin	fertilization envelope	1990	(*22*)
bean/soybean	cell wall	1992	(*23*)
man	lens	1993	(*24*)
worm	cuticular proteins	1993	(*25*)
green algae	cell walls	1993	(*26*)
yeast	sporulating cells	1994	(*27*)
tomato	extensin	1997	(*28, 29*)
man	atherosclerotic plaques	1997	(*30*)
mouse	cardiac and skeletal muscle	1997	(*31*)
man	alzheimer affected brain	1998	(*32*)
man	brain	1999	(*33*)
man	blood (hemodialysis patients)	1999	(*34*)
cockroach	leg	2000	(*35*)

Table 1: Occurence of dityrosine in various tissues

Currently, dityrosine fluorescence properties have become a tool in the identification and localization of resilin to investigate the anatomical arrangement of ligaments involved in movements. Using this tool, cockroach leg functioning was studied by localization of dityrosines (*35*).

Dityrosine was also identified in the cell wall of the yeast *Ascopore* and demonstrated to be sporulation-specific (*21*). Since dityrosine has been found predominantly in tissues providing structural integrity, it was recognized that tyrosine cross-linking has the specific function of adding stiffness to structural proteins (Figure 3).

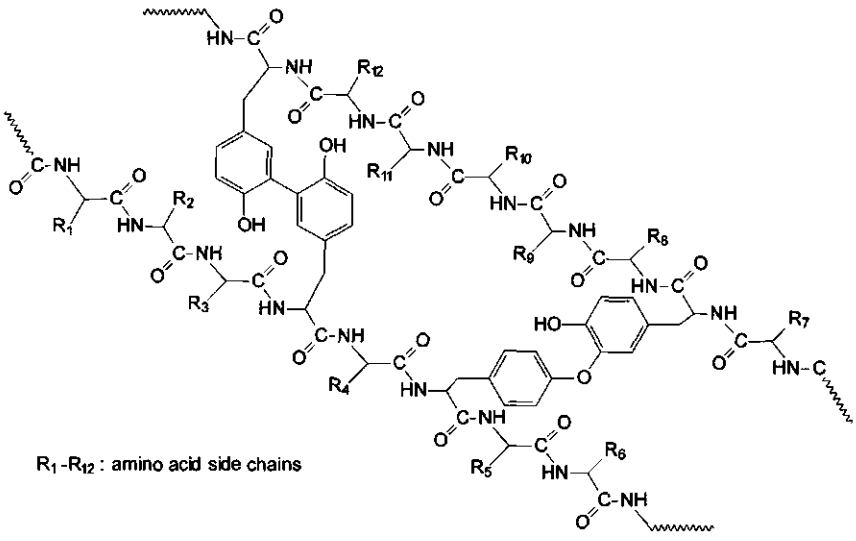

Figure 3 : Model of isodityrosine and dityrosine bridges between two peptide chains

1.3 Involvement of peroxidase in dityrosyl protein coupling

Crucial for oxidative coupling of tyrosines is the presence of an oxidative agent. In biology, this can be a peroxidase iron-oxo compound, a compound that results from reaction of H_2O_2 with the active site of a peroxidase. Peroxidases are oxidative enzymes that are already known since the ninetienth century. The fertilization of egg envelopes has played a crucial role in the exploration of peroxidase involvement in tyrosine coupling over

several decades. The idea that an oxidative process was responsible for the hardening of the fertilization membrane of sea urchin eggs was first proposed by Motomura in 1954, before even the first *in vitro* or *in vivo* identification of dityrosine (*36*). This relation between the hardening of the fertilization membrane of sea urchin eggs and the activity of peroxidase was actually demonstrated by treatment of the eggs with or without known peroxidase inhibitors (*12*). In these inhibition studies it was shown that inhibitors of the ovoperoxidase that is associated with the fertilization, inhibited hardening. In the hardened fertilization membranes di- and tri-tyrosines were identified that were absent in the fertilization membranes before hardening, unequivocally proving the role of peroxidase mediated tyrosine coupling in the hardening process. From hydrolysates of the hardened egg chorion of *Aedes aegypti* dityrosine and trityrosine were isolated and peroxidase activity was detected in the mosquito's ovaries, containing the developing eggs, 24 to 48 h after blood feeding (*37*). Only for the yeast spore wall an exception was reported in the assembly of the structural proteins to intermolecularly cross-linked networks *via* local oxidation of tyrosines and subsequent radical coupling (*27*). The sporulation specific gene, DIT2, a member of the cytochrome P450 superfamily, was demonstrated to be responsible for the dityrosine containing precursor proteins. The epimerization of the LL-dityrosine to DL-dityrosine of the precursors took place after incorporation into the spore wall, but dityrosine formation did not take place in the spore wall. In the exploration of fertilization envelopes also knowledge on the products of tyrosine coupling was gained; in the fertilization envelope of the sea urchin embryo, pulcherosine, an isomeric trimer of trityrosine was identified for the first time (*22*). Altogether, three isomeric trimers of tyrosine have been isolated from animal tissue (Figure 4).

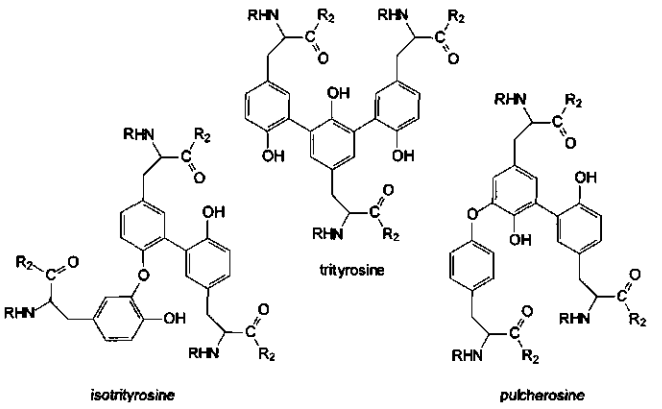

Figure 4 : Three isomeric trityrosines

Besides the trityrosine that Andersen identified from insect resilin (*2*), isotrityrosine, was identified in Ascaris cuticle collagen (*38*). In activated neutrophils of human phagocytes, trityrosine, pulcherosine and isodityrosine were generated and identified in a mammalian system for the first time (*39*). In addition, in these studies, the precursor of trityrosine was deduced to be dityrosine and the precursor of pulcherosine to be isodityrosine.

1.4 Tyrosine coupling in plant proteins

The first identified coupled tyrosines were always from tissue of animal origin (*vide supra*), but plant proteins were also increasingly demonstrated to be dityrosyl cross-linked. In plant cell wall extensins, dityrosines are responsible for the formation of a solid protein network. In extensin from tomato, intramolecular isodityrosines were identified (*20*). Another function of tyrosine coupling, as a first aid defense mechanism, was discovered in further studies on plant proteins; after wound healing of potato tubers, dityrosine formation was demonstrated (*40*). Bradley showed that in bean, a stimulus-dependent oxidative burst resulted in protein cross-linking via tyrosines as a rapid defense response (*23*). In hydrolysates of primary cell walls of a tomato cell culture also pulcherosine was identified (*28, 41, 42*). This might well be an intermediate for di-isodityrosine (Figure 5) that was demonstrated in plant cell wall protein.

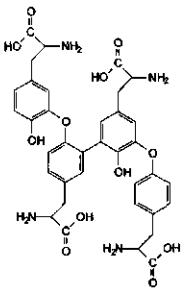

Figure 5 : Di-isodityrosine

1.5 Tyrosine coupling in non-structural proteinaceous tissue

Besides adding functionality, the cross-linking of proteins can also cause damage to tissue and dityrosine formation has been increasingly hypothesed to contribute to physiological damage in various tissues.

1.5.1 Eye-lens

The first identification of dityrosine in the eye was in cataractous human lens, in the insoluble protein fraction (*14*). This insoluble protein is found in the capsular layer that surrounds the exterior of the tissue and represents approximately 1 percent of the lens weight. Though, it was not unambiguously demonstrated that the identified dityrosine stems from interprotein linkage of tyrosines. It was stated by Garcia-Castineiras (*14*) that the conditions known –at that time- to generate dityrosine, namely photolysis or peroxidase/H_2O_2, may occur in the eye lens. Nonetheless, the fluorescence methods used to identify dityrosine were later found to overestimate the participation of dityrosine in the characteristic 318 nm --> 410 nm fluorescence emission. This was noticed when GC-MS was applied to measure dityrosine content in normal human eye-lenses of all ages (*24*). According to these studies, dityrosine content is increased in the eye-lens between 1 and 78 years with 33% and was thus concluded to play a minor role in the normal aging of lens proteins *in vivo*. In cataractous eye-lens, DOPA, *o*- and *m*-tyrosine, 3-hydroxyproline and 5-hydroxyleucine are found rather than dityrosine. The post-translational modification of crystallins in eye-lenses was found to be dominated by hydroxyl radical and Fenton chemistry rather than tyrosine coupling (*43*). An interesting study with the dityrosine linked dimer of γB-crystallin revealed changes in the tertiary structure of the dimer as apparent from CD-studies on monomer and dimer. The secondary structure of the dimer appeared to be maintained but the

tertiary structure was altered subtlely and as a consequence, the dityrosine linked dimer denatured more readily than the parent monomer and was also less soluble (*44*).

1.5.2 Brain

In recent years, dityrosine coupling has been inceasingly reported in relation to brain malfunctioning. Oxidative stress has been implicated in pathogenic mechanisms of Parkinson's disease and many other neurodegenerative diseases (*45-47*).

Dityrosine was demonstrated in lipofuscin granules in the pyramidal neurons of the aged human brain by reaction with an antibody (*48*). Quantification of 3-nitrotyrosine and dityrosine in four regions of the human brain that are differentially affected in Alzheimer's disease revealed 5 to 8 fold greater quantities of dityrosine in the hypocampus and neocortical regions and in the ventricular fluid (*32*). In the striatum and midbrain of mice, *o-o'*-dityrosine levels were markedly increased upon administration of MPTP, a model to mimic oxidative stress (*33*). Incubation of brain proteins with myeloperoxidase resulted in increased levels of *o-o'*-dityrosine *in vitro* as well. Myeloperoxidase may well be involved in dityrosine formation *in vivo*, since it is expressed by myeloid cells and can therefore be present at low levels in brain macrophages and microglial cells. Dityrosine formation might therefore be a key step in Parkinson's disease. Since α-synuclein has been shown to be a major component of several pathological intracellular inclusions, including Lewy bodies in Parkinson's disease, studies with recombinant α-synuclein have been performed (*49*). Dityrosine was demonstrated to be responsible for the formation of stable α-synuclein polymers after incubation with either peroxynitrite/CO_2 or myeloperoxidase/H_2O_2/(nitrite). α-Synuclein is a 140 amino acid protein with four tyrosine residues and is, in contrast to most globular proteins, rather unstructured in aqueous solution. Therefore, it was reasoned to have readily accessible tyrosines, prone to modification. In the same studies, β-synuclein and RNase A did not form aggregates when incubated under the conditions applied with α-synuclein. The fact that only α-synuclein could be dityrosine cross-linked was related to its aggregation in pathological lesions, whereas other synucleins, such as β-synuclein, are not found in Lewy bodies and glial cell inclusions.

Very recently, oxidative formation of the α-synuclein dimer was found to be the rate-limiting step for Parkinson's disease fibrilogenisis. The dimer was found to be a prenuclear species during 40 days of fibrilogenisis as

dimers increased to 2% of the total protein on day 15 and then rapidly decreased until day 35, after which no longer dimer was found (*50*).

1.5.3 Blood

Incubations of high density lipoprotein (HDL) and tyrosine with myeloperoxidase or horseradish peroxidase and H_2O_2 strongly increased protein associated fluorescence, presumed to stem from *o,o'*-dityrosines (*51*). The conversion of HDL tyrosines to dityrosines required the presence of free tyrosine, implying that tyrosyl radical is a diffusible messenger radical that conveys oxidizing potential from the active site of the heme enzyme to protein tyrosyl residues. The role for tyrosine as a messenger radical between myeloperoxidase in activated human neutrophils and low density lipoprotein (LDL) was further proven from *in vitro* studies (*52*). Whereas addition of L-histidine or L-tryptophan to an incubation of LDL and activated neutrophils induced no lipid peroxidation, addition of free L-tyrosine did cause significant increase in hydroxyoctadecadienoic acid and cholesteryl ester hydroperoxide. In atherosclerotic plaques from human vascular tissue, dityrosine levels were increased 100-fold compared to circulating LDL (*30*). The modification of LDL to LDL˙ was found to stem from a reaction with oxidized hemoglobin species and involved dityrosine formation (*34*). Dityrosine was found to be an endogenous marker of organismal oxidative stress after the discovery of selective proteolysis of oxidatively modified red blood cell hemoglobin by (the 19 S) proteasome. (*53*). A potential physiological catalyst of lipoprotein oxidation is, again, myeloperoxidase.

1.6 Tyrosine cross-link formation *in vitro*

1.6.1 Free tyrosine and tyrosine containing peptides

The avalanche of dimeric and trimeric tyrosines isolated from plant and animal origin since the 1960's gave rise to in tandem exploration of oligomerization of tyrosine and tyrosine containing peptides. The incubation product of tyrosine with horseradish peroxidase and H_2O_2 was first determined by Gross in 1959 and concluded to be dityrosine and possibly trityrosine (*54*). A dityrosine comprises two tyrosines connected via a covalent bond that replaces a hydrogen atom in each of the constituting, original, tyrosine monomers. The reaction therefore requires the abstraction of an electron and a proton from the tyrosine monomer.

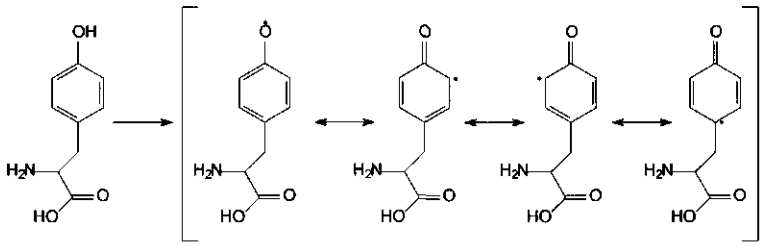

Figure 6 : Tyrosine and the mesomers of its radical

Many plant and animal peroxidases have a sufficient high electron potential to abstract an electron from the tyrosine hydroxyl. After subsequent acceptance of a proton from the tyrosyl radical cation, the resulting tyrosine free radical is stabilized by four mesomeric resonance contributors (Figure 6). Those mesomers, in which the radical is localized at the *ortho* position, or at the oxygen of the aromatic nucleus, are sterically capable of combining with another radical. Such radical combination can therefore lead to 2 isomeric forms of the tyrosine dimer: dityrosine and isodityrosine (Figure 7).

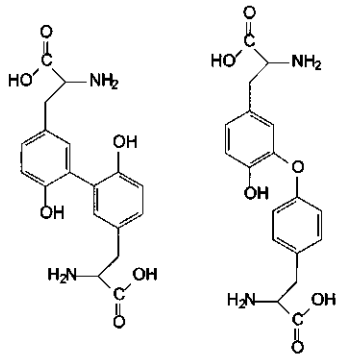

Figure 7 : Dityrosine (left) and isodityrosine

These dimers have been known since 1959 when Gross and Sizer synthesized tyramine and tyrosine dimers by incubation with horseradish peroxidase and H_2O_2 (*54*). They were the first to determine the characteristic strong fluorescence emission at 410 nm upon irradiation at 318 nm. Studies on the reaction of tyrosine and tyrosine containing peptides in subsequent

decennia demonstrated that also higher oligomers result from incubation with horseradish peroxidase and H_2O_2. In 1995, Marquez and Dunford showed that dityrosine is a good substrate for myeloperoxidase, therewith explaining further oligomerization via enzymatic oxidation of the dimer (*55*). Michon and coworkers in 1997, however, found that a pentapeptide, after dimerization *via* tyrosine coupling, inhibited horseradish peroxidase, and no higher oligomers than dimers were found (*56*). Oligomerization of tyrosine leads to a twofold increase in possible dimers for each subsequent oligomer due to coupling at the *ortho* or oxygen position of the next coupled monomer. Although in industrial polymer chemistry of phenolic resins much is known about the regiochemistry of the polymeric chain, not much is known about the regioselectivity of tyrosine oligomers. The only information comes from RP-HPLC-MS, unambiguously showing many isomers of the higher oligomers regarding retention differences with the same mass. By MALDI-TOF mass spectrometry an incubation of free tyrosine was shown to yield dimers up to nonamers (*57*). Pulse radiolysis of free tyrosine in solution also generates tyrosyl radicals and was employed to study the effects of molecular oxygen and anti-oxidants on tyrosyl radical formation (*58*). Whereas molecular oxygen had no effect on dityrosine formation between pH 4-12, ascorbate, urate and trolox (the soluble form of vitamin E) strongly inhibited dityrosine formation.

The elaborate and extensive studies of Michon and coworkers elegantly demonstrated the difference of oligomerization of tyrosine as free amino acid and tyrosine in different pentapeptides (*56*). Furthermore, the difference between horseradish peroxidase and manganese peroxidase catalysis with respect to the type of oligomerization products was demonstrated. From this a conclusion was drawn about the effect of neighboring amino acids on tyrosine oxidation. These studies clearly revealed that cross-linking becomes more complicated from tyrosine to peptide to protein; effects of neighboring amino acids but also plain steric hindrance and its effect on tyrosine accessibility are increasingly met.

1.6.2 Protein dityrosines

The accessibility of tyrosines in proteins is imperative with respect to interprotein cross-linking via tyrosine coupling. This is illustrated by the cross-linking of sperm whale metmyoglobin, that has tyrosines at positions 103, 146 and 151. In the presence of H_2O_2, this protein dimerizes exclusively via a dityrosine bond between Tyr103 of one myoglobin chain and Tyr151 of the other (*59*). The mutual orientation of Tyr103 and Tyr151

was concluded to be the only sterically allowed conformation adjoining two tyrosines.

Lactoperoxidase (LPO) has been dityrosyl cross-linked to myoglobin from sperm whale (SwoMb) or horse (HoMb) in the presence of H_2O_2 (*60*). Incubations with HoMb yielded a heterodimer and a LPO1-HoMb2 trimer in addition to LPO and HoMb homodimers. By kinetic control it was demonstrated that the reaction was due to radical transfer from the myoglobin to LPO; addition of H_2O_2 to myoglobin followed by catalase to quench excess H_2O_2 before addition of LPO still yielded dityrosyl cross-linked products. The involvement of two tyrosine residues of LPO in the cross-linking was deduced from the fact that heterotrimers were formed with 2 HoMb molecules, that have only one tyrosine for cross-linking, unlike SwoMb that has two.

Another thorough investigation on the actual tyrosine(s) responsible for protein dimerization, was performed with the brain protein α-synuclein (*61*). By alternately replacing each of the four tyrosines with phenylalanine, it was demonstrated that one particular tyrosine of α-synuclein, Tyr125, was responsible for the dimerization after nitration.

From 1987 till 1998 intra- and inter-molecular coupling of tyrosines in the bovine brain protein calmodulin was extensively investigated by Malencik and Anderson. Calmodulin is a major intracellular sensor of Ca^{2+} in plants and animals. This 148 amino acid protein contains two tyrosines, both in Ca^{2+} binding regions. Tyr99 faces into the third Ca^{2+}-binding loop and is partially exposed to the solvent, Tyr138 points outward from the fourth Ca^{2+}-binding site into a hydrophobic pocket. Initially, intramolecular dityrosine cross-links were generated by irradiation of calmodulin at 280 nm (*62*). Subsequently, irradiation of calmodulin in the presence of a synthetic derivative of the calmodulin binding peptide substance P, [Tyr^8] substance P was performed. Irradiation led to dityrosine bridged adducts of calmodulin Tyr138 and [Tyr^8] substance P, effectively competing with the intramolecular cross-bridging between Tyr99 and Tyr138 (*63*).

Interprotein cross-linking of calmodulin occurs only in the presence of superoxide dismutase (*64*). This was attributed to the generation of superoxide radicals during irradiation, because these radicals repair tyrosine radicals by donating the superoxide free electron. Such repair is inhibited by superoxide dismutase and therefore prolongs the lifetime of the tyrosine radical and facilitates interprotein coupling. The enhancement of dimer formation by superoxide dismutase applies to irradiations in the presence of Ca^{2+} as well as in the absence of Ca^{2+}. Intraprotein coupling however only

occured in the presence of Ca^{2+}; apparently Ca^{2+}-binding is required to keep the 2 tyrosines in a conformation that spatially allows coupling.

In 1996 a peroxidase was found that could catalyze the formation of dityrosines between calmodulins. This peroxidase from *Arthomyces ramosus* catalyzed the efficient (>40%) cross-linking of bovine brain calmodulin, whereas attempts with horseradish peroxidase, lactoperoxidase and soybean peroxidase failed. Myeloperoxidase caused a very slow increase in dityrosine fluorescence in incubations with calmodulin (*65*).

The accessibility of protein tyrosine residues is decisive for participation in covalent bond formation. This is reflected in the lack of tertiary structure in proteins involved in tissue firmness. In such proteins, presumably due to the organization of the tandem repeat motifs, neighboring hydrophilic amino acids ensure a sufficient exposure of the hydrophobic tyrosines to the exterior to enable participation in cross-linking reactions.

A clear relation between protein structure and tyrosine cross-linking of proteins was elegantly demonstrated in incubations of wheat prolamines with horseradish peroxidase, soybean peroxidase and manganese peroxidase in either aqueous or organic solvent (*66*). In 25% dioxane, the yield of dityrosine after incubation with horseradish peroxidase or soybean peroxidase, was five times higher than in aqueous medium. This difference was attributed to the increased solvent exposure of tyrosines in 25% dioxane as compared to water. This relation was further demonstrated by using manganese peroxidase (MnP), which requires no direct contact between the enzyme active site and the protein tyrosine, but exerts its oxidative power via diffusion of a lactate-Mn complex (*66*). Dityrosine formation in aqueous medium in the presence of MnP was nearly tenfold higher than with soybean peroxidase, indicating that tyrosine oxidation is limited due to lowered accessibility of the enzyme active site.

1.6.3 Stability effects of dityrosyl protein cross-linking

Calmodulin, bovine pancreatic ribonuclease A and bovine eye-lens γB-crystallin were all dimerized via dityrosyl cross-linking. The secondary and tertiary structures of the dimerized proteins appeared to be largely maintained. However, the thermal stability and unfolding properties were changed. The melting temperature (T_m) of the dimer of RNase A was lowered from 60 °C to 54 °C, and GdmCl induced denaturation started at 2.6 M instead of 3 M for the monomer and the activity of the dimer was lowered by 25%. For calmodulin, a similar effect was found for the dimer as compared to the monomer, but only in the absence of Ca^{2+}, that is known to stabilize the structure of calmodulin. For γB-crystallin the T_m of the dimer

was 59 °C, whilst the monomer denaturated at 66°C and the dimer precipitated out of solution at 62 °C while the monomer coagulated only beyond 72 °C. Altogether, a similar trend was observed for the dityrosine linked proteins, lowered conformational stability. Possible consequences of these phenomena for protein polymerization are described in **Chapter IV** of this thesis.

1.7 Covalent coupling of ferulic acid

In 1925, Durham reported that addition of H_2O_2 to a flour-water slurry increased the viscosity of the slurry (*67*). In 1943, the water-soluble pentosans in such a slurry were demonstrated to cause this H_2O_2-induced increased viscosity (*68*). In 1963, the moiety responsible for oxidative gelation in these pentosans was suggested to be ferulic acid (*69*). Yet, diferulic acid was first identified in saponified grass cell walls by Markwalder and Neukom in 1976 (*70*). Subsequently, diferulic acid was isolated from *Lolium multiflorum* (*71*), from arabinoxylans in bamboo shoot cell walls (*72*) and from many other plants (*73-84*). In Figure 8 a typical dehydrodiferulic acid bridging two arabinoxylans is shown.

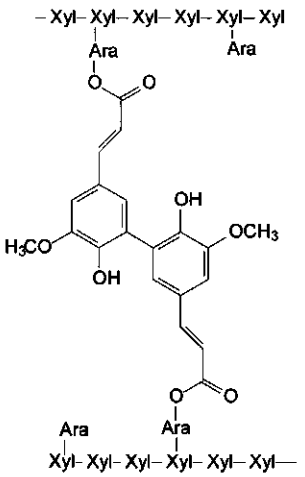

Figure 8 : 5-5 Dehydrodiferulic acid bridging two xylose chains

In the 1970's, decisive *in vitro* work on the formation and identification of diferulates was conducted by Markwalder and Neukom (*85, 86*). The water-soluble polysaccharide guaran was synthetically esterified with caffeic acid, ferulic acid, *p*-coumaric acid, homovanillic acid, vanillic acid, protocatechuic acid or *p*-hydroxybenzoic acid. Incubations with horseradish peroxidase and H_2O_2 of all esterified guarans led to gelation of the solution (*87*). Subsequently, in a fraction of water-soluble wheat flour pentosans diferulic acid was identified upon addition of peroxidase after addition of H_2O_2, whereas addition of H_2O_2 without peroxidase did not result in gelling of the solution.

Monomeric ferulic acid was first identified in the nineteenth century. Over 1.5% ferulic acid was isolated from a commercial resin of *Ferula foetida*, an umbelliferous fennel-like plant (*88*). Ferulic acid was later found in numerous plants (*89-91*).

In the plant cell wall, ferulic acid and related *p*-coumaric acids can be bound to the arabinoxylans of the grass family (gramineae) or to the pectins of dicotyledons through an ester linkage. From the 1990's, the chemistry of ferulic acid has been an exciting field of investigation. Ralph and coworkers explored the nature of diferulic acids in grass cell walls and discovered that besides the initially identified 5-5-coupled diferulic acid (Fig 8), six other isomers occur in cell walls of cocksfoot, switchgrass and suspension cultured corn (*92*). The population of the various dimers was found to differ largely between different sources. In barley and processed barley the predominant 8-O-4'-diferulic acid along with 5,5-, 8,5'-benzofuran and the 8,5'-open form-diferulic acid were identified (*93*). In sugar beet pulp the 8,5' diferulic acid was preponderant followed by 5-5'-, 8-8'- and 8-O-4'-diferulic acid (*94*). In subsequent years additional isomers of the FA-dimer were discovered (*95, 96*). Figure 9 shows all the FA-dimers currently known.

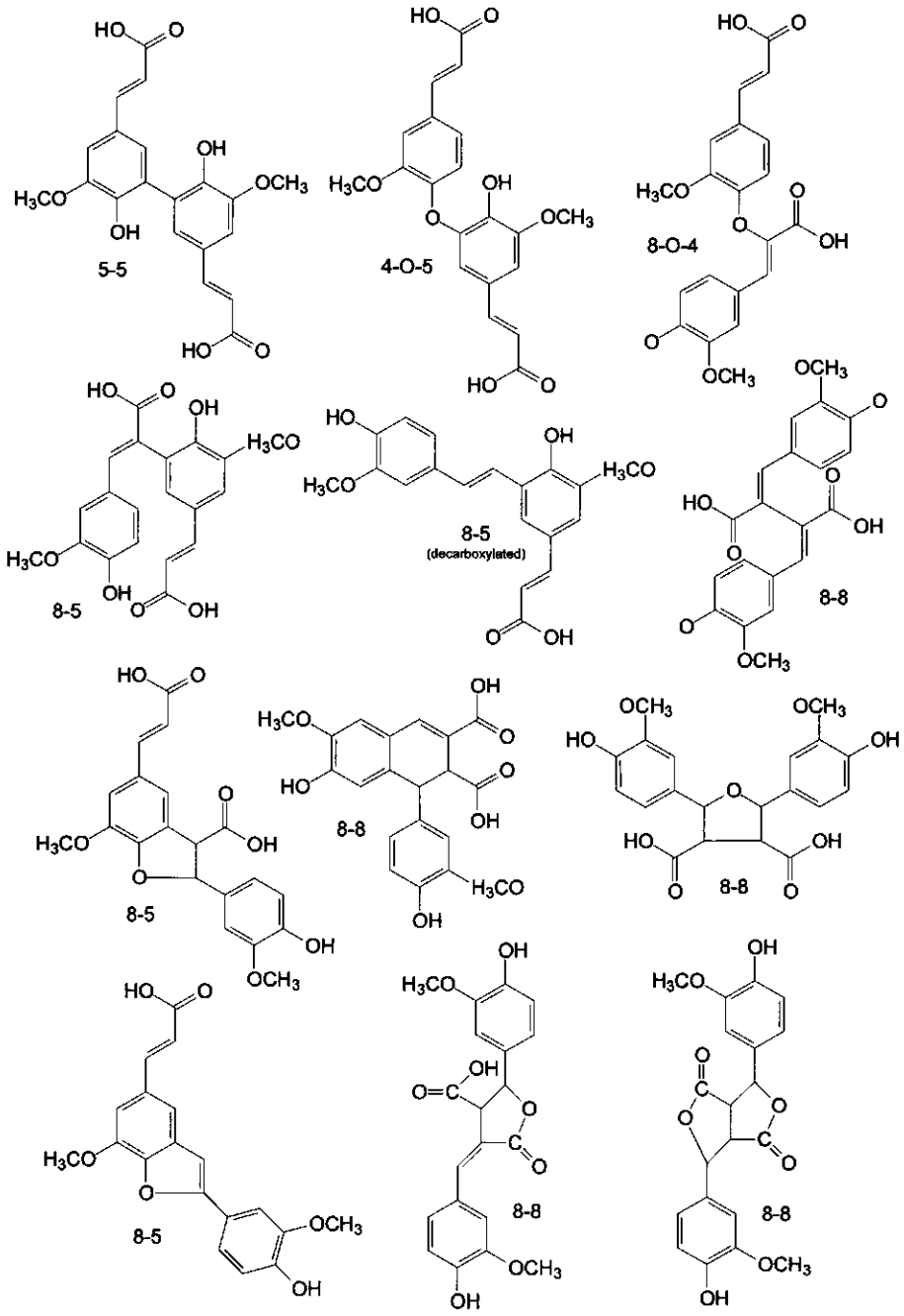

Figure 9 : All FA-dimers currently known

Also after *in vitro* coupling of FA-containing polysaccharides, the population of isomeric dimers bridging the polysaccharides varies akin to diferulic isolates from plant material. Predominantly 8-5 and to a lesser extent 8-O-4, 8-8 and 5-5 diferulate bridges, were found when feruloylated arabinoxylans from wheat flour and wheat bran were cross-linked by incubation with horseradish peroxidase and H_2O_2 (*97*). After incubations of sugar beet pectin with horseradish peroxidase and H_2O_2, predominantly 8-5 and 8-O-4 dimers were identified (*98*). The horseradish peroxidase/H_2O_2 induced gelation of corn bran hemicellulose was discovered to stem predominantly from 8-O-4'-diFA, 8,5'-diFA benzofuran form, 8,8'-diFA open form, 8,5'-diFA open form and 8,8'diFA aryltetralin form dimerization of ferulic acid (*99*). A protein that guides stereoselectivity of E-coniferyl alcohol coupling has once been identified (*100*) and use of surfactants led to a significant shift in population of the different dimers of FA (*101*).

A diferulate comprises two ferulic acids connected via a covalent bond that replaces a hydrogen atom in each of the constituting, original, FA monomers. Similar to other phenols, the reaction requires the abstraction of an electron and a proton from the FA monomer. FA is an excellent substrate for many plant and animal peroxidases that have a sufficient electron potential to abstract an electron from the ferulic acid hydroxyl and subsequently accept a proton from the FA radical cation. The resulting FA free radical is stabilized by five resonance contributors (Figure 10).

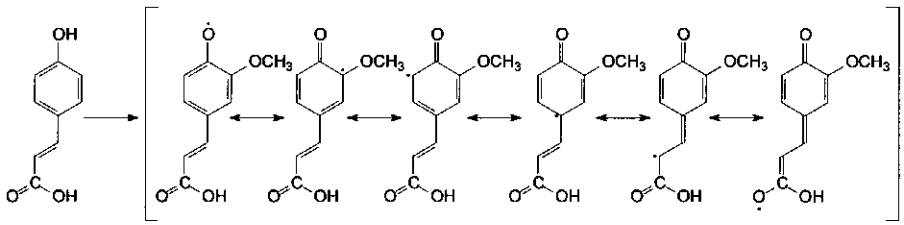

Figure 10 : Ferulic acid and its radical mesomers

A major distinction of ferulic acid as compared to tyrosine, is the propionylic acid side chain, that further stabilizes the free radical and introduces additional chemical reactivity of both unoxidized and oxidized FA. Up to 2001, dimers were the only *known* covalently coupled FA oxidation products, although some suggestions were made for the formation

of higher oligomers. These were first identified by Ward in studies of the initial steps of ferulic acid polymerization by lignin peroxidase (*96*). After separation by gel permeation, two trimers were identified by NMR spectroscopy (Figure 11).

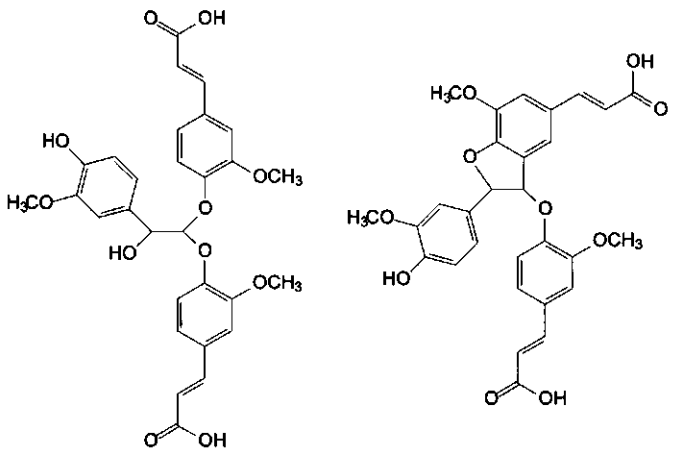

Figure 11 : Two trimers of ferulic acid (Ward)

Both of these trimers are decarboxylated indicating an essential role of decarboxylation in the formation of the trimer. Based on this finding, a mechanism was proposed in which a third ferulic acid free radical attacks the vinylic bond in a decarboxylated dimer. Further research on this mechanism of trimer formation is described in **Chapter III** of this thesis. Since these first reports of decarboxylated FA trimers, two reports on the isolation of trimeric FA *in vivo* have been published (*102, 103*).

1.8 Phenols in plant cell wall architecture

1.8.1 Biosynthesis of lignin

Diferulic acid and dityrosine, bridging polysaccharides and proteins respectively, are not the only cross-linked phenols that contribute to the architecture of the plant cell wall. Two other types of plant cell wall tissue, lignin and suberins, comprise complex phenolic polymers. In fact these tissues are the most abundant aromatic biopolymers on earth. Lignin is traditionally considered to result from polymerization of three alcohol

monomers: the monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 12).

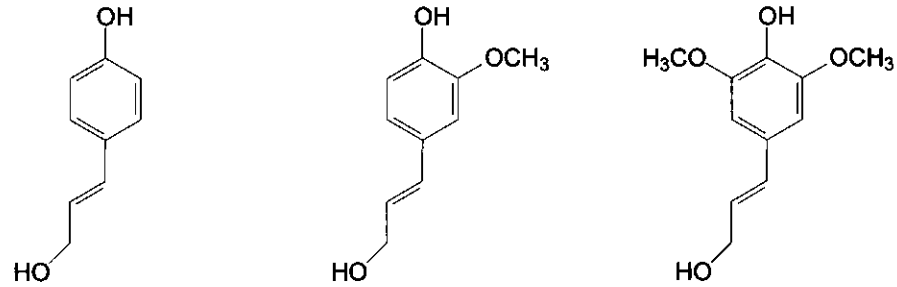

Figure 12 : The monolignols

Many features of lignin biosynthesis are presently still to be explored (*104*). This includes localization of growing lignin and the temporal and spatial deposition of lignins in the cell wall. The question of how plant cells regulate the deposition of lignin in the cell wall has been considered important since early workers identified differences in lignin subunit composition and cross-linking patterns between different parts of the cell wall. For the initiation of lignin deposition, a radical nucleation site has been supposed, though the nature of this site remains unclear (*105, 106*). Interestingly, many plant cell wall proteins are extremely rich in tyrosines (*42, 107-112*). Only once was the suggestion made that these phenols might play a role in the anchoring of lignins (*113*). Model studies showed that synthetic proteins rich in tyrosine could be cross-linked into lignin-like dehydrogenation products (*113*). Recently, sinapate dehydrodimers and sinapate-ferulate heterodimers were identified in cereal dietary fiber (*114*). Furthermore, for (hydroxy)proline-rich structural proteins in the cell wall of bean or soybean cells, it was shown that tyrosine-coupling occurs upon treatment with fungal elicitors (*23*). This stimulus-dependent oxidative cross-linking of structural cell wall proteins has an important function in cell wall maturation and toughening of cell walls in the initial stages of plant defense. This study clearly demonstrated the role of free radical coupling of tyrosines in plant cell wall build-up. Furthermore, a lysine/tyrosine rich protein associated with lignification in tomato has been identified (*115*). Another indication of tyrosine-hydroxycinnamic acid dimerization was

obtained from retained, alkali-resistant autofluorescence of proteins from the wall of wheat aleurone cells (*116, 117*). In addition, arabinofuranoside esterified with ferulic acid could be incorporated into a synthetic lignin dehydrogenation polymer of coniferyl alcohol by a kinetically controlled incubation of both substrates with horseradish peroxidase (*118, 119*).

1.8.2 Peroxidase involvement in lignin and suberin synthesis

In early studies on lignin biosynthesis, the dehydrogenated bonds between phenolic precursor moieties in plant tissues were often circumstantially deduced to stem from peroxidase action. Direct evidence for peroxidase involvement in nascent polyphenolic tissue has later been increasingly provided. An anionic peroxidase was shown to be temporally and spatially associated with the wound-induced suberization process in potato tubers (*120-122*). This 45 kD class III (plant secretory) peroxidase has a preference for feruloyl (o-methoxyphenol)-substituted substrates such as those that accumulate in tubers during wound healing. From tomato a cell-wall peroxidase was purified and demonstrated to cross-link extensins *in vitro* (*123*). An interesting link between peroxidase expression and lignification evolved from a *Arabidopsis* mutant with increased lignin levels (*124*). This mutant showed increased levels of mRNA encoding ATP A2 peroxidase and was, therefore, concluded to be involved in a complex regulation of covalent cross-linking in the plant cell wall. The co-substrate of peroxidases, H_2O_2, was found to be co-localized with zones of lignification (*125*). Adding potassium iodide, an H_2O_2 scavenger, to a cell suspension of *Pinus taeda* terminated the formation of lignin that is normally induced by exposure to 8% saccharose solution (*126*). Addition of H_2O_2 and peroxidases extracted from maize cell suspensions to primary maize walls acylated with 2% ferulate led to incorporation of the ferulates into lignin at over 90% (*95*). Ferulic acid was incorporated in the propanoid side chains of the lignin polymers as could be demonstrated after feeding [^{13}C]ferulic acid to seedlings of *Triticum aestivum* over extended durations (*127*). More suggestions were made about the possible role of ferulic acid in lignification. Based on maize cell wall architecture it was suggested that the heteroxylans are presumably cross-linked to the cell wall proteins. The nature of this linkage was, however, not determined (*128*).

1.8.3 Biosynthesis of suberin

Suberin is an abundant, complex, intractable biopolymer found between the primary cell wall and plasmalemma in plants (*129*). Suberin is comprised of

both aromatic and aliphatic components. Analysis of the aromatic components revealed a mixture of feruloyl esters that were suggested to function as suberin precursors (*40, 130*) and furthermore tyrosine, tyramine and substituted benzaldehydes/benzoates. Intact suberin from potato wounded periderm was examined *in situ* using solid-state ^{13}C NMR spectroscopy after uptake of [1-^{13}C]-, [2-^{13}C]- or [3-^{13}C]phenylalanine (*131*). This revealed that the phenolic domain of suberin is primarily composed of a hydroxycinnamic acid-derived phenolic polymer, though, the sequential stages in its assembly remained unclear.

In developing wheat seedlings a remarkable variation of ferulic acid and diferulic acid content was found in relation to the stage of development (*132*). At the start of lignification, the concentration of ferulic and *p*-coumaric acid esters was increased significantly, which further indicated a crucial role for these molecules in plant cell wall assembly. A proposed role for ferulic acid in lignin anchoring is described in **Chapter III** of this thesis.

1.9 Oxidative enzymes involved in phenol coupling

1.9.1 Horseradish peroxidase

The radical coupling reactions of tyrosine and ferulic acid are always initiated by the formation of a free radical from the phenol moiety (*vide supra*, 1.3). In plants and animals, peroxidases are the class of enzymes that perform this reaction. Already as early as 1810, Planche reported that a tincture of guaiacum developed a stronger color when a piece of fresh horseradish root was soaked in it (*133*). The peroxidase from horseradish (oxidoreductase EC 1.11.11 : donor) is one of the best-studied peroxidases. The first report of horseradish peroxidase was in 1937, in which the existence of horseradish peroxidase compound II is described (*134*). In 1941, compound I was first identified (*135*) and in 1976 it was the first peroxidase of which the primary sequence was determined (*136*).

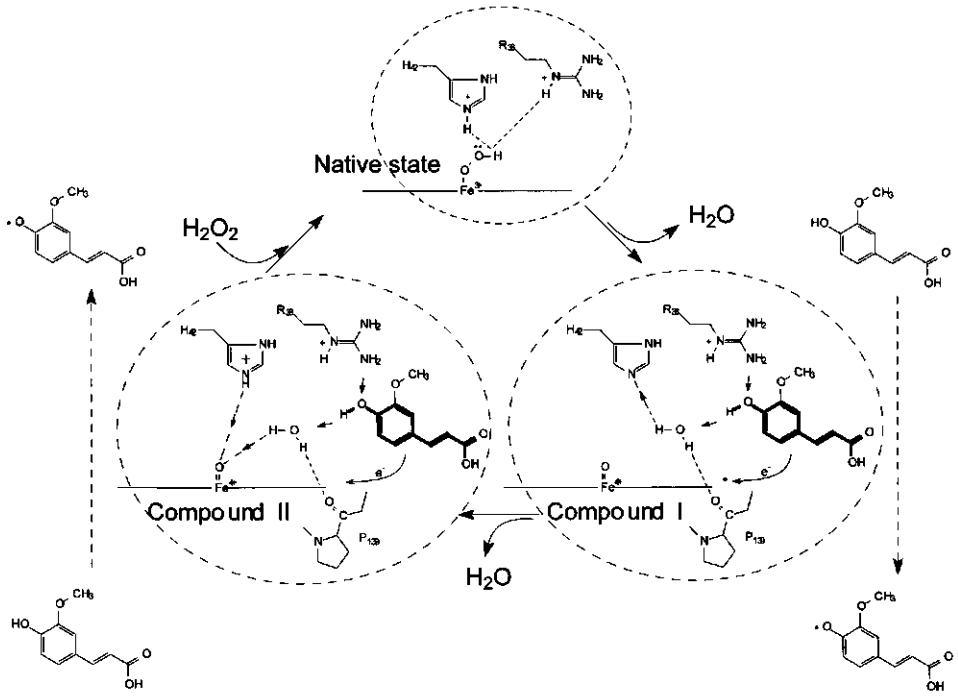

Figure 13 : Reaction cycle of horseradish peroxidase during oxidation of two ferulic acids

George (*137-140*) formulated the reaction cycle of horseradish peroxidase, in which an equivalent of the cosubstrate H_2O_2 is utilized for the oxidation of two phenols. First, a peroxocomplex is formed by reaction of H_2O_2 with the heme iron. The peroxocomplex, called compound I, then reacts with a phenol present in the active site by abstracting an electron from the HOMO of this substrate. From the resting enzyme an activated complex is initially formed, in which the distal histidine accepts a proton from H_2O_2 and the distal arginine stabilizes the charge. In a subsequent step water is released, a double bond is formed between Fe^{3+} and the second oxygen of H_2O_2 resulting in a radical on the porphyrin ring. Compound I of horseradish peroxidase is very stable compared to that of other peroxidases: It exists for at least 20 minutes after it has reacted with a stoichiometric amount of H_2O_2. After reaction with a reducing substrate, Compound II is formed that generally reacts with the same reducing substrates as Compound I. At pH values > 9 Compound II is no longer reactive and accumulates as a stable compound up to 24 hours. After the first electron uptake by the iron center, a second electron is abstracted from a second substrate donor by the so called Compound II, upon which the enzyme can return to the native state and perform another cycle oxidizing two phenols. Horseradish peroxidase can attain 5 different oxidation states during a reaction cycle, as shown in the pentagonal diagram in Figure 14 (*141*). Recently, the details of the catalytic pathway of horseradish peroxidase were studied by X-ray cryo-crystallography (*142*).

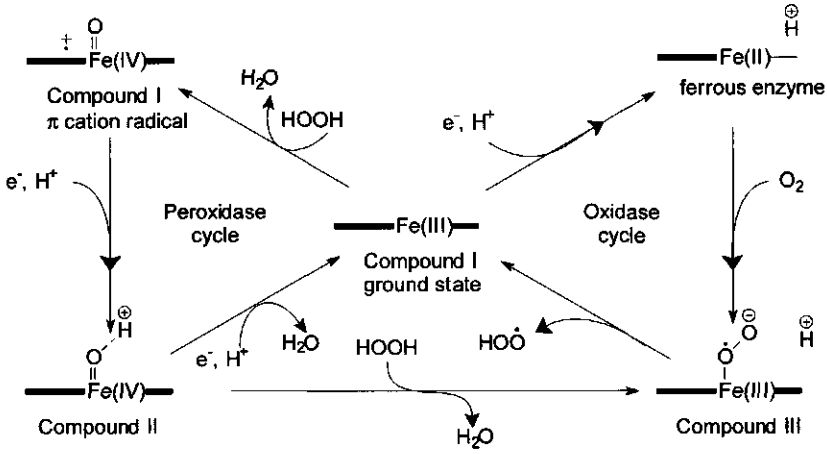

Figure 14 : Oxidation states of horseradish peroxidase in peroxidase and oxidase cycle (*142*)

The outer substrate channel of horseradish peroxidase is hydrophobic and contains three phenylalanine residues. These aromatic residues constitute an aromatic substrate-binding site that facilitates the facile oxidation of phenolic substrates. The crystal structure of horseradish peroxidase compound I in complex with ferulic acid nicely shows the binding of this substrate near the heme iron center (*143*). A Michaelis-Menten-like complex between horseradish peroxidase compound II and di-(N-acetyl-L-tyrosine) was evidenced from stopped flow studies (*144*). Within their range of solubility, saturation of horseradish peroxidase was observed for the substrates Gly-Tyr and $(NAT)_2$. A kinetic model including enzyme-substrate complex formation prior to each of the two reduction steps was derived from stopped flow kinetic data.

In vitro use of horseradish peroxidase requires carefully chosen incubation conditions to keep the enzyme catalytically active. Horseradish peroxidase loses activity at $[H_2O_2]$ above 250 mM and is irreversibly inactivated at temperatures above 50 °C. The specific activity of Ca^{2+}-depleted horseradish peroxidase was found to be 50 % of that of the native form (*145*). Horseradish peroxidase has a relatively low redox potential of –278 mV when compared to cytochrome c peroxidase (-194 mV) and lignin peroxidase (-130 mV) (*146-148*).

1.9.2 Myeloperoxidase

In the nineteenth century, it was discovered that pus could oxidize guaiacol. Early in the twentieth century, leukocytes were found to oxidize guaiacol as well. (*149*). The enzyme responsible was later found to be myeloperoxidase (verdoperoxidase), which causes the green color of pus. The characteristic green colour of this peroxidase stems from the extraordinary attachment of the heme to the protein, via a methionine residue. Myeloperoxidase catalyses the formation of dityrosine from tyrosine (*150*) as well as proteins (*151*) and is often suggested to cause dityrosine cross-linking in human tissues (*vide supra*).

1.10 Covalent oxidative coupling of proteins and polysaccharides

1.10.1 Applications in food industry

As described, protein cross-linking via dityrosine is known since 1964 and diferulic acid bridges coupling polysaccharides since 1978. Because the formation of both protein and arabinoxylan homocoupling is based on the combination of free radicals, Neukom and Markwalder suggested that the formation of a covalent adduct between a protein tyrosine and an arabinoxylan ferulic acid might be feasible as well (*152*). Whereas diferulates and di- or tri-tyrosines have been isolated from plant and animal sources, a hetero ferulic acid-tyrosine complex has never been isolated. Since Neukom's suggestion, many efforts were made to achieve this heterocoupling by incubating proteins and arabinoxylans with several peroxidases. Cross-linking of ferulic acid(-esters) with tyrosine has remained a topic of interest. Besides the relevance of such linkage in plants, it might also yield potentially interesting products for the food industry and provide an explanation for the beneficial effect of the addition of peroxidase to, e.g., bread dough.

Already in 1939 it was reported that the dough forming properties and the baking performance of wheat flours can be improved by addition of H_2O_2 (*153*). This was later reconfirmed (*154-157*) (*158*) and further established by the addition of peroxidase, peroxidase with H_2O_2 (*159-161*) and peroxidase/H_2O_2 with catechol (*162*). Also in the large protein complex known as "gluten" the formation of dityrosines contributing to the gluten network was evidenced during dough-mixing and bread-making processes (*163*). Incubation of whey protein isolate with microbial peroxidase and H_2O_2 led to almost full conversion to cross-linking products of β-lactoglobulin, but not of α-lactalbumin (*164*). Interestingly, when whey protein isolate was incubated with laccase, neither α-lactalbumin nor β-lactoglobulin were converted to oligomers. However, when chlorogenic acid was added to these reaction mixtures, exclusively α-lactalbumin polymerized.

Proteins might be linked to arabinoxylans or pectins via phenolic bridges comprising the protein tyrosine and the polysaccharide esterified phenolic acid. When equimolar amounts of protein and polysaccharide are present during the cross-linking reaction, the majority of product will be, statistically, that stemming from homocoupling. This is solely due to the fact that the collision events leading to homoproducts outnumber those leading to heteroproducts, i.e. protein-protein or polysaccharide-polysaccharide collisions dominate the less probable protein-polysaccharide collisions. This

even holds when the number of reactive sites per biopolymer is equal and the protein reactive sites are equally good substrates for the enzyme as the polysaccharide active sites. The amount of hetero-product is increased with an increasing number of reactive sites per protein or polysaccharide molecule since heterocoupling at one reactive site turns all homocoupling at other sites into a hetero-product. In this case a network consisting of protein and polysaccharide results.

In 1999, Figueroa-Espinoza and coworkers reported on attempts to cross-link feruloylated arabinoxylans and proteins using a fungal laccase. In these studies, no evidence was found for any coupling between the arabinoxylan feruloyl and the protein tyrosines (*165*). However, in this study the difference in reactivity of the two substrates for the enzyme active site was not taken into account neither was the rate of reaction of the formed radicals. In another study, Figueroa-Espinoza and coworkers found that the presence of cysteine but not tyrosine affects the gelation of arabinoxylans with manganese peroxidase or horseradish peroxidase/H_2O_2. Cysteine at high concentrations delayed gelation, whereas tyrosine accelerated the consumption of ferulic acid (*166*).

The general function of the coupling of phenols in proteins or polysaccharides is the concomitant structural integrity of the tissue in which these biopolymers are cross-linked to a solid polymeric network. The cross-linking of proteins with polysaccharides is supposed to result in novel emulsifying and foam-stabilizing properties of food ingredients. Proteins give nutritional value to food and contribute to the texture and mouth feel of the food product. These physicochemical properties can be altered, and possibly improved, by cross-linking arabinoxylans, a natural constituent of grain products, with proteins. The cross-linking of proteins with proteins and the cross-linking of arabinoxylans with arabinoxylans is known to occur in nature and has been performed *in vitro*. The hetero-cross-linking of proteins with polysaccharides is expected to further improve the textural properties of foods. A schematic picture of the intended complex of proteins and polysaccharides is shown in Figure 15.

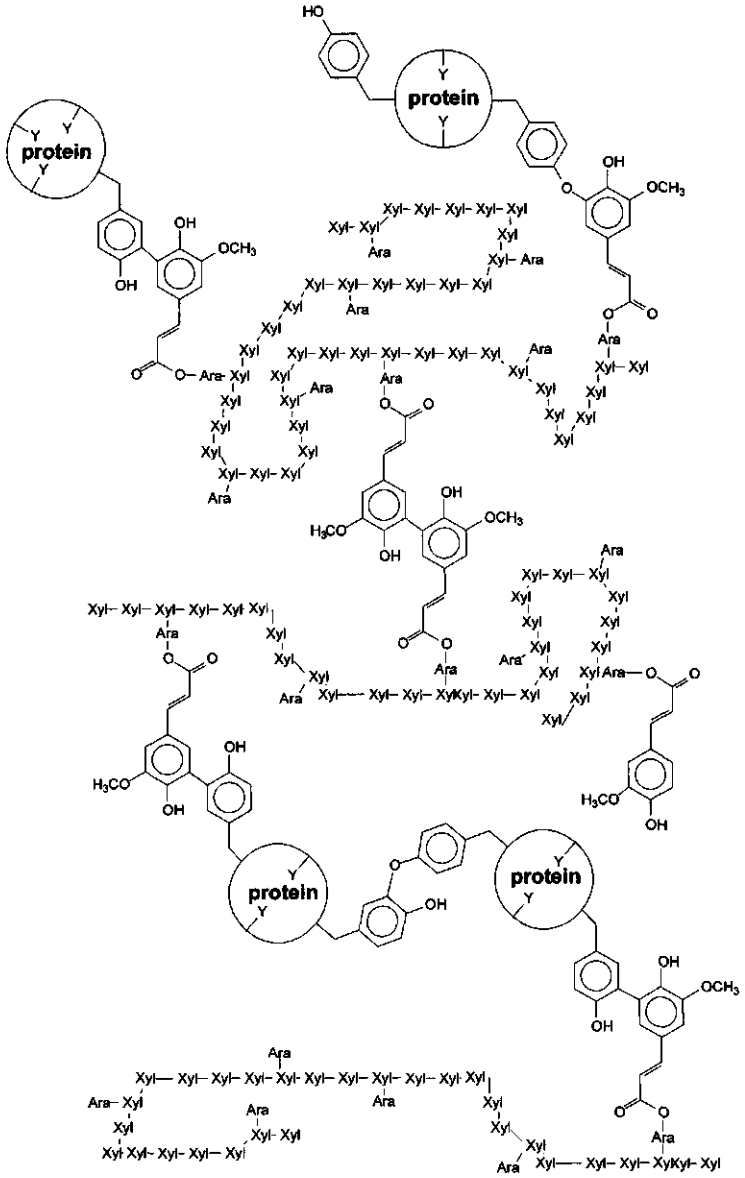

Figure 15 : Schematic view of covalently linked network of proteins and arabinoxylans

Means for cross-linking are limited since foods must meet requirements with respect to food safety and the appearance of the resulting product. Therefore the use of an exogenous cross-linking reagent is not acceptable and the use of endogenous moieties of the polymers is preferred. Proteins give nutritional value to food and contribute to the texture and mouth feel of the food product. A potential cross-linked food ingredient can therefore best be regarded as a network of homo- and hetero-cross-linkages, altogether causing an altered, and possibly improved physicochemical behavior as compared to the non-cross-linked biopolymers.

Peroxidase is naturally occurring in many foods of plant and animal origin and is also present in humans. Peroxidases may therefore have potential for use in foods. In order to cross-link proteins and arabinoxylans, the major challenge is the hetero-cross-linking of proteins with polysaccharides, instead of homo-cross-linking of proteins with proteins or polysaccharides with polysaccharides (Figure 16).

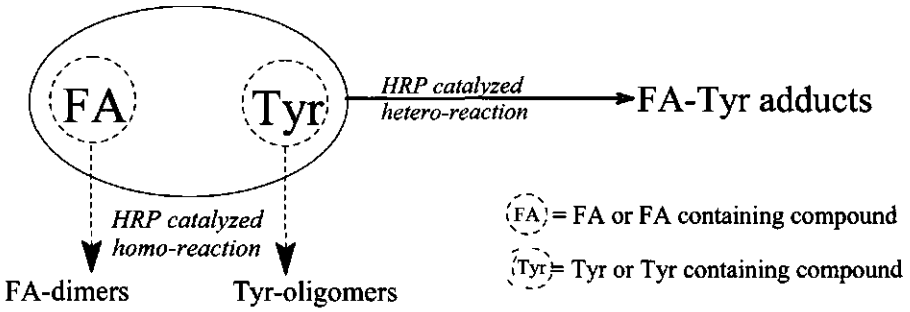

Figure 16 : Scope of the research towards hetero-cross-linking

Several factors influence the ratio of homo-cross-linking to hetero-cross-linking. The bottom-line is the incident population of reactive tyrosine radicals in the protein and FA reactive radicals in the polysaccharide present in the incubation mixture. When equimolar amounts of protein and polysaccharide reactive sites are present, the difference in the rate of conversion plays a crucial role in the ratio of homo- to hetero-products. In reverse, a known difference in rate of conversion of both substrates can be utilized to maximize the amount of hetero-products. The incubation should then be carried out by kinetic control i.e. the molar ratio of the substrates should continuously be compensated for the difference in conversion rate. A

successful method to perform such incubation is described in **Chapter II** of this thesis. Successful application of this method is describer in **Chapter VI** of this thesis.

1.11 Aim of the research

The research described in this thesis was embedded in the scientific program 'Industrial Proteins' from the Dutch Ministery of Economic Affairs. The goal of this program was the development of knowledge in the field of application of industrial proteins between industry, institutes and academia. This project was part of a collaboration between Wageningen University and the institutes TNO and ATO, aiming at the development of a method to cross-link proteins and polysaccharides by use of peroxidase to improve the properties of food products. The role of the ATO comprised predominantly the application of the findings from model studies on the level proteins and polysaccharide. An important part of the project within the university comprised the investigation of the cross-linking properties of the moieties in proteins and polysaccharides potentially responsible for such cross-linking. Furthermore, the development of a general method to perform the cross-linking reaction was aimed at.

Outline of the thesis

The introduction of this thesis describes the developments that gave rise to this project; the first discoveries of oxidative protein and polysaccharide cross-linking, the subsequent *in vitro* exploration of the cross-linking reaction and the suggestion that not only homo-coupling but also hetero-coupling might be feasable. In chapter II and III studies on the peroxidase-catalyzed modification of the reactive sites in proteins (tyrosine) and polysaccharides (ferulic acid) are described. To this end incubations with a tyrosine-containing peptide and ferulic acid were performed (Chapter II and III). The findings from these model studies were applied in the modification of a model protein, α-lactalbumin. The crucial differences between cross-linking α-lactalbumin and cross-linking a tyrosine containing tripeptide are described in Chapter IV. With this knowledge on extended cross-linking of α-lactalbumin, the protein was modified with ferulic acid (Chapter V). Attempts to identify the tyrosine in α-lactalbumin that is cross-linked with either FA or FAAE are described in Chapter VI, as well as efforts to identify the cross-linked peptides in α-lactalbumin. In Chapters VII and VIII, cross-linking by use of exogenous phenols is described. These studies comprise

model studies to explore which amino-acids can react with catechol (Chapter VII) followed by the application of catechol, and other exogenous phenols (Chapter VII) in incubations of α-lactalbumin, ß-casein or BSA with HRP and H_2O_2.

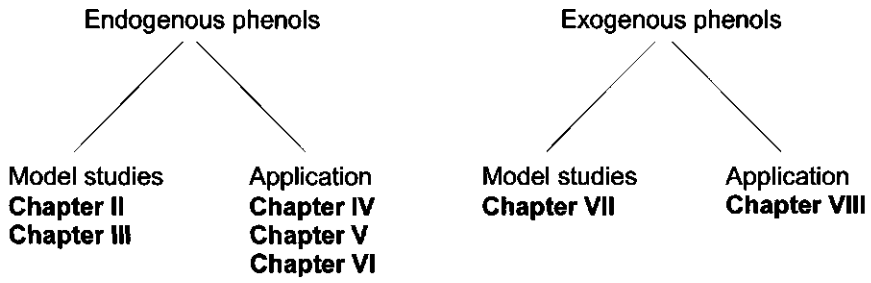

Figure 17: outline of the thesis

Each chapter decribes a different stage of the research, that can be summarized as follows.

1. **Introduction**
   Literature overview of the covalent cross-linking of proteins, *via* tyrosines, and polysaccharides, *via* ferulic acid. Examples of the occurence of oxidative phenolic cross-linking in plants and animals are described.

2. **Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid**
   The demonstration, by means of mass spectrometry, of a characteristic range of covalent adducts of tyrosine and ferulic acid, after kinetically controlled incubation of the tripeptide Gly-Tyr-Gly with ferulic acid. A mechanism that explains the idiosyncratic range of products obtained is proposed.

3. **Horseradish peroxidase-catalyzed oligomerization of ferulic acid on a template of a tyrosine-containing tripeptide**
   The demonstration of an additional, deviating, range of covalent adducts of tyrosine and ferulic acid, by tandem mass spectrometry. A mechanism that is new-fangled in the chemistry of ferulic acid is proposed.

4. **Cross-linking of α-lactalbumin by horseradish peroxidase**
   The demonstration of the time-dependent formation of α-lactalbumin oligomers dependent on conditions affecting protein structure. A role of oligomer formation in the formation of higher polymers is proposed

5. **Ferulic acid traps intermediates during horseradish peroxidase catalyzed polymerization of α-lactalbumin**
   The demonstration of the formation of covalent adducts of ferulic acid with α-lactalbumin based on the findings in chapters II, III and IV. A new role of ferulic acid and other anti-oxidants in the termination of protein oligomerization is proposed.

6. **Horseradish peroxidase catalyzed cross-linking of feruloylated arabinoxylans with β-casein**
   The demonstration of the formation of heteropolymers comprising β-casein covalently coupled with arabinoxylans.

7. **Catechol is cross-linked with peptidyl tyrosine and histidine by horseradish peroxidase via different reaction mechanisms** The demonstration of the formation of covalent adducts of catechol with tripeptides containing tyrosine or histidine after incubation with HRP and H_2O_2. Two mechanisms for the peroxidase catalyzed cross-linking of catechols with proteins are proposed.

8. **Cross-linking of proteins by peroxidase mediated oxidative dehydrogenation in the presence of exogenous phenols**
   The demonstration of the enhancement of formation of high molecular mass protein-polymers of β-casein and BSA at room temperature only by use of catechol in incubations with HRP and H_2O_2.

9. **General discussion**

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Chapter II

Peroxidase-Mediated Cross-Linking of a Tyrosine-Containing Peptide with Ferulic Acid

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The tyrosine-containing peptide Gly-Tyr-Gly (GYG) was oxidatively cross-linked by horseradish peroxidase in the presence of hydrogen peroxide. As products, covalently coupled di- to pentamers of the peptide were identified by LC-MS. Oxidative cross-linking of ferulic acid with horseradish peroxidase and hydrogen peroxide resulted in the formation of dehydrodimers. Kinetic studies of conversion rates of either the peptide or ferulic acid revealed conditions that allow formation of heteroadducts of GYG and ferulic acid. To a GYG-containing incubation mixture was added ferulic acid in small aliquots, therewith keeping the molar ratio of the substrates favorable for hetero-cross-linking. This resulted in a predominant product consisting of two ferulic acid molecules dehydrogenatively linked to a single peptide and, furthermore, two ferulic acids linked to peptide oligomers, ranging from dimers to pentamers. Also, mono- and dimers of the peptide were linked to one molecule of ferulic acid. A mechanism explaining the formation of all these products is proposed.

Keywords: *Horseradish peroxidase; tyrosine; ferulic acid; cross-linking; dehydropolymerization*

INTRODUCTION

Peroxidases are able to oxidize a wide variety of phenolic compounds. Typically two molecules of the phenolic substrate are oxidized, whereas one molecule of hydrogen peroxide is consumed. The types of radicals that are formed by the enzyme and the resonance contributors thereof are reflected in the type of products that are obtained. The coupling of tyrosine (Tyr), present in many proteins and peptides, as well as ferulic acid (FA), a phenolic esterified to arabinoxylans and pectins, by peroxidase has been the subject of many studies both in vivo and in vitro. Theoretically, the incubation of the two substrates could lead to homoproducts (Tyr oligomers and FA dimers) and heteroproducts (FA-Tyr adducts).

Peroxidase-Mediated Cross-Linking of Tyrosines in Vitro and in Vivo. In vivo, dityrosine cross-links are found in a wide variety of organisms. This was first shown for the insect cuticulum protein resilin (*1*). In vitro, proteins and peptides can be cross-linked via their tyrosine residues by peroxidase (*2*−*5*).

Tyrosine is converted into a phenolic radical after one-electron oxidation by peroxidase and can react at three positions on the aromatic ring (*6*). Either an ether linkage between the phenolic oxygen and the ortho-carbon of the aromatic ring or a linkage between aromatic carbons at either one of the ortho positions of the two tyrosines is formed. Cross-linking of free tyrosine by peroxidase leads among others to two isomeric dimers: the isodityrosine ether dimer, the ortho−ortho coupled product (*6*), and several isomeric trimers (*2*, *7*). The kinetics of the cross-linking of tyrosine-containing peptides via their tyrosines is greatly dependent on the type of amino acid adjacent to the tyrosine (*8*).

Peroxidase Cross-Linking of Ferulic Acid in Vitro and in Vivo. In vivo, peroxidases are supposedly involved in the formation of plant cell walls by interchain cross-linking of polysaccharides via ferulic acid moieties esterified to these carbohydrates (*9*). The resulting dehydrodimers of ferulic acid have been identified in grass cell walls (*10*). In vitro, arabinoxylans and pectins have been cross-linked via their ferulic acid moieties by incubation with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) or with laccase (*11*−*13*). Ferulic acid (FA) is converted into a semiquinone radical upon one-electron oxidation by peroxidase and H_2O_2. This radical has five mesomeric resonance contributors (*14*) and can react at two positions on the aromatic ring and one on the vinylic position. Therefore, the vinylic, aromatic, and oxygen radicals can couple to each other in all possible combinations, resulting in five products (*10*) reflecting the parental radical compounds.

Peroxidase-Mediated Cross-Linking of Tyrosines and Ferulic Acid. Enzymatic modification of food proteins is of interest for the food industry. Modification with carbohydrate moieties will probably influence the gelling, emulsifying, and foaming properties of the protein. Addition of such modified proteins to food

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systems is expected to have beneficial effects on their properties. To obtain such modified proteins, hetero-cross-linking of proteins via their tyrosines to carbohydrates via ferulic acids seems a logical step, in analogy to the homo-cross-linking of both tyrosines and FA via radical combination. The oxidative cross-linking of proteins and carbohydrates by peroxidase has been suggested (*12*, *15*, *16*). Cross-linkages between the protein tyrosine and the FA moiety of the feruloylated arabinoxylan were outlined, and possible structures of these adducts have been proposed (*12*). Neither in vivo nor in vitro have such cross-links been identified.

Figueroa-Espinoza and Rouau reported (*17*) that cross-linking of feruloylated arabinoxylans to proteins with a fungal laccase was not successful. Our studies described here concern a model system for the attachment of arabinoxylans (and pectins) to proteins via Tyr–FA linkages. A tyrosine-containing peptide may be considered as a model for proteins, whereas ferulic acid could mimic feruloylated arabinoxylans and pectins. As most tyrosines are located at an internal position in the amino acid sequence of a protein, Gly-Tyr-Gly (GYG) was chosen as a model peptide. Because peroxidase is able to use both Tyr and FA as substrates, in both cases leading to the formation of reactive radicals, we decided to investigate the kinetic parameters and resulting products for both compounds as model substrates in homoincubations. On the basis of information obtained in these studies kinetically controlled heteroincubations were performed.

MATERIALS AND METHODS

Materials. The peptides Tyr-Gly-Gly (YGG), Gly-Tyr-Gly (GYG), and Gly-Gly-Tyr (GGY) were obtained from Bachem, Bubendorf, Switzerland. HRP (type VI-A) and FA were obtained from Sigma. Hydrogen peroxide was obtained from Merck. All other chemicals were of analytical grade.

General. All incubations and control experiments were carried out in triplicate at 25 °C in a stirred solution. A 40 μM stock solution of HRP was prepared in 50 mM potassium phosphate buffer, pH 7.4, and kept on ice. A 25 mM stock solution of hydrogen peroxide was always prepared freshly before incubation. Stock solutions of 4 mM ferulic acid in 100 mM KPi buffer, pH 7.4, were always stored in the dark. This stock solution was used in all incubations to which aliquots of FA were added sequentially. For each peptide incubation a 50 mM stock solution in 100 mM KPi, pH 7.4, was used. The enzymatic reaction was monitored at 318 nm (at which dityrosines absorb maximally) and 348 nm (at which diferulates absorb maximally) using a Hewlett-Packard 8453A diode array spectrophotometer. Initial rates of conversion for the different peptides and FA were analyzed in terms of Michaelis–Menten parameters. Apparent values of V_{max}/K_M [$(V/K)_{app}$] were determined using the hyperbolic regression modulus of Sigmaplot 4.0.

Incubations containing initially 140 nM HRP, 250 μM H_2O_2, and final concentrations of peptide ranging from 1 to 5 mM or FA ranging from 20 to 80 μM in 100 mM KPi, pH 7.4, were allowed to react until no further spectral changes occurred. To ascertain that neither depletion of hydrogen peroxide nor inactivation of the enzyme was causing the absorbance to become constant, additional aliquots of HRP and H_2O_2 were added, to allow full conversion of the substrate. For each substrate, the change in extinction coefficient ($\Delta^{318}\epsilon_{app}$) for the conversion of the substrate into whatever product was determined from the slope of the plot of the final absorbance at 318 nm (corrected for increase of volume) versus the initial concentration of the substrate in the incubation mixture. This was done analogously to the method used in ref *18*.

Homoincubations of GYG, YGG, and GGY. To determine the initial rates of reaction of YGG, GYG, and GGY, 0.25, 0.5, 1.0, 2.5, 5, 10, and 20 mM of each peptide were incubated in total volumes of 1 mL of 100 mM KPi, pH 7.4, containing 140 nM HRP and 250 μM H_2O_2. The rate of conversion of the peptides was calculated from the linear part of the curve using the $\Delta^{318}\epsilon_{app}$ determined as described above.

For product identification by LC-MS, the incubation of 10 mM GYG was allowed to react for 900 s. When during the incubation no more changes in absorbance were observed, an aliquot of H_2O_2 was added. An aliquot of HRP was added when no changes in absorbance were observed upon addition of H_2O_2. In total, during the 900 s five aliquots of H_2O_2 and one aliquot of HRP were added. Furthermore, a control incubation of 10 mM GYG without HRP was carried out.

Homoincubation of Ferulic Acid. To determine the initial rate of reaction, FA was incubated at initial concentrations of 20, 40, 60, 100, 140, 200, and 300 μM in 100 mM KPi buffer, pH 7.4, containing 140 nM HRP and 250 μM H_2O_2 in a stirred solution in a total volume of 1 mL. The conversion rate of FA was calculated from the linear part of the curve using the $\Delta^{318}\epsilon_{app}$ that was determined as described above.

For product identification with LC-MS 17 10-μL aliquots of FA were added over 900 s to 1 mL of 100 mM KPi buffer, pH 7.4, containing 140 nM HRP and 250 μM H_2O_2 in a stirred solution. Five times a 10 μL aliquot of hydrogen peroxide was added. Twice a 10 μL aliquot of HRP was added. The sequence of addition of H_2O_2 and HRP was in analogy to the hetero-incubation described below. In a control experiment HRP was omitted.

Heteroincubations of GYG and Ferulic Acid. Batchwise heteroincubations were performed in 100 mM KPi buffer, pH 7.4, containing 140 nM HRP, 16.7 mM GYG, 250 μM H_2O_2, and 400 μM FA in a total volume of 1 mL. Eight aliquots of hydrogen peroxide and three aliquots of HRP were added during the incubation.

Kinetically controlled heteroincubations were performed in 100 mM KPi buffer, pH 7.4, containing 140 nM HRP, 16.7 mM GYG, and 250 μM H_2O_2 initially in a total volume of 1 mL. To the dityrosine-forming incubation mixture was added the first aliquot of FA. Over a 900 s period 17 10-μL aliquots of FA were added while the reaction process was monitored. In total, seven 10-μL aliquots of H_2O_2 and two 10-μL aliquots of HRP were added when no spectral changes at 318 and 348 nm occurred upon addition of FA.

Analysis and Characterization of the Incubation Products. *HPLC.* Chromatographic separation of the reaction products was carried out immediately after the incubations. Substrates and reaction mixtures were analyzed by injecting 100 μL of the crude incubation mixture onto an Inertsil ODS-2 5 μm column (Alltech, Breda, The Netherlands) in a Spectrophysics apparatus (Thermo Separation Products) equipped with a SpectraSYSTEM UV 3000 photodiode array detector. The eluate was monitored at 280 and 318 nm. Eluents of 0.01% TFA in water and 0.01% TFA in acetonitrile were used for elution at a flow rate of 1 mL/min in a linear gradient with the second eluent rising from 10 to 100% over a 40 min period.

LC-MS. For LC-MS analysis, 20 μL of reaction mixture was separated on a 150 × 2.1 mm Alltima C18 column (Alltech, Breda, The Netherlands). Eluents of 0.01% TFA in water and 0.01% TFA in acetonitrile were used for elution at a flow rate of 0.2 mL/min in a linear gradient with the second eluent rising from 10 to 100% over a 40 min period. Mass spectrometric analysis (LCQ ion trap, Finnigan MAT 95, San Jose, CA) was performed in the positive electrospray mode using a spray voltage of 2.5 kV and a capillary temperature of 200 °C. The apparatus and data were controlled by XCalibur software. The accuracy of the mass determinations is ±0.3 Da.

The mass data were processed using the algoritm

$$m\,(\text{product})/z\,(\text{product}) = \{[aM(\text{peptide}) + bM(\text{ferulic acid})] - 2(a + b - 1) + q\}/q \quad (1)$$

with a = the number of oxidatively linked molecules of GYG, b = the number of oxidatively linked molecules of FA, q = the

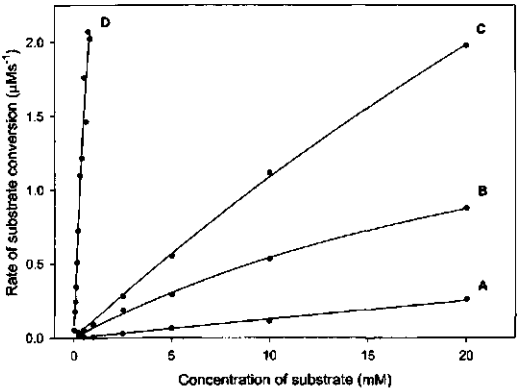

Figure 1. Rate of tyrosine conversion for GYG (A), YGG (B), GGY (C), and FA (D) as a function of the substrate concentration upon incubation with HRP and H_2O_2.

Table 1. Overall Extinction Coefficients at 318 nm for the Products of the Complete Conversion ($\Delta^{318}\epsilon_{app}$) and Apparent Values of V_{max}/K_M for the Conversion of GYG, YGG, GGY, and FA

	substrate			
	GYG	YGG	GGY	FA
$\Delta^{318}\epsilon_{app}$ (M^{-1} cm^{-1})	310	590	715	7000
$(V/K)_{app}$ (s^{-1})	0.014	0.078	0.13	1.66

number of protons, and therefore the charge, on the adduct, and z = the total charge of the adduct.

MS/MS was performed in triple-play mode with a relative collision energy of 35%. Data were analyzed using the program Protein Prospector MS Digest by P. R. Baker and K .R. Clauser (http://prospector.ucsf.edu).

RESULTS

Rates of the Conversion of YGG, GYG, GGY, and FA. The $\Delta^{318}\epsilon_{app}$ values for the full conversion of substrates into products were determined as described under Materials and Methods. The results are reported in Table 1. For the products of the conversion of FA a decrease of 7000 M^{-1} cm^{-1} was found, which agrees well with a previously reported value (*18*).

Spectrophotometric monitoring of the incubations of YGG, GYG, and GGY at 318 nm always resulted in an increase in absorbance, which was linear for 30 s at least. Spectrophotometric monitoring of incubations of FA showed a rapid decrease in absorbance at 318 nm and an increase in absorbance at 348 nm. The velocities of tyrosine oligomerization differed significantly between the different peptides and an order of magnitude with the FA conversion (Figure 1). $(V/K)_{app}$ values are listed in Table 1. These data indicate that tyrosine-containing peptides are poor substrates for HRP and must be present in large excess over FA to allow formation of similar concentrations of each radical.

Product Identification in GYG and FA Homoincubations. In incubations of 10 mM GYG with HRP and H_2O_2 a new absorption with a maximum at 318 nm was observed. The incubation mixture turned clear brown/yellow during the incubation. Analysis of the reaction mixture with RP-HPLC (data not shown) revealed several nearly coeluting reaction products that were not present in the control reactions. Characterization by LC-MS of the products in the unresolved peaks led to the identification of oxidatively linked GYG ranging from dimers to hexamers. Di-, tri- and tetramers were the predominant products. Table 2 shows the *m/z* values of the singly and doubly charged compounds, which absorb both at 280 and at 318 nm. All masses were in accordance with a polymerization mechanism as for a one-electron oxidation followed by dehydrogenative coupling of the radical compounds. The control incubation of 10 mM GYG in which HRP was omitted showed neither new products in the HPLC chromatogram nor compounds of new masses at any time during elution.

Table 2. Assignment of the *m/z* of the Compounds Resulting from Incubation of GYG with HRP and H_2O_2

compd	mass(es) found	theor value	rel intensity
$(GYG)_2$	589.5	589.4	high
$(GYG)_3$	882.5	882.6	high
$(GYG)_4$	1175.7	1175.8/588.4	high
$(GYG)_5$	1468.9/735.3	1469.0/735.0	medium
$(GYG)_6$	1761.8	1762.0/881.6	low

Table 3. Assignment of the *m/z* of the Compounds Resulting from Incubation of GYG and FA with HRP and H_2O_2

compd	mass(es) found	theor value	rel intensity
FA-GYG	488.2	488.4	low
FA-$(GYG)_2$	781.7	781.6	low
FA-$(GYG)_3$	1075.4	1074.8/537.9	low
FA-GYG-FA	680.5	680.6	high
FA-$(GYG)_2$-FA	973.4	973.8/487.4	medium
FA-$(GYG)_3$-FA	1267.8/634.3	1267.0/634.0	medium
FA-$(GYG)_4$-FA	1560.9/780.1	1560.2/780.6	low
FA-$(GYG)_5$-FA	927.0/617.9	927.2/618.5	low
FA-$(GYG)_6$-FA	716.9	1073.8/716.2	low

During the homoconversion of FA an increase in absorption with a maximum at 348 nm was observed. Analysis of the reaction products with RP-HPLC revealed several new compounds (data not shown). LC-MS led to the identification of two resolved dehydrodimers, singlefold positively charged species of 387 *m/z*, corresponding to the product of two ferulic acids after one-electron oxidation. No higher oligomers of FA were found in mass spectrometric analysis. The control incubation of FA in which HRP was omitted showed neither new products in the HPLC chromatogram nor products of new masses at any time during elution.

Product Identification in the Heteroincubation of GYG and FA. Because the kinetic studies showed that GYG must be present in large excess over FA to generate approximately equal amounts of each radical, FA was added in 10 μL aliquots over time (kinetic control of the reaction). When GYG was kinetically controlled incubated with FA, the UV absorbance of the incubation mixture differed from that of the incubations with GYG alone. The absorbance at 318 nm always increased before addition of FA. After each addition of FA, an increase of the absorbance at 318 nm, proportional to the amount of FA added, was observed. Subsequently, a rapid decay to a level slightly above that before the addition was observed, followed by a 15–20 s period of no spectral changes, after which time the absorbance at 318 continued to increase, slightly more slowly than before the addition of ferulic acid. The incubation mixture turned yellow. RP-HPLC analysis of these incubations revealed a broad range of products absorbing at 318 nm, which were not present in control incubations of GYG or FA alone.

Characterization of these products by LC-MS led to the identification of many different compounds. The

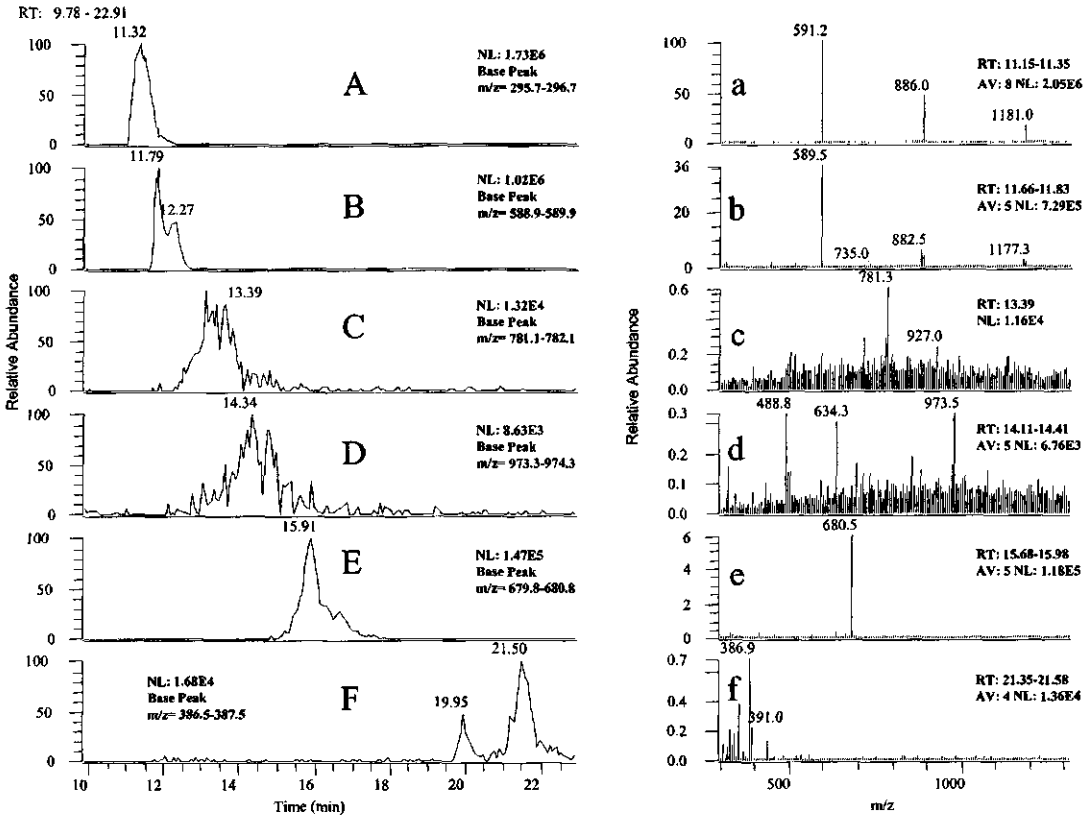

Figure 2. Base-peak RP-HPLC chromatograms of the kinetically controlled heteroincubation of GYG and FA (A–F), with RP-HPLC elution of the mass indicated in each chromatogram and corresponding selected mass spectra of these eluates (a–f): (A/a) GYG; (B/b) $(GYG)_2$; (C/c) FA-$(GYG)_2$; (D/d) FA-$(GYG)_2$-FA; (E/e) FA-GYG-FA; (F/f) $(FA)_2$.

masses of all compounds that could be attributed directly as a linear combination of the masses of GYG and FA are shown in Table 3 together with the theoretical values using the algorithm in eq 1. Figure 2A shows the elution of all compounds with an *m/z* ratio between 295.7 and 296.7, for GYG *m/z* 296.2, and the concomitant mass spectrum 2a shows the noncovalently bound oligomers of GYG as *m/z* species of 591.2 and 886.0. The covalently coupled dimer of GYG elutes slightly later (Figure 2B) as two different isomers, and the concomitant mass spectrum (Figure 2b) shows the mass of 589.5 in agreement with a dehydrogenatively coupled dimer. Also, a significant amount of coeluting dehydrogenatively coupled trimer of GYG, *m/z* 882.5, is revealed in the averaged mass spectrum of the dimer base peak. Figure 2c, a mass spectrum recorded from the eluate of the base peak of FA-$(GYG)_2$, shows the first eluting heteroadducts, the GYG dimer dehydrogenatively coupled with one FA and the GYG pentamer dehydrogenatively coupled with two FA molecules. Figure 2D shows the base peak of the dehydrogenatively linked GYG dimer that is dehydrogenatively linked to two FA molecules. The concomitant mass spectrum in Figure 2d reveals the coelution of FA-GYG and the dehydrogenatively linked trimer of GYG, dehydrogenatively linked to two FA molecules. Figure 2E shows the most predominant products, which yielded the highest signal to noise ratio, with an *m/z* ratio of 680.5 eluting at 15.9 and 16.7 min,

Table 4. Assignment of the *m/z* of the Ions Resulting from Fragmentation of FA-GYG-FA

fragment	mass found	theor value
b-2 ion	605.0	605.18
y-2 ion	577.3	577.18
a-2 ion	623.2	623.19

consisting of two FA molecules dehydrogenatively linked to one GYG. MS/MS of FA-GYG-FA yielded fragment ions. Both GY [b_2 and a_2 ions (*19*)] and YG (y_2 ion) fragments modified with two FA were identified (Table 4), proving that it is the tyrosine moiety that has been modified.

In the RP-HPLC chromatogram of the heteroincubation also two dimers of FA (Figure 2F) elute after all GYG-containing adducts.

When GYG was incubated batchwise with FA at a GYG/FA ratio that would yield approximately equal concentrations of radicals, a lower amount of new products compared to the kinetically controlled heteroincubations was found according to the products absorbing at 318 nm during elution. Also, the population of heteroproducts was less extended than in the kinetically controlled incubation, only FA-GYG-FA and FA-GYG-GYG-FA were found.

DISCUSSION

Rates of the Reactions. In the present study an overall extinction coefficient for all products formed was

the fact that it stems directly from GYG and no preceding combinations are required. In case the dimeric FA-GYG radical fuses with a GYG radical instead of an FA, a possible "growing chain" analogue to that for the GYG homoincubation product formation results. The length of these chains is statistically spread just like in the GYG homoincubations and explains the other heteroadducts found. As can be seen in the base peak chromatograms in Figure 2 the elution pattern obeys the proportion of the assigned oligomer that comprises the "hydrophobic FA foot" relative to the hydrophilic peptide moiety.

In Figure 4 all possibly occurring radical reactions in the incubation mixture have been outlined in a tentative model. As long as FA has not fused with another radical, it will be present in the reaction mixture as a radical. GYG radicals will only be generated at maximum velocity when all FA has reacted. The fact that only a slight amount of heteroproduct can be attributed to a FA-GYG, FA-$(GYG)_2$, and FA-$(GYG)_3$ adducts and not to higher hetero-oligomers with only one FA can be explained by the sequential additions of FA. Once such an adduct is formed, it is present in the mixture during the remainder of the incubation in which radicals are continuously generated. Therefore, it is susceptible to hydrogen abstraction by any radical present for a long period of time. It is thus more likely that further polymerization will take place when such an adduct is formed than that the adduct remains unreacted and can be detected. If the ratio of the two substrates is not continuously controlled and equal amounts of FA and GYG would be present, the much higher $(V/K)_{app}$ of FA would not allow the range of products as found in these studies to be formed.

The results obtained suggest the possibility of similar covalent linking in proteins and carbohydrates. Suggestions have been made about the possibility of cross-linking of arabinoxylans and proteins (*12*, *21*, *22*). Attempts to cross-link proteins and carbohydrates in vitro by peroxidase in model studies have failed thus far (*11*). The addition of tyrosine to a mixture of arabinoxylans did not affect the gelling caused by dehydrogenative cross-linking of the ferulic acids esterified to these polysaccharides (*11*, *23*). The fact that attempts to cross-link the tyrosine and FA in proteins and arabinoxylans have failed (*17*) can now be attributed to the fact that substrates were brought together with fixed initial conditions, often with the FA-containing polymer in excess or in stoichiometric amounts. Results presented here indicate that kinetic control is needed to achieve heterocoupling. Furthermore, the fact that tyrosine can couple to two ferulic acids suggests a role as a spacer, rather than preventing cross-linking of carbohydrate chains.

Conclusion. The cross-linking of GYG and FA can be achieved by kinetic control of the reaction catalyzed by HRP. In the polymerization reaction of GYG that is taking place, the reaction is terminated by the FA radicals that are generated batchwise in a kinetically controlled fashion. According to the mechanism proposed, the covalent cross-linking of tyrosine-containing proteins with arabinoxylans by HRP and H_2O_2 must certainly be possible utilizing the same approach.

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Chapter III

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Horseradish Peroxidase-catalyzed Oligomerization of Ferulic Acid on a Template of a Tyrosine-containing Tripeptide*

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Ferulic acid (FA) is an abundantly present phenolic constituent of plant cell walls. Kinetically controlled incubation of FA and the tripeptide Gly-Tyr-Gly (GYG) with horseradish peroxidase and H_2O_2 yielded a range of new cross-linked products. Two predominant series of hetero-oligomers of FA linked by dehydrogenation to the peptidyl tyrosine were characterized by electrospray ionization (tandem) mass spectrometry. One series comprises GYG coupled with 4–7 FA moieties linked by dehydrogenation, of which one is decarboxylated. In the second series 4–9 FA moieties linked by dehydrogenation, of which two are decarboxylated, are coupled to the tripeptide. A third series comprises three hetero-oligomers in which the peptidyl tyrosine is linked to 1–3 FA moieties of which none is decarboxylated. Two mechanisms for the formation of the FA-Tyr oligomers that result from the dualistic, concentration-dependent chemistry of FA and their possible role in the regulation of plant cell wall tissue growth are presented.

Plant peroxidases (donor: hydrogen-peroxide oxidoreductases, EC 1.11.1.7) have often been suggested to be involved in the biosynthesis of complex plant cell wall macromolecules constituting lignin and suberin tissues. Lignins are located in the primary and secondary walls of specific plant cells and are synthesized for mechanical strength, defense, and water transport in terrestrial vascular plants. The temporal and spatial correlation between lignin synthesis and peroxidase catalysis has been suggested based on studies of the enzyme, the hydroxycinnamic acid substrates, and the hydrogen peroxide co-substrate *in vivo*. In lignin tissues of transgenic plants, the *Arabidopsis* ATP A2 peroxidase promoter directs GUS reporter gene expression, indicating involvement of the peroxidase in the assembly of the lignin polymer (1). After administration of the reducing substrate [^{13}C]ferulic acid to wheat cell walls for extended duration, elevated levels of ferulic acid in the lignin-like tissues were revealed by *in situ* solid state ^{13}C NMR (2). In *Pinus taeda* cell suspension cultures, lignin synthesis is correlated with the generation of the oxidizing co-substrate H_2O_2 (3). Suberin provides a physical barrier to moisture loss in plants as well as a defensive shield against pathogens. In the potato, a H_2O_2-generating system with NAD(P)H-dependent oxidase-like properties is temporarily associated with suberin synthesis (4), and the polyaromatic domain of suberins in wound-healing potato (*Solanum tuberosum*) tubers is constituted of polymers derived from hydroxycinnamic acid (5).

Ferulic acid (FA,[1] 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid), a hydroxycinnamic acid that is often found to be esterified to plant cell wall polysaccharides (6, 7) is a good substrate for most plant peroxidases. The x-ray structure of horseradish peroxidase C (HRP C) in complex with FA (8) provides molecular insight into the initial abstraction of the hydroxyl hydrogen via one-electron oxidation and subsequent deprotonation of the cation radical, resulting in the FA free radical. Dehydrodimers of FA stemming from radical combination have been identified in plant cell walls (9–13). *In vitro*, similar FA dehydrodimers have been identified after incubation of peroxidase with either free FA or FA esterified to pectins and arabinoxylans (14). Recently, it was reported that in incubations with lignin peroxidase, higher polymers of ferulic acid are formed, and the structures of two dehydrotrimers were assigned by ^{1}H NMR (15).

Tyrosine, both as free amino acid and in peptides or proteins, is also a substrate for many plant peroxidases. After one-electron oxidation and subsequent deprotonation, a tyrosine radical species is formed that can combine with another tyrosine radical at either the phenolic oxygen or at one of the *ortho* positions of the aromatic ring (16). Dityrosines and higher oligomers resulting from peroxidase-mediated combination of tyrosine radicals have been characterized for tyrosine-containing peptides (17–19). The *in vivo* occurrence of dityrosines in proteins was first demonstrated in insect cuticulum (20). Upon

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[1] The abbreviations used are: FA, ferulic acid; HRP, horseradish peroxidase; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MS^2, tandem mass spectrometry; MS^3, subsequent CID after spectrometry MS; CID, collision-induced dissociation.

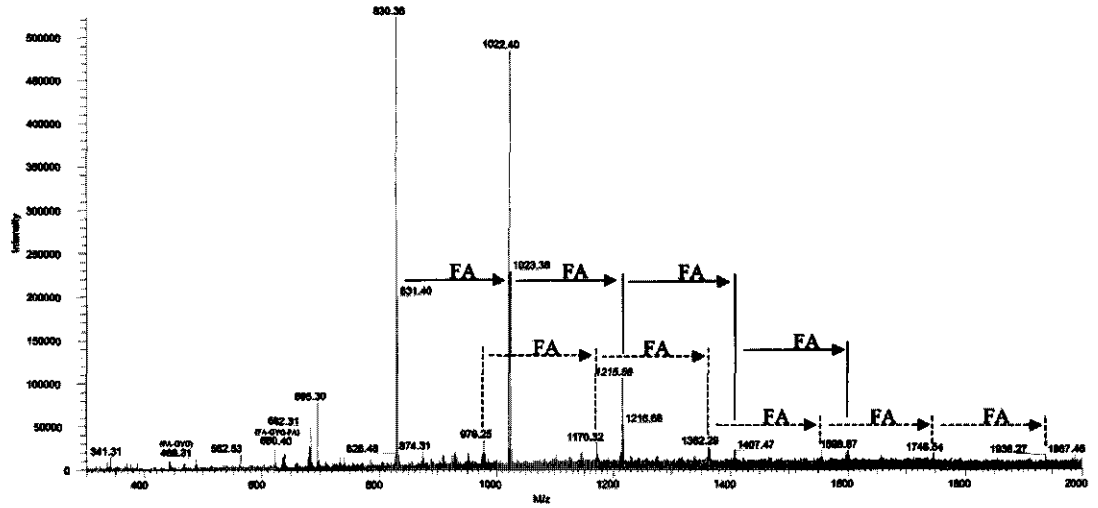

FIG. 1. **Nano-electrospray mass spectrum of the redissolved incubation mixture.**

elicitor and wound induction of bean and soybean cell walls, a rapid oxidative cross-linking of the (hydroxy)proline-rich glycoproteins was demonstrated (21). These structural proteins contain highly repetitive peptide sequences, among which the tyrosine-containing Val-Tyr-Lys-Pro-Pro pentapeptide. *In vitro*, dityrosines stemming from peroxidase catalysis in proteins were demonstrated in soluble collagens and in wheat prolamine (22).

Because both FA and tyrosine are subject to peroxidase-mediated oligomerization, we rationalized that hetero-coupling of these substrates should be feasible (19). Such peroxidase-mediated hetero-coupling could provide an explanation for the occurrence of protein-carbohydrate complexes in plant cell walls and the incorporation of FA and other hydroxycinnamic derivatives into lignin and suberin tissues on a protein template. From an earlier study with the tripeptide Gly-Tyr-Gly (GYG) and FA, we established conditions that resulted in covalent linkage of FA and the peptidyl tyrosine (19). Evidence was provided for the peroxidase-mediated formation of GYG oligomers linked by dehydrogenation, ranging from dimers to pentamers that are oxidatively cross-linked to either one or two molecules of FA (19). In the present study we have further explored the mechanism of hetero-adduct formation of GYG and FA. Complementary to the aforementioned studies (2, 3, 5, 9, 10, 13) in which the constituting precursor moieties in plant cell wall tissue were deduced from the *in vivo* synthesized biopolymer, we now present the *in vitro* synthesis of the biopolymer by HRP and H_2O_2 from the hypothesized FA precursor on a tyrosine template.

MATERIALS AND METHODS

Glycine-tyrosine-glycine (GYG) was obtained from Bachem, Bubendorf, Switzerland. HRP (type VI-A) and FA were obtained from Sigma. Hydrogen peroxide (30% v/v) was obtained from Merck. All other chemicals were of analytical grade.

Incubation—Solutions of 12.44 mM ferulic acid and 12.44 mM H_2O_2 (kept on ice) in 50 mM sodium phosphate, pH 8.0, containing 10% (v/v) acetonitrile were continuously and simultaneously added to the incubation mixture using two peristaltic pumps (LKB Bromma 2232 Microperpex S). The incubation mixture, thermostatted at 20 °C, contained 25 mM GYG and 1.25 mg of HRP in an initial volume of 10 ml of 50 mM sodium phosphate, pH 8.0. 20 ml of the FA solution and 20 ml of the H_2O_2 solution were added over a 15-min period. After another 10 min of incubation, the reaction mixture was frozen in liquid nitrogen, freeze-dried, and stored at 4 °C until analysis.

HPLC-MS—For liquid chromatography-MS analysis, 20 μl of the reaction mixture (10 mg of lyophilisate redissolved in 100 μl of MilliQ water) was separated on a 150 × 2.1-mm Alltima C18 column (Alltech, Breda, The Netherlands) running in 0.03% (v/v) trifluoroacetic acid in water at a flow rate of 0.2 ml/min. Elution was performed with a linear gradient of 0–40% acetonitrile in 0.03% (v/v) trifluoroacetic acid in water over a 45-min period.

Mass spectrometric analysis was performed with a LCQ ion trap (Finnigan MAT 95, San José, CA) with the use of electrospray ionization and detection in the positive ion mode. The capillary spray voltage was 2.5 kV, and the capillary temperature was 200 °C. The instrument was controlled by Xcalibur software. The accuracy of the mass determinations is ±0.5 Da. After a full MS scan, ions with a mass to charge (*m/z*) ratio within 10 *m/z* units of the most abundant ion were selected and subjected to collision-induced dissociation (CID, MS^2). The five most abundant product ions resulting from the first stage of CID were fragmented further by a second stage of CID (MS^3). For nanospray MS, 10 μl of the redissolved reaction mixture was diluted with acetonitrile (1:1) and loaded in a conductive metal-coated (coating 3AP) nanospray tip (2-mm internal diameter) (NewObjective, Woburn, MA) using a Gelloader pipette tip (Eppendorf). Nanospray conditions used were 25 units of nitrogen backing pressure and 1.2-kV ionization spray voltage, and the heated desolvation capillary was held at 200 °C. 500 scans detected in the positive-ion mode were averaged.

RESULTS

After freeze-drying the incubation mixture, a yellow/orange powder was obtained. Nanoelectrospray MS of the redissolved lyophilisate revealed two dominant series of products (Fig. 1). The most abundant ion series starts at *m/z* 830.36 and proceeds with average *m/z* increases of 192 to ions with apparent *m/z* values of 1022.40, 1215.58, 1407.47, and 1598.87. The other series starts at *m/z* 978.25 and proceeds with average *m/z* increases of 192 to ions with apparent *m/z* values of 1170.32, 1362.29, 1554.42, 1746.54, and 1938.27. The difference of 192 between the ions in both series corresponds to FA linked by dehydrogenation. According to the total ion current after reverse phase HPLC of the redissolved incubation mixture, species with *m/z* 829.8, 1021.9, and 978.0 are the three most abundant ions (Fig. 2*a*). Figs. 2, *b–g*, show the single ion chromatograms of the reverse phase HPLC elution of these and three other selected species with a *m/z* of 341.0, 1213.8, and 873.9 (mono-isotopic), respectively. All these species are singly charged, as apparent from the *m/z* difference of 1 between the first 3 ^{13}C isotopes in the partial mass spectra obtained by

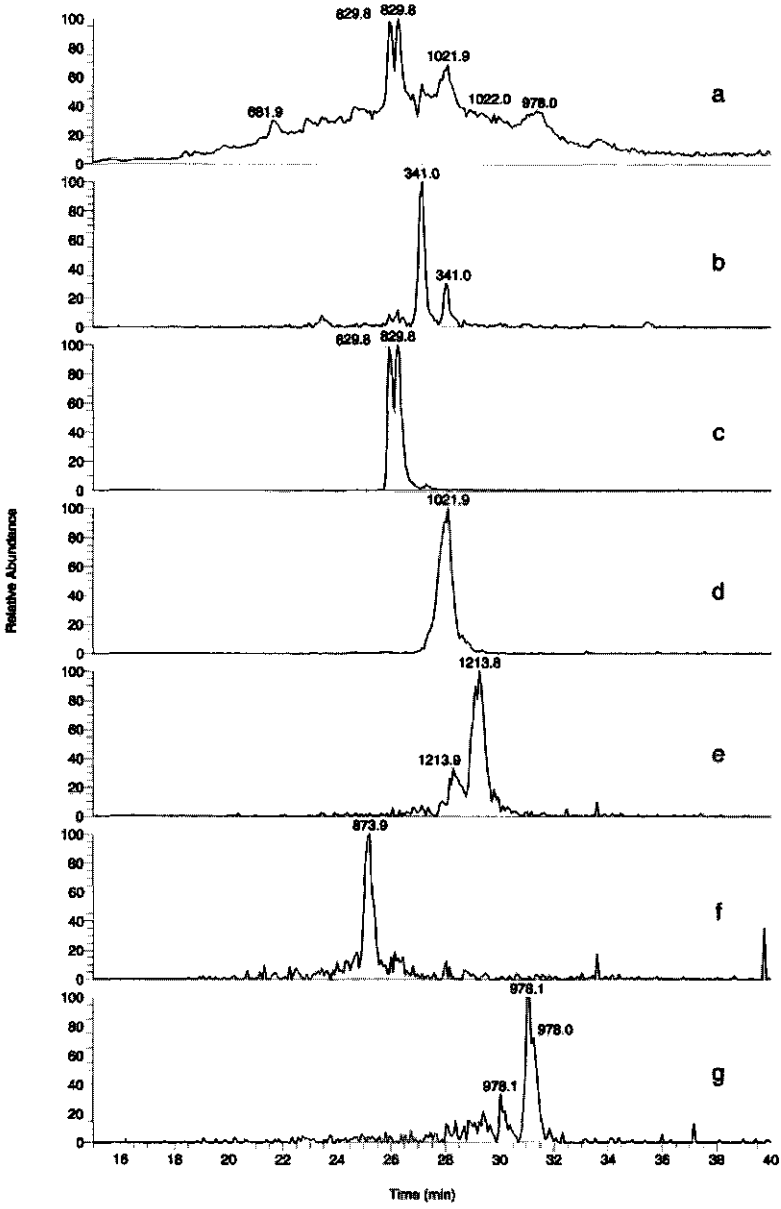

FIG. 2. **Reverse phase-HPLC-MS chromatogram of the crude incubation mixture.** *a*, total ion current. Single ion chromatograms of *m/z* species of 341.0 (*b*), 829.9 (*c*), 1021.9 (*d*), 1213.8 (*e*), 873.9 (*f*), and 978.1 (*g*).

selection of ions within 10 *m/z* units from the ion of interest (data not shown). Therefore, the molecular mass of the compounds is considered equal to the apparent *m/z* values.

The most abundant species is the ion with an apparent *m/z* value of 829.8 (mono-isotopic). The mass corresponds to a hetero-tetramer of 1 GYG linked by dehydrogenation to a decarboxylated trimer of FA. In the single ion chromatogram of this compound (Fig. 2, *panel c*), two main isomers, nearly base line-resolved, were detected. Upon collision-induced dissociation of the *m/z* 829.8 ions, positively charged product ions with *m/z* 636.0 and 441.8 result (Fig. 3, *panel c*), stemming from consecutive loss of 2 FA moieties (194 Da each). The fragment ions with *m/z* 366.8 and 339.0 originate from further dissociation of the *m/z* 441.8 ion after consecutive loss of Gly and CO from the peptide moiety, resulting in the b2 and a2 ion of the modified tripeptide, as is proved by collision of the isolated *m/z* 441.8 ion during a second stage of CID (MS^3).

The second predominant ion, *m/z* 1021.9 (mono-isotopic), corresponds to dehydrogenation linkage of another FA moiety to the hetero-tetramer with a *m/z* of 829.8 and is assigned to a

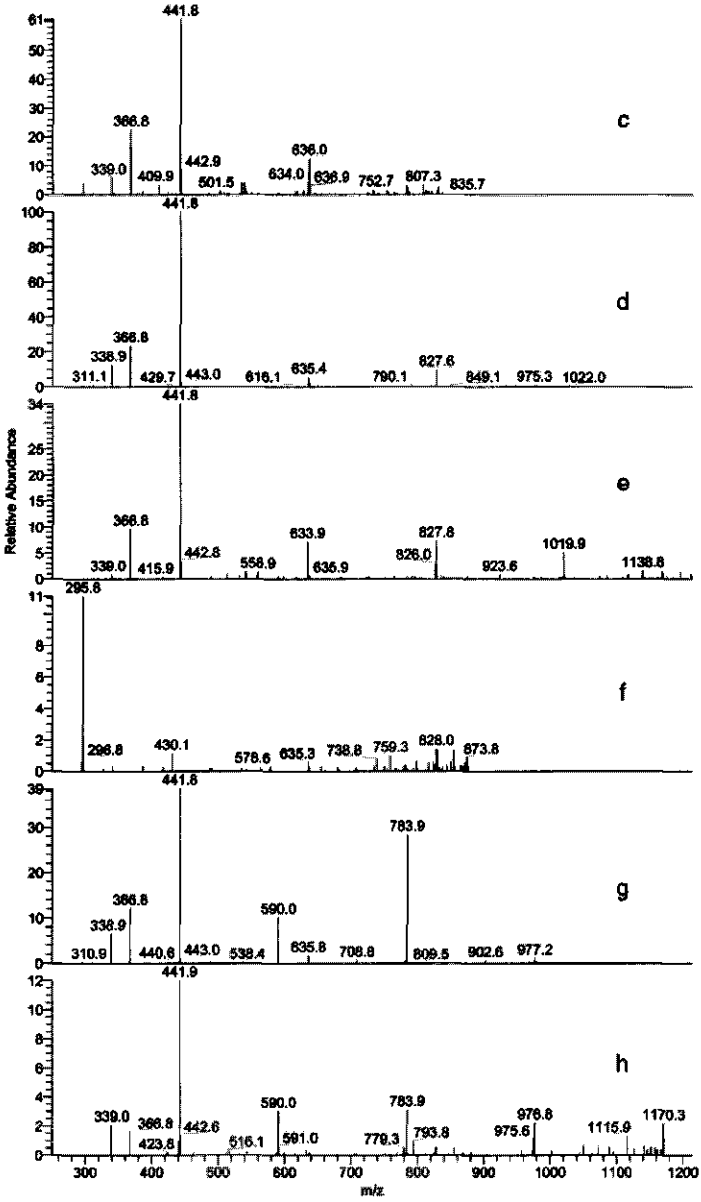

FIG. 3. **Fragmentation spectra of CID (MS^3) of *m/z* species of 829.9 (*c*), 1021.9 (*d*), 1213.8 (*e*), 873.9 (*f*), 978.1 (*g*), and 1170.3 (*h*); for convenience, the letter designating each fragmentation spectrum corresponds to the letter indicating the concomitant single ion chromatogram in Fig. 2 and therefore starts at c.**

hetero-pentamer of 1 GYG linked by dehydrogenation to 4 FA molecules, of which one is decarboxylated. In the single ion chromatogram of this compound, several co-eluting isomers were detected (Fig. 2, *panel d*). Upon CID of any of these *m/z* 1021.9 ions, positively charged product ions with *m/z* 827.6, 635.4, and 441.8 result (Fig. 3, *panel d*), stemming from consecutive losses of 3 FA moieties (194, 192, and 194 Da). A second stage of CID (MS^3) on both the isolated *m/z* 827.6 and isolated *m/z* 635.4 fragment ions results in the same *m/z* 441.8 fragment ion as resulted from CID of the hetero-tetramer, indicating the same structural element in the hetero-pentamer.

In the single ion chromatogram of the third consecutive hetero-oligomer in this series of hetero-oligomers, *m/z* 1213.8, 1 minor isomer and 2 co-eluting major isomers were detected (Fig. 2, *panel e*). These are assigned to a hetero-hexamer of 1 GYG peptide linked by dehydrogenation to 5 FA, of which 1 is decarboxylated. Upon CID of any of these isomers, fragment ions with *m/z* 1019.9 (loss of one FA, 194 Da), *m/z* 827.8 (loss of second FA, 192 Da), *m/z* 635.9 (loss of third FA, 192 Da) and predominantly fragment ions of *m/z* 441.9, 366.8, and 339.0 result (Fig. 3, *panel e*). The three "fingerprint" ions again demonstrate a constituting moiety similar to that in the preceding compounds in this series.

One higher hetero-oligomer in this series was found (Fig. 1),

and this *m/z* 1407.3 species is assigned to a hetero-heptamer of 1 GYG and 6 FA, of which 1 is decarboxylated. CID of this ionized compound predominantly yielded the fragment ions with *m/z* 825.9 (loss of 3 FA moieties) and 441.8 (loss of 5 FA moieties) and is concluded to contain the same structural element. The full ion series with *m/z* values of 829.9, 1021.9, 1214.4, and 1405.8 are assigned to GYG-FA oligomers linked by dehydrogenation containing 3, 4, 5, or 6 FA moieties respectively, each containing the same structural element consisting of a covalent adduct of 1 GYG and decarboxylated FA. The base line-resolved-eluting *m/z* 341.0 species (Fig. 2, *panel b*) are assigned to isomeric decarboxylated dehydrodimers of FA.

The third predominant ion in the total ion current, *m/z* 978.1 (Fig. 2, *panel a*), is assigned to a hetero-pentamer of 1 GYG linked by dehydrogenation to 4 FA moieties, of which 2 are decarboxylated. Several minor isomers elute separated from two major isomers that co-elute (Fig. 2, *panel g*). CID of these isomers results in fragment ions with *m/z* 783.4 and 590.0 (Fig. 3, *panel g*), corresponding to the consecutive loss of two FA moieties (194 Da). Loss of a third, decarboxylated, FA (148 Da) leads to the main fingerprint fragment ion with *m/z* 441.8 and the other two fingerprint ions with *m/z* 366.8 and 339.0 (Fig. 3, *panel g*). Therefore the core structural element is concluded to be the same as for the single fold decarboxylated series of hetero-oligomers.

The second predominant product in this ion series, *m/z* 1170.3 (Fig. 1), corresponds to dehydrogenation linkage of another FA moiety to the 2-fold decarboxylated hetero-pentamer with *m/z* 978.1 and is assigned to a hetero-hexamer of 1 GYG linked by dehydrogenation to 5 FA molecules, of which 2 are decarboxylated. In the single ion chromatogram of this compound (data not shown), several co-eluting isomers were detected at a retention time similar to that of the *m/z* 978.1 species. Upon ESI and CID of any of these *m/z* 1170 species, positively charged ions with *m/z* 976.8, 783.9, 590.0, and 441.8 result (Fig. 3, *panel h*), originating from consecutive losses of 4 FA moieties (194, 193, 194, and 148). The full series of ions starting at *m/z* 978.3 (Fig. 1) are therefore assigned to hetero-oliogomers of 1 GYG coupled by dehydrogenation with 4–9 FA moieties, of which 2 are decarboxylated.

The *m/z* 873.9 ion (Fig. 1 and the chromatogram in Fig. 2*f*) is assigned to a hetero-tetramer of 3 nondecarboxylated FA moieties and 1 GYG. This *m/z* value of this species corresponds to 2 FA molecules linked by dehydrogenation and 1 FA molecule not linked by dehydrogenation to GYG. CID of these ions (Fig. 3, *panel f*) results in different product ions as compared with the series of 1- and 2-fold decarboxylated hetero-oligomers, and the fingerprint ions from the former series are completely absent. This implies that the ion with a *m/z* of 873.9 cannot contain the *m/z* 441.9 moiety like the former series of hetero-oligomers. The main fragment ion is that with *m/z* 295.8, corresponding to the GYG ion after loss of the FA moieties. No higher hetero-oligomers containing exclusively nondecarboxylated FA moieties were found. However, two smaller nondecarboxylated hetero-oligomers, *m/z* 488 (hetero-dimer) and *m/z* 680 (hetero-trimer), were formed in minor amounts (Fig. 1). Interestingly, these compounds were main products in a previous incubation (19).

DISCUSSION

In this study it is shown that the kinetically controlled incubation of FA and GYG with peroxidase and H_2O_2 results in a complex range of (isomeric) products of different degrees of polymerization. Because isolation of these products for NMR characterization is not straightforward, mass spectrometry including a first and second stage of collision-induced dissociation was the method of choice to elucidate the range of products and their compositional structural elements.

In both the series of 1-fold decarboxylated and the series of 2-fold decarboxylated hetero-oligomers the difference in mass between the compounds was always 192 Da, a mass difference expected for dehydrogenation linkage of FA to each preceding compound in a constitutive fashion. Unambiguous assignment of the ions of the compounds to the constituting moieties of the two series of hetero-oligomers is provided by CID. Upon CID of a hetero-oligomer, the covalent linkages in the proximity of the C-C bond that connects the four aromatic moieties A, B, C, and Tyr (Fig. 4, *structure 5h e.g.*) are most susceptible to fragmentation due to the high probability of the presence of the proton in this acetal-type structural moiety. After loss of two neutral FA moieties, the 441.9 fragment results (Fig. 4, *structure* $5h_4$). Further fragmentation of this fragment (MS^3) resulted in the b2 and a2 ions (Fig. 4, *structures* $5h_6$ and $5h_7$) that further validates the assignment of the 441.9 fingerprint ion as GYG linked to the decarboxylated FA moiety. The presence of the fragment ion of 441.9 (Fig. 3, *panels c, d, e, g,* and *h*) in all MS^2 spectra of compounds of the one- or 2-fold decarboxylated hetero-oligomers and the fragments of 367 and 339 in the MS^3 spectrum of the parent mass 441.9 clearly indicates that the GYG peptide bound to the decarboxylated FA moiety (Fig. 4, all structures, *ring C*) is always one of the constituting moieties of at least 1 isomer of the 1- and 2-fold decarboxylated hetero-oligomers. Most of the other, isomeric hetero-tetramers (Fig. 6*b*, *structures 5a–h*) are unlikely to lead to the fingerprint fragment ions as outlined for isomer *5h* in Fig. 4. However, these species may well contribute to the total ion current of the compound of interest but not participate in the fragmentation pattern of a co-eluting isomer.

In contrast to the 1- and 2-fold decarboxylated hetero-oligomers, the fragmentation of the 873.9 species (Fig. 3, *panel f*) does not lead to product ions corresponding to the *m/z* 441.9 structural element (Fig. 4, *structure* $5h_4$). This nondecarboxylated hetero-oligomer results mainly in an *m/z* 295.8 product ion (Fig. 3, *panel f*), which corresponds to GYG with no decarboxylated FA moiety attached. This distinct product ion pattern was also found for nondecarboxylated FA-GYG-FA and FA-GYG in a previous study, where GYG was kept in excess over FA (19) during the incubation. Higher hetero-oligomers of this type were not found in those studies nor in the present one.

This discrepancy between the wide range of decarboxylated, further oligomerized hetero-oligomers and the limited range of nondecarboxylated, maximally tetrameric hetero-oligomers suggests two different mechanisms for the covalent attachment of FA to the tyrosine template. Under the conditions applied, the main route occurs via radical attack on the vinylic bond of the decarboxylated FA dimer (Fig. 5*a*, structures *2a* and *2b*), whereas the minor route proceeds via de- or nondehydrogenation addition of the non-decarboxylated monomeric FA radical. The main route deduced from the present data is supported by a recently proposed mechanism for the lignin peroxidase-catalyzed homo-trimerization of FA (15). This mechanism comprises an attack by a phenoxy-FA radical (Fig. 5*a*, *structure 1a*) on the vinylic bond of a decarboxylated FA dehydrodimer (Fig. 5, *structures 2a* and *2b*). The initially formed trimeric FA radical species resulting from this attack (Fig. 5*b*, *structures 3a–d*) can be oxidized and, after deprotonation, result in a FA trimer, as was authenticated by one- and two-dimensional ^1H NMR (15). Decarboxylated dehydrodimers of FA were also formed in the present HRP-mediated incubation of GYG with FA (Fig. 2, *panel b*).

Because GYG radicals are continuously present (Fig. 6*a*, *structures 4a* and *4b*) in the hetero-incubation, as follows from the presence of GYG dimers (data not shown), two routes can

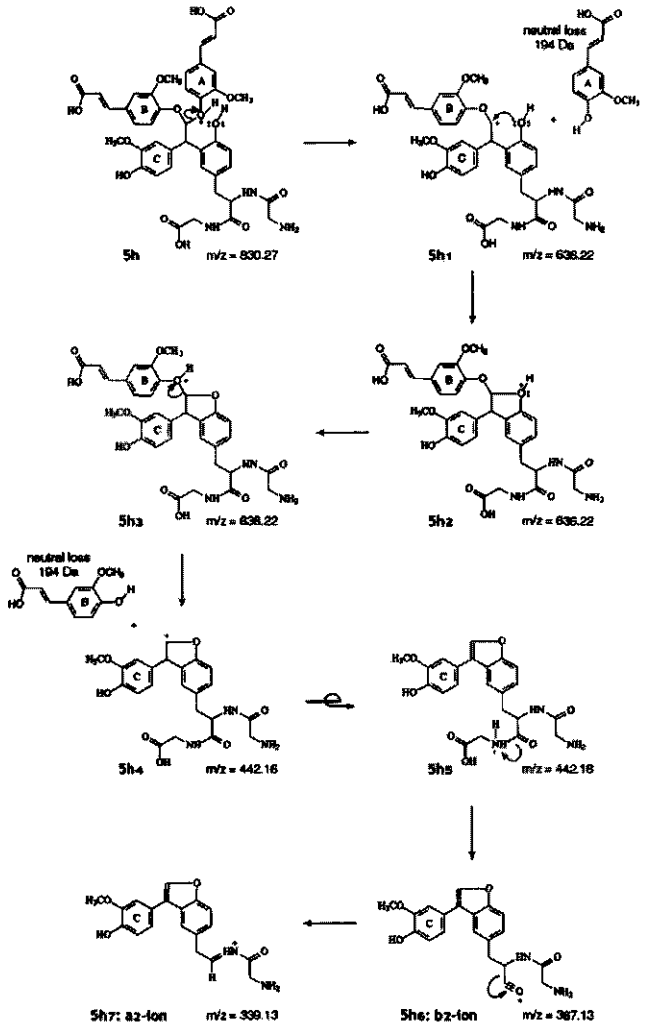

FIG. 4. **Mechanism of collision-induced dissociation of hetero-tetramer 5h.**

directly lead to a hetero-oligomer. Combination of the trimeric FA radical (Fig. 5*b*, *structures 3a–d*) with the peptidyl tyrosine radical of an oxidized GYG (Fig. 5*a*, *structures 4a* or *4b*) results in a hetero-tetramer comprising 3 FA and 1 GYG (Fig. 6*b*, *structures 5a–h*). Attack of the GYG radical on the vinylic bond of the decarboxylated FA dimer and subsequent combination of the resulting hetero-trimeric radical with another FA radical also leads to the hetero-tetramer, whereas attack of the hetero-trimeric radical on another decarboxylated FA dimer leads to a 2-fold decarboxylated hetero-pentamer. These routes, both via decarboxylated FA dimers, appear to be important in the oligomerization to higher FA oligomers on the peptide since the higher hetero-oligomers always are 1- or 2-fold-decarboxylated. Any of the 1-fold decarboxylated hetero-tetramers (Fig. 6*b*, *structures 5a–h*) or 2-fold decarboxylated hetero-pentamers evolved from this reaction path still has several sites for further oligomerization to higher hetero-oligomers.

On the basis of the similar product pattern of polymerization of FA by either Lignin peroxidase or HRP, it was suggested that FA oligomerization is governed by radical chemistry rather than enzyme specificity (15). These findings are in agreement with our data, which show that it is the chemistry of the enzymatically primarily formed radical products and especially the chemistry of the decarboxylated dehydrodimers of FA that leads to oligomerization. The GYG-FA hetero-oligomers found in the present study are clearly distinct from the ones found in a previous study (19). This shows that the type of oligomerization is strongly dependent on the substrate concentrations and kinetic control of the reaction.

Our present findings confirm the suggestion of Ward *et al.* (15) that the peroxidase-induced cross-linking of FA leads to higher oligomers than the trimers they identified by NMR. Furthermore, the attachment of higher oligomers of FA bound to a peptidyl tyrosine would supply a spatial basis for the covalent anchoring of easy diffusible small phenols on a tyrosine template in a plant cell wall protein and supports the often hypothesized template-guided *in situ* polymerization process of lignin (23–25). During growth or stress, increased levels of FA in the plant can result in differentiation of the type of tissue formed in a similar fashion as the kinetically and stoichiomet-

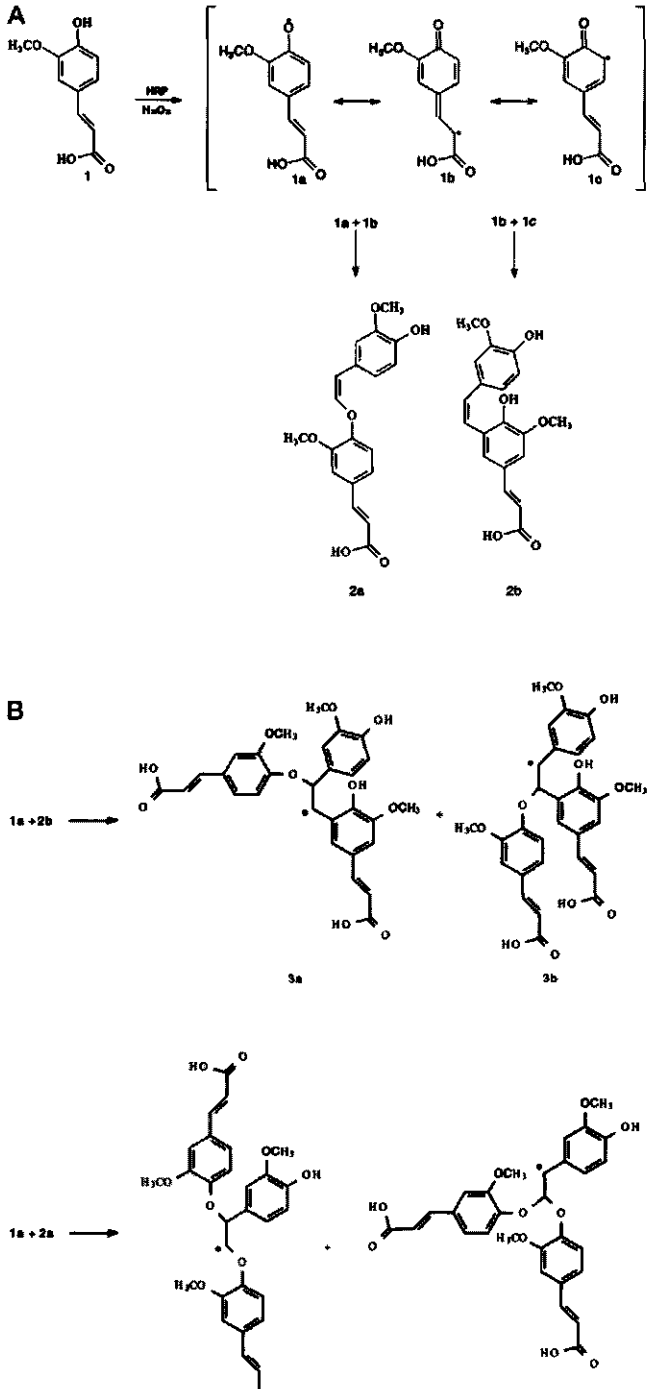

FIG. 5. **HRP-catalyzed oxidation of FA, combination of monomeric FA radicals to decarboxylated dehydrodimers, and attack of monomeric FA radicals on the vinylic bond of the decarboxylated dehydrodimers leading to trimeric FA radicals.**

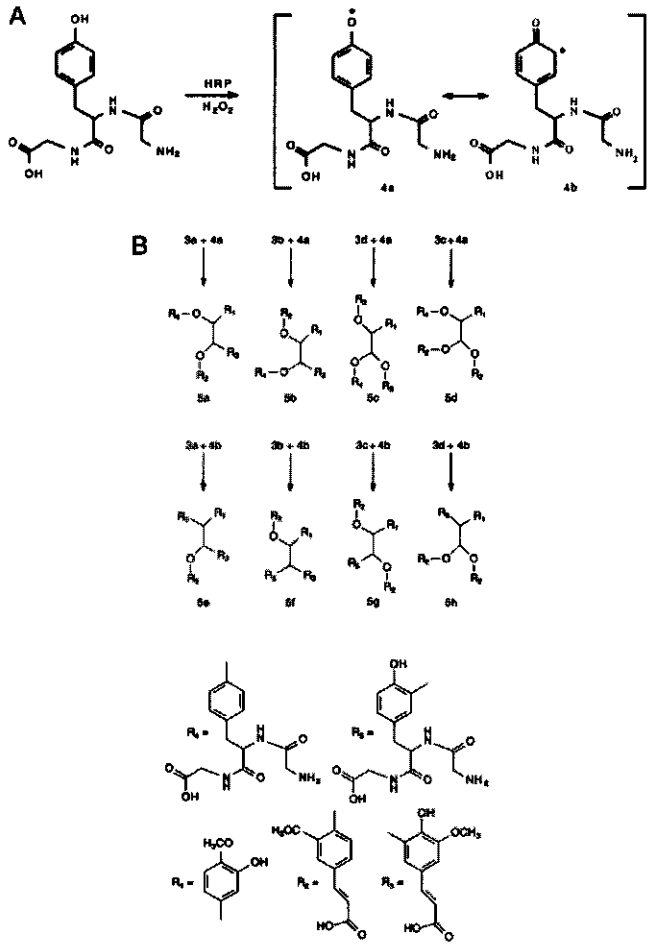

FIG. 6. *a*, HRP-catalyzed oxidation of GYG. *b*, eight possible hetero-tetrameric radicals resulting from radical combination of two isomeric GYG radicals and four isomeric trimeric FA-radicals.

rically controlled polymerization of FA described in this paper. The phenolic moiety enables oxidation of the molecule as a trigger for oxidative polymerization, whereas the vinylic moiety stabilizes the radical and allows further polymerization after decarboxylative dimerization.

Just as the peptide oligomers in a previous study always appeared to be blocked for further oligomerization by two non-decarboxylated FA molecules linked by dehydrogenation to the growing peptide oligomer (19), low concentrations of the FA monomer in plants can block possible sites for growth either on an esterified FA or on a protein-tyrosine template. High levels of FA statistically enhance dimerization to the decarboxylated FA dimers that enable further polymerization as established in the present study. The regulation of plant cell wall growth via control of the level of FA would therefore be amplified by an intrinsic feature of FA chemistry inevitably associated with FA concentration in the presence of peroxidases. We suggest that FA and possibly related cinnamic acid derivatives can exert two opposite effects on the growth of plant cell wall tissue, solely dependent on the incident concentration.

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Chapter IV

Conformation α-lactalbumin governs tyrosine coupling by horseradish peroxidase and changes during polymerization

Abstract

Tyrosine coupling of proteins is essential to function in many types of proteinaceous structural tissue. In contrast, tyrosine coupling of globular proteins can be highly undesirable and is increasingly implicated in disease. Here, the role of protein conformation on tyrosine-tyrosine coupling and protein cross-linking during incubation of α-lactalbumin with horseradish peroxidase and H_2O_2 was investigated. In the absence of the conformation stabilizing Ca^{2+}, the rate of dityrosine formation was strongly dependent on temperature. The presence of Ca^{2+} inhibited the increase in rate of dityrosine formation at higher temperatures. Incubations of α-lactalbumin in urea confirmed the direct relation between protein conformation and extent of oligomerization. Higher oligomers were obtained only at full denaturing urea concentrations. Prolonged incubation of α-lactalbumin at 40 °C in the absence of Ca^{2+} resulted in large covalently linked protein polymers. It is proposed that a distortion of α-lactalbumin conformation is induced by the initial coupling of two structured monomers, rendering the other tyrosines available for extended cross-linking.

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Introduction

Dityrosine and trityrosine cross-linkages between proteins were firstly demonstrated *in vivo* after isolation from resilin of insect cuticula (*1, 2*). These covalent interprotein bonds were supposed to stem from oxidation of the protein tyrosines by peroxidase. *In vitro* both tyrosine (*3, 4*), tyrosyl peptides (*5, 6*) and tyrosines in a limited number of proteins have been cross-linked by horseradish peroxidase (HRP) and H_2O_2 (*7-14*). Upon one electron oxidation by peroxidase and subsequent hydrogen abstraction of the phenolic hydrogen, a free tyrosine radical is formed. This radical or one of its resonance contributors can combine with another tyrosine free radical, which results in a covalent bond between the tyrosine side chains at either the oxygen or the *ortho* position of the aromatic ring.

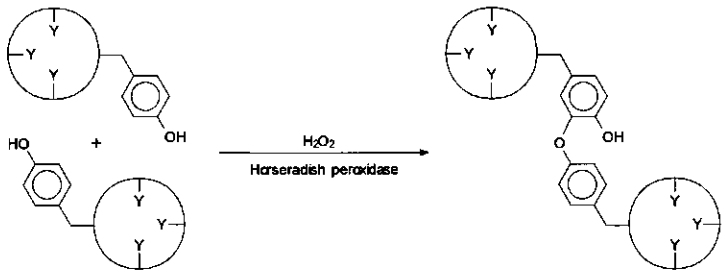

Figure 1 : Model representing HRP mediated cross-linking of two protein molecules, each with one of four tyrosines exposed, by an iso-dityrosine bond

The feasibility of this oxidative cross-linking reaction of highly structured proteins is biologically of interest since oxidative conditions are associated with many pathogenic protein aggregations (*15-19*). In addition, this reaction can be of industrial relevance in altering the properties of proteins after cross-linking. Therefore, we have investigated this cross-linking reaction pertaining to bovine α-lactalbumin in incubations with horseradish peroxidase and H_2O_2.

Bovine α-lactalbumin is a monomeric globular, lactose synthase regulating protein. The primary structure of bovine α-lactalbumin contains 123 amino acids amongst which are 4 tyrosines, at positions 18, 36, 50 and 103. The α-domain of α-lactalbumin is rich in α-helices and contains

disulfide bridges between Cys6-Cys120 and Cys28-Cys111. The β-domain is rich in β-sheet and has Cys61-Cys77 and Cys73-Cys91 disulfide bonds. Furthermore, α-lactalbumin contains a calcium binding site that is formed by three aspartate side-chains.

The conformation of α-lactalbumin governs the accessibility and solvent exposure of its 4 tyrosines. The conformation strongly depends on pH, temperature, the presence of Ca^{2+} and the concentration of unfolding agents (*19-35*). At low pH in the absence of Ca^{2+}, α-lactalbumin forms a molten globule, which radius of gyration is 10% larger than that of the native Ca^{2+}-bound protein (*36*). Upon increasing the concentration of an unfolding agent, the structure of apo-α-lactalbumin changes from native *via* the molten globule to the fully unfolded state. At neutral pH, the molten globular state of apo-α-lactalbumin can be obtained upon mild heating at 45°C. At low pH (*37-39*), elevated temperature (*38, 40*) or at low concentrations of unfolding agent like Guanidine chloride (*41, 42*) holo-α-lactalbumin also unfolds to a molten globule-type state.

Contradictory reports have appeared on the accessibility of the tyrosines in native α-lactalbumin and the molten globule. In chemically induced dynamic nuclear polarization (CIDNP) studies on native α-lactalbumin, it appeared that of the three histidine, four tryptophan and four tyrosine residues, only Tyr18, His68 and Trp118 were sufficiently exposed to react efficiently with the photosensitizer (*43-48*). However, in the molten globule state, Tyr50 (β-domain) and Tyr36 (close to the interface of the α-domain) became also accessible for labeling by the photosensitizer (*49*). In other studies, the implications of conformational changes on the exposure of amino acids in bovine α-lactalbumin to the solvent at pH2 were investigated by NMR exchange experiments. All 4 tyrosines in α-lactalbumin were found to gradually participate in the exchange event when the concentration of urea was increased. The first appearing tyrosine signal, and therewith exposure to the solvent, was that of Tyr36 at 1 M urea. Subsequent tyrosine NMR-signals were observed for Tyr50 (4 M urea), Tyr18 (5 M urea) and Tyr103 (8 M urea) (*21*).

To explore the inclination of tyrosine residues of α-lactalbumin in cross-linking by HRP and H_2O_2, we varied the conditions that have been implicated to influence the conformation of α-lactalbumin. The initial rate of tyrosine coupling and the range of formed products dependent on variations in temperature, the presence or absence of Ca^{2+} and the concentration of urea were probed.

Materials and methods

Bovine α-lactalbumin type III (Ca^{2+} depleted), horseradish peroxidase (HRP) type VIa and hydrogen peroxide were obtained from Sigma. Criterion precast gels for SDS-PAGE were obtained from Biorad. The tripeptide gly-tyr-gly (GYG) was obtained from Bachem, Bubendorf, Swiss. All other chemicals were of analytical grade. All incubations were performed in 0.1 M ammonium acetate pH 6.8. The commercial preparation of apo-α-lactalbumin contains 0.3 moles of Ca^{2+} per mole of α-lactalbumin. Therefore, a mixture of 30% holoprotein and 70% apoprotein is referred to whenever the apo-α-lactalbumin is indicated. For reconstitution of the holoprotein, 10 mM $CaCl_2$ was included in the incubation mixture. In such incubation, the protein is referred to as holo-α-lactalbumin.

Initial rate studies

Initial rate cross-linking studies were carried out in a stirred and thermostated cuvet in a Hewlett Packard 85220a photodiode array spectrophotometer. Incubations with α-lactalbumin contained 5 mg α-lactalbumin and 0.5 mg HRP (molar ratio 30:1) in a volume of 1 mL. Incubations with GYG contained 2 mM GYG and 0.05 mg HRP in a volume of 1 mL. Reactions were started and prolonged with 10μL aliquots of H_2O_2 that contained 33.3 nmol of H_2O_2. Incubations of both apo-α-lactalbumin and GYG were performed at 20, 30, 40 or 50°C in duplicate. In another series of incubations, holo- or apo-α-lactalbumin and GYG, either in the presence or absence of Ca^{2+}, were incubated at 20°C or 40°C in triplicate.

Incubations in urea

For cross-linking experiments in the presence of urea, 5 mg apo-α-lactalbumin was equilibrated in 1 mL urea containing solution overnight at 25°C. The urea concentrations were 1, 3, 3.75, 4.25, 4.75, 5.25, 6 and 8 M. After the equilibration, HRP (0.05 mg) was added followed by 10μL aliquots of H_2O_2, the first immediately after addition of HRP and a second after one hour. Samples (10 μL) withdrawn from the incubation mixtures were heated in the presence of β-mercaptoethanol prior to SDS-PAGE analysis on a 15% T precast gel. HRP activity in urea was tested by incubations of GYG monitored at 318 nm.

Monitoring and product analysis of prolonged incubations

HRP-mediated cross-linking of α-lactalbumin was monitored at 318 nm (dityrosine), 410 nm (native HRP) and 425 nm (HRP Compound I/II). The reaction was started by addition of H_2O_2. After decrease of the absorption at 425 nm to the initial value and when the absorbance at 318 nm no longer increased, a 10 µL sample for SDS-PAGE analysis was withdrawn from the incubation mixture, followed by the addition of another 5 µL aliquot of H_2O_2. Prior to SDS-PAGE, samples were heated in the presence of β-mercaptoethanol. The incubations of apo-α-lactalbumin and holo-α-lactalbumin at 40°C were analyzed on 15% T gels, the incubation of holo-α-lactalbumin at 20°C on a 10.5 – 14% gradient gel. Staining was performed with Coomassie Biosafe obtained from Biorad.

Results

Kinetic studies

In a first approach, the initial rate of formation of *ortho-ortho* tyrosine cross-links in incubations of α-lactalbumin with HRP and H_2O_2 was studied at 318 nm as a function of temperature and compared with that of GYG.

Temperature (°C)	v (apo-α-lactalbumin) [ΔmAU/s]	v (holo-α-lactalbumin) [ΔmAU/s]	v (GYG) without Ca^{2+} [ΔmAU/s]	v (GYG) in 10mM Ca^{2+} [ΔmAU/s]
20	0.10	0.08	1.58	1.29
30	0.34	n.d.*	2.39	n.d.
40	0.90	0.38	3.10	2.84
50	1.92	n.d.	3.83	n.d.

*n.d.:not determined

Table 1: Initial rate of increase in absorbance at 318 nm in incubations of GYG and α-lactalbumin at different temperatures with or without added Ca^{2+}.

From Table 1, it is clear that the augment in the increase of the rate of dityrosine formation in each 10°C interval is bigger for apo-α-lactalbumin than for GYG. The effect of Ca^{2+} binding on the initial rate of dityrosine formation was measured at 20°C and 40°C. As can be seen in Table 1, the presence of Ca^{2+} only slightly affected the rate of dityrosine formation for GYG at both 20°C and 40°C . However, Ca^{2+} addition considerably slowed down the rate of dityrosine formation in α-lactalbumin at elevated temperature (40°C).

Spectrophotometrically monitored incubations

The incubation of apo-α-lactalbumin with HRP and H_2O_2 at 40°C resulted in strong absorption changes in the near UV and visible regions. Figure 2 shows the typical course of an incubation, monitoring the HRP oxidation state (425 nm) and *ortho-ortho*-tyrosine bond formation (318 nm).

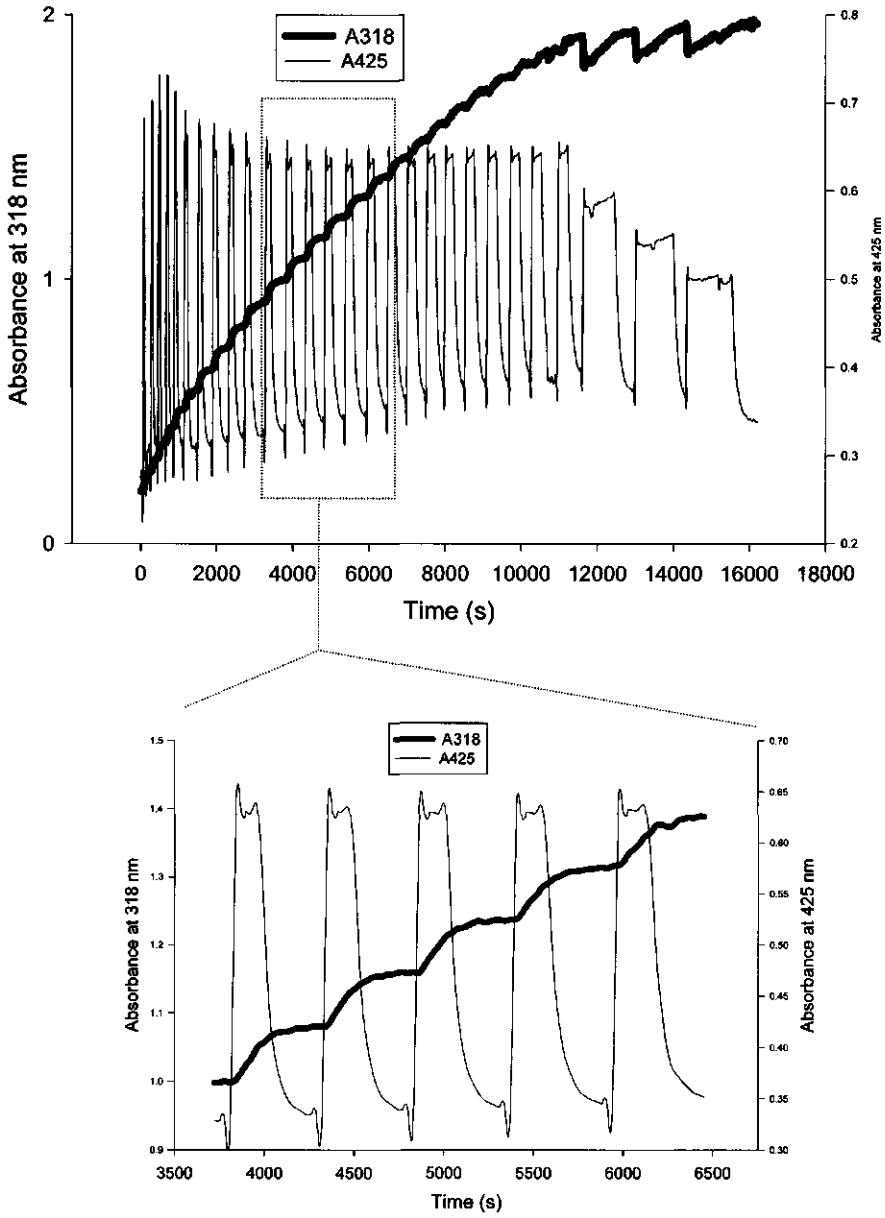

Figure 2 : Changes in absorbance at 318 and 425 nm during incubation of apo-α-lactalbumin with HRP and H_2O_2 at 40°C, below a magnification of the designated area above.

After addition of the first aliquot of H_2O_2, immediate rise of the absorption at 425 nm to a constant level was observed, indicative for formation of active HRP Compound I. Subsequently, the absorbance at 318 nm increased reaching a constant level shortly after the return of the Soret absorption to its initial level, indicative for the coincidence of dityrosine formation exclusively during the presence of iron-oxo HRP. Addition of another aliquot of H_2O_2 resulted in a repeat of this cycle (Figure 2). A detail of these absorption traces (Figure 2 below), clearly shows the relation between the activated enzyme (425 nm) and the course of dityrosine formation as monitored by the increase in absorption at 318 nm.

Similar, but drastically slower changes in absorbances were observed during the incubations of holo-α-lactalbumin at 40°C or apo- and holo-protein at 20°C. Figure 3 shows one of the characteristics, the Soret absorption of HRP, for apo-α-lactalbumin at 40°C and holo-α-lactalbumin at 20°C and furthermore HRP after addition of one aliquot of H_2O_2 in the absence of α-lactalbumin.

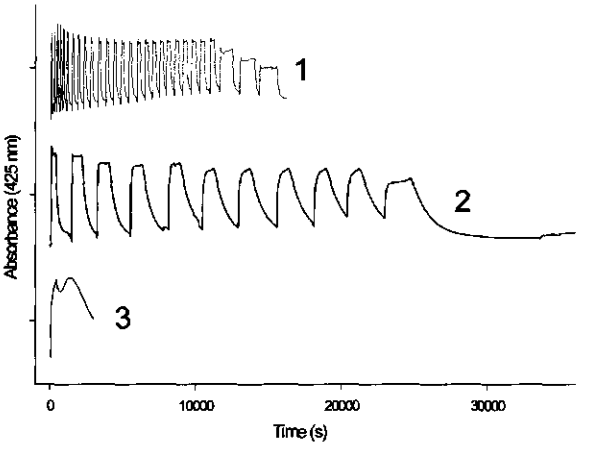

Figure 3: UV absorbance changes at 425 nm in the incubation with HRP and H_2O_2 of 1) apo-α-lactalbumin at 40°C, 2) holo-α-lactalbumin at 20°C 3) no α-lactalbumin at 40°C

Table 2 shows a few characteristics of these incubations of α-lactalbumin with HRP and H_2O_2. These characteristics are defined in terms of the average time it takes for the enzyme to return to its native state and the concomitant absorbance at 318 nm after addition of 13 aliquots of H_2O_2. The

number of 13 aliquots was chosen since this was the amount of H_2O_2 added in the slowest incubation, that of holo-α-lactalbumin at 20°C, after more than 10 hours of incubation.

	apo 40	holo 40	apo 20	holo 20
total aliquots H_2O_2	28	22	15	13
total incubation time (s)	16000	26000	36000	37000
absorbance after 13 aliquots H_2O_2	1.15	0.70	0.73	0.67
average time first 5 Soret returns (s)	200	1200	1850	2000

Table 2 : Some characteristics of the incubations of holo-α-lactalbumin and apo-α-lactalbumin with HRP and H_2O_2 at 20°C and 40°C

In the incubation of holo-α-lactalbumin at 40°C, the absorbance at 318 nm (dityrosine) did only reach a value of 0.7 after the addition of 13 aliquots of H_2O_2 and more than 4 hours of incubation. With the apoprotein at 40°C, the absorption at 318 nm was over 1.0 AU after the same amount of H_2O_2 added, after an incubation period of less than 1.5 hours. Moreover, based on the absorbance changes at 425 nm, the average time for the active enzyme to return to its initial state increased in the incubation of the holoprotein at 40°C compared to the apoprotein at 40°C, from 200 s for apo-α-lactalbumin to 1200 s for holo-α-lactalbumin.

In the incubation of holo-α-lactalbumin at 20°C a value of 0.7 AU for the absorbance at 318 nm was only reached after about 10 hours of incubation. Incubation of apo-α-lactalbumin at 20°C showed a similar time-course for the absorption changes at 318 and 425 nm as for holo-α-lactalbumin (Table 2). No increase of absorbance at 318 nm was observed throughout the incubation of HRP with 1 aliquot of H_2O_2 in the absence of α-lactalbumin (Figure 3, trace 3). The Soret absorbance at 425 nm decays, but slower than in all incubations in the presence of α-lactalbumin, proving that under all conditions applied, it was α-lactalbumin that reduced the active enzyme.

SDS-PAGE monitoring

In order to relate the absorbance changes to the formation of products, samples were withdrawn from the incubations of α-lactalbumin and analyzed by SDS-PAGE. Figure 4 shows the incubation of apo-α-lactalbumin with HRP and H_2O_2 at 40°C.

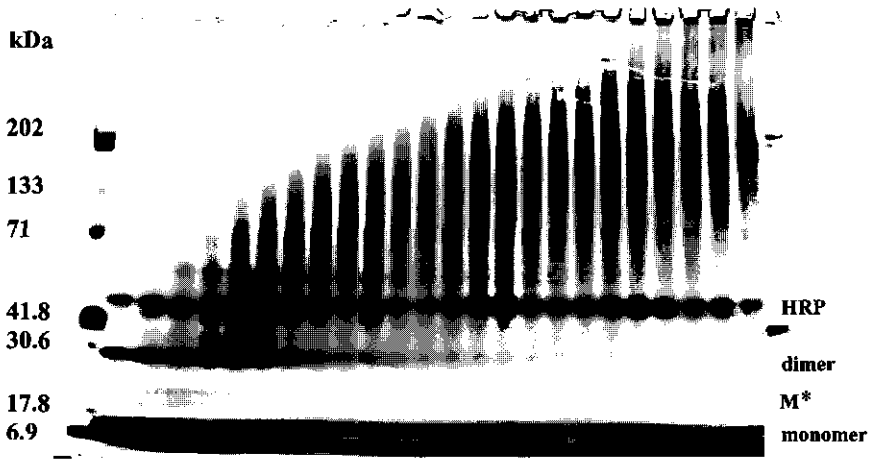

Figure 4: SDS-PAGE analysis of the incubation of the apo-α-lactalbumin with HRP and H_2O_2 at 40°C, left lane t=1 progressive to the end of the incubation in the right lane (t=200 min)

From Figure 4 it can be seen that the α-lactalbumin monomer (apparent molecular mass 14.4 kDa) disappears with time. Furthermore, in the first part of the incubation, a relatively high amount of α-lactalbumin-dimer is formed. This dimer is gradually transformed into higher molecular mass species. At the end of the incubation no α-lactalbumin dimer is present and only a small amount of monomer is left. Instead, very high molecular mass species are observed, some of which can even not enter the gel. Apart from the appearance of dimeric α-lactalbumin additional, minor, distinct protein bands (M*,D*) with an apparent molecular mass between 17 and 37 kDa are observed in the first part of the incubation.

SDS-PAGE analysis of the incubation of holo-α-lactalbumin with HRP and H_2O_2 at 40°C (Figure 5) shows that in this case most of the monomeric protein persists until the end of the incubation. In this case, only small amounts of dimer and higher polymers are formed. No polymerization beyond 200 kDa takes place, in contrast to what is observed for the

apoprotein at 40°C. Again minor additional protein bands (M*) are observed between 17 and 37 kDa, in this case persisting longer than those in the incubation of apo-α-lactalbumin at 40°C.

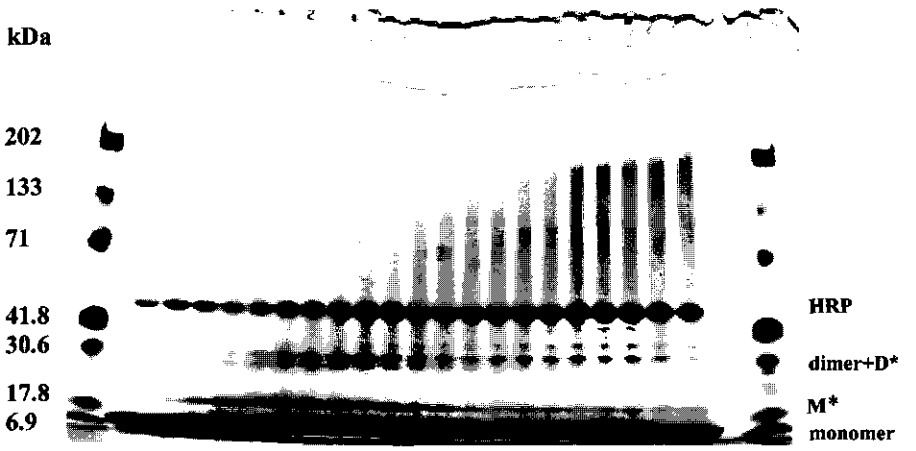

Figure 5: SDS-PAGE analysis of the incubation of the holo-α-lactalbumin with HRP and H_2O_2 at 40°C, left lane t=0 progressive to the end of the incubation in the right lane (400 min)

When holo-α-lactalbumin is incubated at 20°C, nearly all monomer persists after 10 hours of incubation (Figure 6). Almost no cross-linking occurs and only small amounts of dimer and oligomers are formed. In this case the pattern of minor protein bands observed between 17 and 45 kDa (M* and D*, respectively) is more intense and in contrast to the incubations at 40°C, these products persist throughout the incubation. A clear protein band with an apparent molecular mass of 20 kDa is observed, indicative for the formation of a modified monomeric species (belonging to M*). The incubation of apo-α-lactalbumin at 20°C (data not shown) resulted in a SDS-PAGE pattern similar to that of the holoprotein at 20°C (Figure 6).

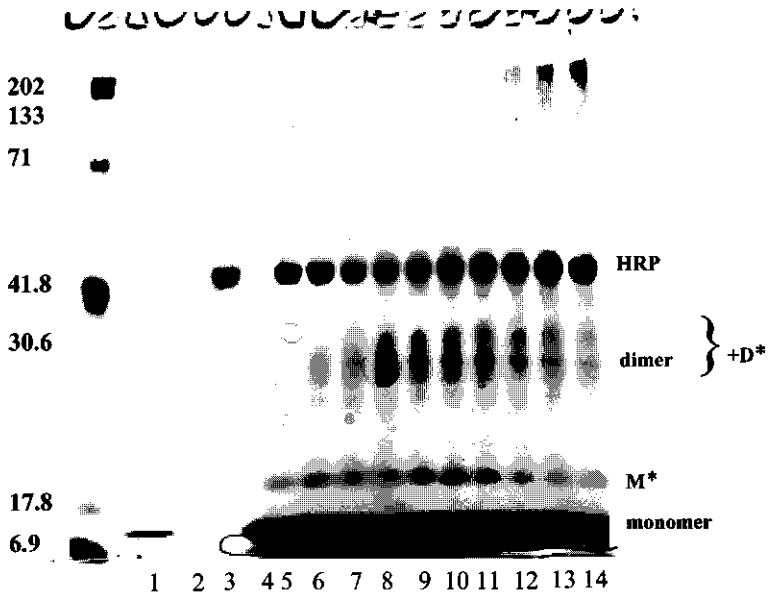

Figure 6: SDS-PAGE analysis of the incubation of holo-α-lactalbumin with HRP and H_2O_2 , at 20°C. Lane 1:α-lactalbumin, lane 3: HRP, lanes 5-14, monitor of incubation from t=0 (lane 5) to t=10 h

To study the effect of urea-induced unfolding on the extent of cross-linking, apo-α-lactalbumin equilibrated in different concentrations of urea was incubated for 2 hours with a catalytic amount of HRP and H_2O_2 at 25°C and analyzed by SDS-PAGE (Figure 7). Lanes 1-5 show that up to 5 M of urea only the amount of M*, dimer and possibly trimer is increased. Above 5 M urea (lanes 6-8) an increasing amount of products with a molecular weight exceeding that of trimeric α-lactalbumin is observed. The incubation at 8M urea (Figure 7, lane 8) shows protein species of higher molecular mass comparable to that seen in the incubation of the apoprotein at 40°C after addition of the same amount of H_2O_2 (cf. Figure 4, Lane 4).

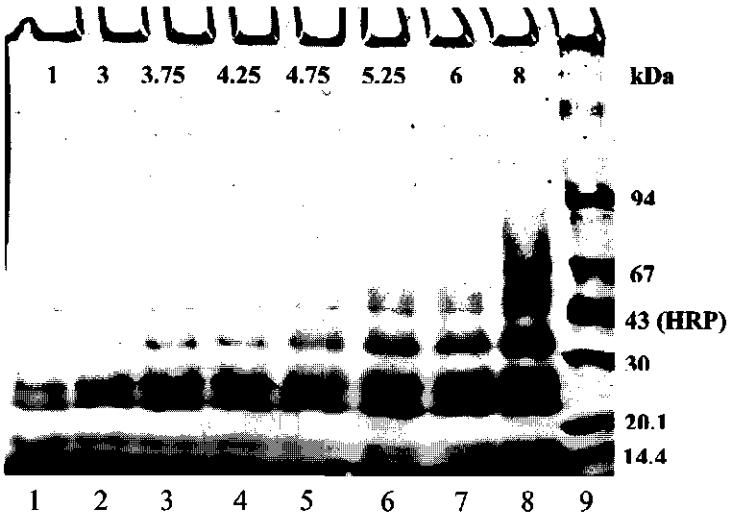

Figure 7: SDS-PAGE analysis of the 2h incubation of apo-α-lactalbumin at 25°C in the presence of urea ranging from 1M (left lane) progressively to 8 M (right lane) as indicated in each lane

Discussion

In this study we have described the cross-linking of α-lactalbumin by HRP and H_2O_2. The extent of dityrosine formation and cross-linking was shown to be strongly dependent on factors affecting protein structure. Moreover, the results also indicate that the conformation of α-lactalbumin changes as a consequence of the cross-linking reaction.

The initial rates of increase in absorption at 318 nm as listed in Table 1 give an indication of the actual rate of dityrosine formation if assumed that the type of dityrosines initially formed –i.e. absorbing *ortho-ortho* bonds or non absorbing oxygen-*ortho* bonds- are not affected too strongly by temperature. Conformational changes of the protein might cause an altered proportion of *ortho-ortho*-dityrosine and isodityrosine or trityrosine formation. Since di- and tri-tyrosine bonds also contribute differently to the absorption at 318 nm, the rate of dityrosine formation can be underestimated. An example of the influence of protein structure on tyrosine coupling evolved from the study of the fertilization envelope of sea urchin embryo (*50*). The molar ratio of dityrosine/trityrosine/pulcherosine in the tissue was 100/38/24, whereas in an incubation of free tyrosine with HRP and H_2O_2 this ratio was 100/3/8. Although the absorption increase at 318 nm might underestimate non-absorbing tyrosine bonds, it does reflect absorbing tyrosine couplings and is therefore a convenient tool to study tyrosine accessibility.

Incubations with GYG were performed to distinguish temperature effects from protein conformational effects. Cross-linking of α-lactalbumin strongly depends on the incubation conditions, i.e. the α-lactalbumin conformation. The overall increase in rate of dityrosine formation for α-lactalbumin from 20°C to 50°C of 19.2 is about 8-fold higher than that of 2.4 for GYG. Clearly, the effect of temperature on protein conformation and concomitant availability of tyrosine for the coupling reaction is demonstrated by this difference in temperature-effect between α-lactalbumin and GYG.

The increased 'availability of α-lactalbumin tyrosine' might well be an increased availability of tyrosine 36 only, since at 40°C the other tyrosines of the apoprotein are still buried in the interior of the protein in analogy to the findings of Dobson et al (*21*). The presence of Ca^{2+} has a somewhat stronger effect on the initial rate of dityrosine formation at 40°C than at 20°C . At 20°C the apoprotein forms dityrosine at a rate only a factor of 1.07 faster than holoprotein whilst at 40°C the apoprotein reaction is about 1.5 times

faster than that of the holoprotein. Possibly, binding of Ca^{2+} in the Ca^{2+}-binding site affects the exposure of the tyrosine responsible for the initial (coupling) rate to some extent, but mainly tyrosines that would participate in the cross-linking reaction of a dimer or oligomer after the first coupling via the best accessible tyrosine has taken place (*vide infra*).

Monitoring the incubation of α-lactalbumin with HRP and H_2O_2 shows an immediate formation of the HRP active form after addition of H_2O_2. Subsequently, the absorption at 318 nm increases linearly and when the enzyme falls back to its initial absorption, the increase of absorption at 318 nm decays rapidly (Figure 2). The rate at which HRP compound I is formed upon each addition of H_2O_2 is nearly constant throughout the incubation and independent of the incubation conditions. However, the time for the enzyme absorption to fall back to its initial absorbance increases, which is also observed in prolonged incubation of GYG with HRP and H_2O_2, and is caused by lowered concentration of substrate. The steepness of the absorbance at 318 nm over the full time interval in the incubation of the apo-protein is, however, significantly higher than that in a similar incubation with GYG, during which the absorbance at 318 nm flattens much faster. Moreover, when the stoichiometry of the HRP catalyzed reaction of the consumption of 1 molecule of H_2O_2 for the oxidation of two tyrosines is taken into account, a salient discrepancy is observed between the amount of H_2O_2 added and the apparently available hydrogen donating substrate to bring the enzyme back in its native form. Theoretically, 5-6 aliquots of H_2O_2 (33.3 nmol/aliquot) would be approximately sufficient to oxidize 350 nmol of α-lactalbumin if one tyrosine is accessible. In the incubation of the apoprotein at 40°C, the enzyme is still rapidly reduced to its native form after 25 aliquots of H_2O_2 and then decays upon further addition of H_2O_2. This amount of H_2O_2 corresponds to the oxidation of all 4 tyrosines in α-lactalbumin. The temperature of 40°C is still below the melting temperature of α-lactalbumin and also below that for the molten globule of the Ca^{2+} -depleted form.

Dobson et al (*21*) reported that in the molten globule of α-lactalbumin at pH 2 the first tyrosine (Tyr36) becomes only exposed after the addition of 1M urea. This suggests that during the incubation of α-lactalbumin at 40°C, the conformation of α-lactalbumin changes due to the cross-linking reaction. The stoichiometry of H_2O_2 and α-lactalbumin therefore circumstantially proves a distortion of the conformation of α-lactalbumin caused by the cross-linking. This is further supported by the incubations in the presence of urea.

In the series of incubations with increasing concentrations of urea the influence of protein structure on the initial product formation is demonstrated. At urea concentrations >5M, significantly increased oligomerization is observed, which is attributed to the unfolding of the protein and the availability of additional tyrosines. The urea experiment demonstrates that higher oligomers are formed, also after addition of only several aliquots of H_2O_2, whenever additional tyrosines are available. An equal amount of H_2O_2 was added at all urea concentrations. Therefore, it can be concluded that if all tyrosines in α-lactalbumin would be available, an oligomerization pattern as in the last lane (8M urea) would be observed. In addition, the incubations at urea concentrations <5M demonstrate clearly that, if the structure of the protein prevents tyrosine availability, no higher oligomers are formed at all. This is illuminative information to bear in mind when evaluating the high oligomers formed in the incubation of the apoprotein at 40°C.

In the incubation of the apoprotein at 40°C (Figure 6), the initial product after the first addition of an aliquot of H_2O_2 is a dimer and not a range of higher oligomers. If the protein under these conditions already had more than one exposed tyrosine, the products would rather look like that of incubation in the presence of >5M urea. The oligomerization of the apoprotein at 40°C in the absence of urea shows a significant different progress of oligomer formation than that in the presence of 8M urea. This is attributed to distortion of the conformation of the initially formed dimer, which fully disappears in the course of the incubation. Therefore, we suggest that after dimer formation, additional tyrosines become more easily available for cross-linking. Decreased conformational stability of dityrosine cross-linked proteins were also found with calmodulin, bovine pancreatic ribonuclease A and bovine eye-lens γB-crystallin (*12*). The tertiary structures of these dimerized proteins appeared to be largely maintained. However, in thermal denaturation studies, the melting temperatures and cooperativity of the melting process were changed. The T_m of the dimer of RNase A was lowered from 60 °C to 54 °C and GdmCl induced denaturation started at 2.6 M instead of 3 M for the monomer. Furthermore, the activity of the dimer was lowered by 25%. For calmodulin, a similar effect was found for the dimer as compared to the monomer but only in the absence of Ca^{2+}, which is known to stabilize the structure of calmodulin. For γB-crystallin, the T_m of the dimer was 59 °C, whilst the monomer denaturated at 66°C and the dimer precipitated out of solution at 62 °C, while the monomer coagulates only beyond 72 °C. Altogether, a similar trend for the three

proteins was observed, lowered conformational stability of dityrosine coupled dimers.

The incubation of holo-α-lactalbumin at 40°C does not show the formation of a distinct dimer as for the apoprotein (Figure 4). The initial availability of the first tyrosine for cross-linking is apparently impeded by the binding of Ca^{2+}. In deamidation studies, modification of α-lactalbumin was also demonstrated to be influenced by Ca^{2+} binding. Enzymatic deamidation of α-lactalbumin by protein-glutaminase at 42°C appeared to be strongly dependent on the presence of Ca^{2+} (*51*). In the presence of Ca^{2+}, the degree of deamidation was 20% after 4 h and 55% after 24 hours, whilst in the absence of Ca^{2+} 61% deamidation was reached after 4 hours and 66% after 24 hours. In the present incubation of holo-α-lactalbumin at 40°C , the formation of some higher oligomers is yet observed but starts only after addition of 5 aliquots of hydrogen peroxide.

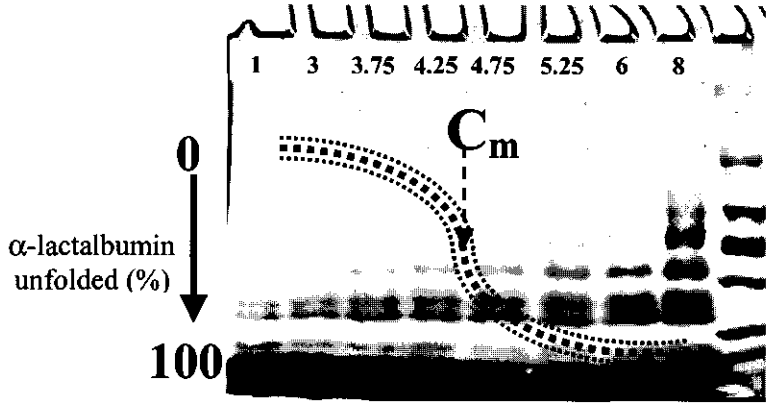

Figure 8: Unfolding curve of apo-α-lactalbumin superposed on SDS-PAGE urea-incubations

Apparently, even the holoprotein has a statistically determined availability of tyrosines i.e. after sufficient time, some unfolding always occurs, despite of the overall (>99%) preferred conformation. This effect is even stronger for the holoprotein at 20°C. The new band appearing in SDS-PAGE just above the monomer (Figure 6) is attributed to intramolecular dityrosine formation, in accordance with some increase in A318, and the low amounts of higher oligomers observed over a drastically longer incubation period.

Taken together, we suggest that the HRP-mediated oxidative cross-linking of α-lactalbumin involves conformational changes after

dimerization, leading to an altered conformation of the cross-linked oligomer (Figure 9). Due to this altered conformation of the oligomer, tyrosines that are initially not available for cross-linking become oxidized and can react with other tyrosines, intra- or inter-molecularly. This explanation is further supported by the incubations performed in the presence of urea (Figure 8). A drastic change in cross-linking products is observed after the C_m, which is known from literature to mark the concentration of urea at which the conformation of α-lactalbumin is drastically changed. An extended cross-linking of α-lactalbumin must therefore be the result of conformational changes.

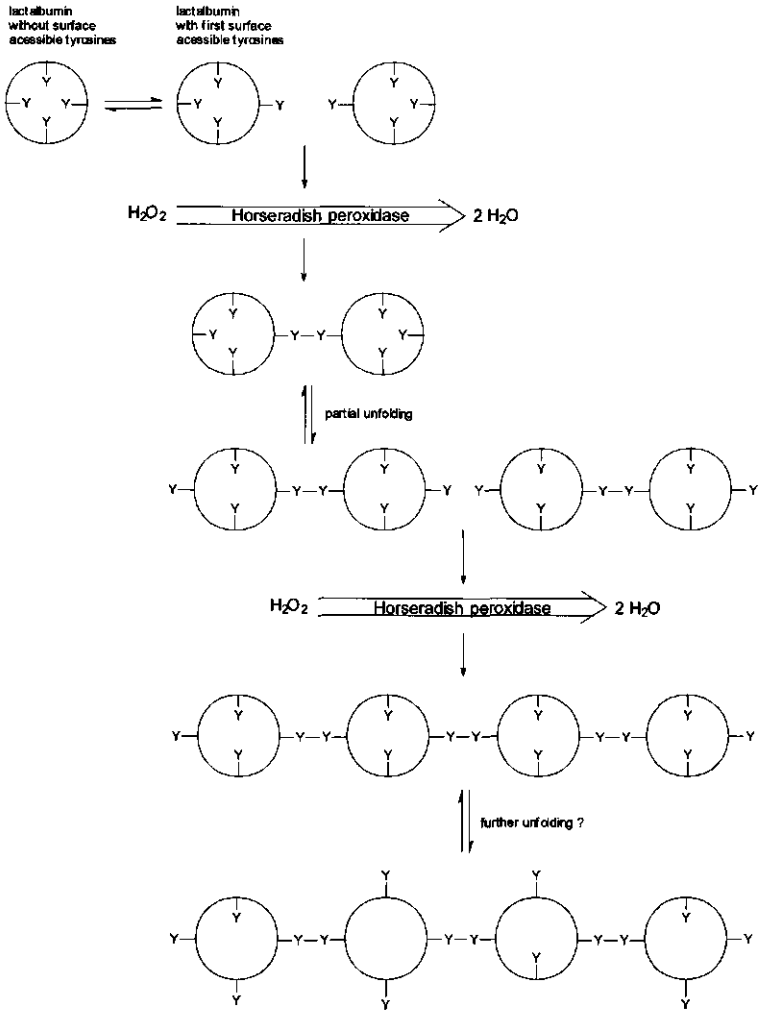

Figure 9: Proposed pathway of polymerization via tyrosine coupling

Oxidative protein oligomerization and the presence of dityrosines is increasingly implicated in the pathogenesis of many diseases involving protein aggregates. α-Lactalbumin has no biological function relating to structural integrity and has certainly no biological purpose to form highly polymerized aggregates. About the role of tyrosine coupling in proteins that

have no role in the organisms physiological structure little has been reported. Whereas tyrosine coupling in structurally important tissues has a clear biological function, tyrosine coupling in globular proteins that have a function in other processes might be an undesired side reaction in organisms that rely on oxidative processes. The present studies demonstrate that under physiological relevant conditions, peroxidase and H_2O_2 can stimulate the polymerization of α-lactalbumin. Therefore, the example provided here with the highly structured α-lactalbumin might contribute to the explanation of the occurrence of biologically unintended protein aggregates by oxidative damage *in vivo*.

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Chapter V

Ferulic acid traps intermediate oligomers during horseradish peroxidase catalyzed tyrosine cross-linking of α-lactalbumin

Abstract

Ferulic acid (FA) is abundantly present in many types of plants and indisputably plays a role in plant cell wall architecture *via* oxidative polymerization. FA makes part of a broad class of phenolic anti-oxidants of which the role in oxidative processes is increasingly explored. In this study we have investigated the effect of the presence of FA in horseradish peroxidase catalyzed oligomerization of α-lactalbumin. After kinetically controlled addition of FA, all FA became coupled with α-lactalbumin or α-lactalbumin-oligomers during the incubation. The degree of polymerization of α-lactalbumin was reciprocal to the incident concentration of free FA; in the presence of high concentrations of FA, mainly α-lactalbumin dimers were formed. In the presence of low concentrations of FA, polymerization of α-lactalbumin proceeded to a large extent like in the absence of FA. We suggest a role of FA to prevent oxidative protein polymerization not by scavenging radicals solely, but also by blocking sites for further polymerization. A tentative model for the conversion of polymerizable α-lactalbumin to non-polymerizable α-lactalbumin by FA is presented.

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Introduction

Oxidative modification of protein tyrosines is involved in many biological processes (*1-7*). Protein tyrosines can be oxidized by peroxidases yielding proteinyl tyr free radicals that can combine with other free radicals. Proteins have been demonstrated to be cross-linked by such mechanism *via* dityrosine bridges (*8-13*).

Ferulic acid (FA), a phenol that is an excellent substrate for most peroxidases, is found to be esterified to certain polysaccharides. The suggestion that tyrosine and FA might shape covalent bridges in protein-carbohydrate hetero-adducts was first made by Neukom and Markwalder (*14*). Such tyrosine-FA adducts, however, have never been demonstrated to occur *in vivo*. Attempts to cross-link feruloylated arabinoxylans and proteins with peroxidase, or laccase *in vitro* were reported to be unsuccessful (*15*). Recently, we reported the horseradish peroxidase (HRP) catalyzed *in vitro* formation of covalent adducts between the tyrosine of the tripeptide Gly-Tyr-Gly (GYG) and FA (*16, 17*) (Figure 1).

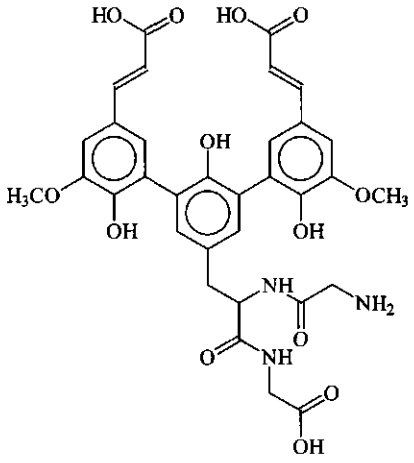

Figure 1: Peptidyl tyrosine coupled with 2 ferulic acid molecules (*16*)

It was shown that kinetic control of the incubation is crucial to obtain adducts of FA with the peptide. FA is oxidized at least three orders of magnitude faster than peptidyl tyrosines by HRP/H_2O_2 (*18*). This difference in rate of oxidation of the tyrosyl compound and FA has to be taken into account throughout the course of an incubation, intended for hetero-adduct

formation (*16*). In experiments with an excess of GYG, covalent adducts of 1 or 2 FA molecules with monomeric to pentameric peptides were synthesized. FA was concluded to terminate further oligomerization of GYG by covalent coupling to the peptidyl tyrosine and therewith blocking the attachment of another peptidyl tyrosine to the tyrosine. In contrast, when FA was added in excess, covalent adducts of 3 to 9 ferulic acid molecules with the peptidyl tyrosine were formed (*17*). In homo-incubations of α-lactalbumin with HRP and H_2O_2, the holoprotein hardly participates in dityrosine formation, whilst the apoprotein is fully converted into high molecular mass polymers at 40°C (Chapter IV).

In the present study, based on the homo-polymerization of apo-α-lactalbumin at 40°C, FA was added at different rates during the incubation of apo-α-lactalbumin with HRP and H_2O_2. By varying the addition rate of FA, it was investigated whether FA can be covalently attached to the protein and what effect the kinetic control of FA addition has on the type of polymers formed.

Materials and methods

Bovine apo-α-lactalbumin type III (70% Ca^{2+} depleted), HRP type VIa, ferulic acid and hydrogen peroxide (H_2O_2) were obtained from Sigma. Criterion precast gradient gels (4-20%) for SDS-PAGE were obtained from Biorad. All other chemicals were of analytical grade. All incubations were performed in 0.1 M ammonium acetate, initial pH 6.8.

Automated modification of α-lactalbumin with FA

Automated incubations of α-lactalbumin with HRP and H_2O_2 with kinetically controlled addition of FA were performed with a home-built rapid-mixing apparatus, equipped with 500 µL gastight Hamilton syringes, HPLC valves (Valco) and PEEK tubing (Upchurch Scientific) to connect the syringes with the reaction vessel. The emptying of the syringes was controlled by 3 software driven engines. These engines were programmed separately to give 100 ms pulses pushing the content of the syringes into the incubation mixture at variable time intervals. The syringe containing the HRP stock solution was programmed to compensate the concentration of HRP for increase in volume of the incubation mixture, keeping the total, active or inactivated, concentration of HRP constant. The syringe containing H_2O_2 (25 mM) was programmed in each incubation to supply the enzyme with sufficient H_2O_2 to oxidize, theoretically, all tyrosines of α-lactalbumin as well as the total amount of added ferulic acid. The third syringe either contained 0.1 M ammonium acetate (blank, homo-incubation of α-lactalbumin) or a stock solution of FA in ammonium acetate. A homo-incubation under addition of 0.1 M ammonium acete was carried out prior to the hetero-incubations with FA. The conditions for the hetero-incubations were based on the results of size exclusion chromatography analysis of this homo-incubation. For higher concentrations of FA, FA was dissolved in 0.5 – 1 M ammonium acetate under gentle heating. Stock solutions of FA (4/8/16 mM) were added to the incubation by 100 ms pulses from the FA-syringe (<1 droplet). The concentration of α-lactalbumin in the reaction vessel at the start of the incubation was always 700 µM and that of HRP 20 µM, in a total volume of 1 mL. All three engines were programmed so that the addition of pulsed aliquots was fast at the start of the incubation and gradually decayed to zero at the end, taking into account the decrease in available α-lactalbumin. Throughout the incubation, 100 µL samples were withdrawn and analyzed by size exclusion chromatography.

Size exclusion chromatography was performed by injection of 100 µL of the incubation mixture onto a Superdex 75 HR 10/30 column, running in 0.1M ammonium actetate at a flow rate of 0.5 mL min^{-1} connected to an Åkta purifier chromatography system at 20°C. The eluate was monitored at 277 and 318 nm.

Spectroscopically monitored incubations of α-lactalbumin and FA

Incubations that were performed by manually adding 10 µL FA-aliquots from stock solutions of either 40, 120, 240 mM FA, were performed in a stirred cuvet at 40°C in 1 mL total volume containing 5 mg α-lactalbumin and based upon monitoring the reaction at 318 and 425 nm. In these incubations an aliquot of FA was added whenever the absorbance at 318 nm increased (dityrosine formation). An aliquot of H_2O_2 was added when the Soret absorption of HRP at 425 nm had returned to its initial level. The concentration of the additional aliquots of H_2O_2 was 25 mM. Throughout the incubations, 10 µL samples were withdrawn after each return of HRP to its resting state, and analyzed by SDS-PAGE. Samples were mixed 1:1 with reducing sample buffer and heated for 1 hour at 85°C prior to electrophoresis. Gels were run at 10 V overnight. Staining was performed using Coomassie Blue R-250 Bio-safe stain (Bio-Rad).

Results

Size exclusion chromatography analysis of the automated incubations

To investigate at what stage of the incubation of α-lactalbumin addition of FA would be germane, the cross-linking of α-lactalbumin in the automated incubation without addition of FA was followed by analyzing samples withdrawn from the homo-incubation at 0, 10, 50, 100 and 250 min by size exclusion chromatography (Figure 2).

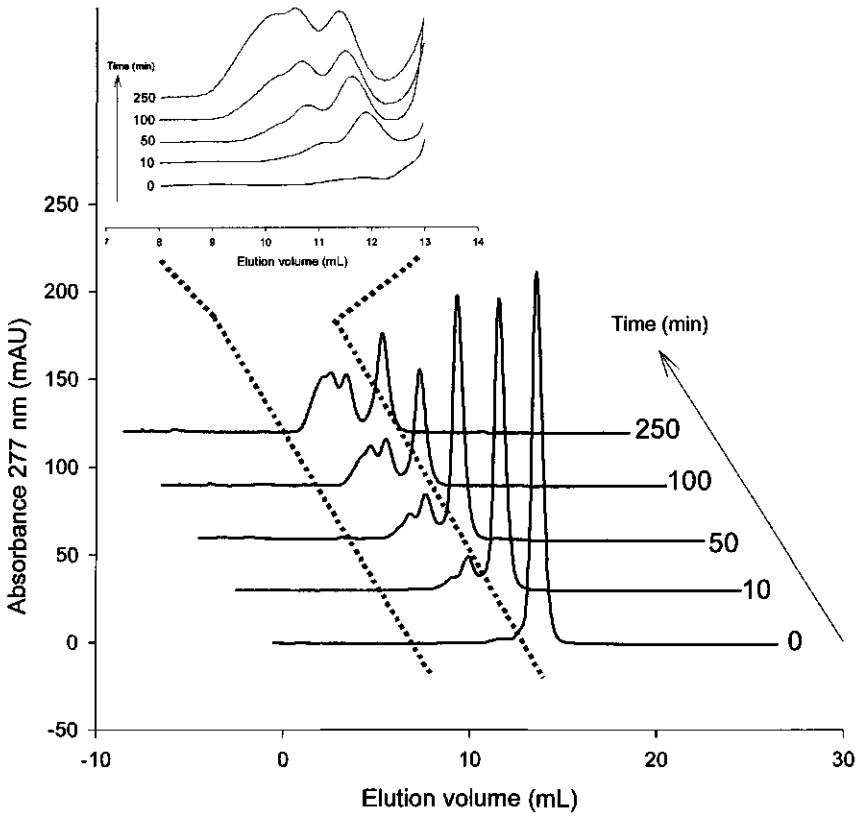

Figure 2: Size exclusion chromatograms of samples withdrawn from the homoincubation of α-lactalbumin. The monomer elutes at 14 mL, newly formed oligomers between 9 and 13 mL.

Clearly, the majority of the monomer is converted into higher oligomers in the homo-incubation. Figure 3a shows the size exclusion chromatogram of the homo-incubation of α-lactalbumin with HRP and H_2O_2 after 5 hours when monitored at 277 and 318 nm.

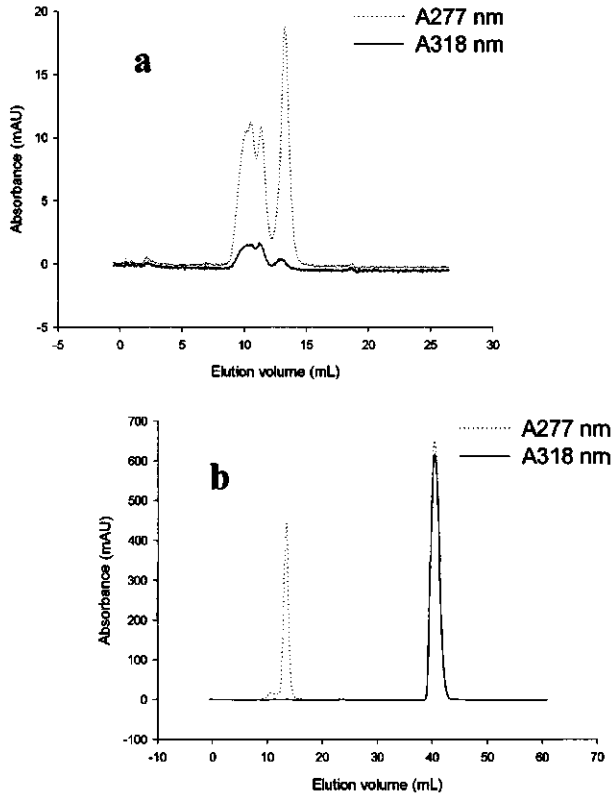

Figure 3 : Size exclusion chromatograms of (a) homo-incubation of α-lactalbumin with HRP in the presence of H_2O_2 after 5 h and (b) α-lactalbumin and FA incubated with HRP in the absence of H_2O_2 after 6 h.

The absorbance at 318 nm stems from dityrosine formation. The ratio of A277/A318 for the cross-linked α-lactalbumin (elution volume 9-13 mL) after the homo-incubation is approximately 5, which is of use to distinghuish the contribution of attached FA to this ratio in hetero-incubations (*vide infra*). When FA was added over a 6 hour period to an incubation of α-lactalbumin with HRP in the absence of H_2O_2, no protein cross-linking

products were observed (Figure 3b). No 318 nm absorbance is associated with the protein that elutes between 10 and 15 mL. At 40 mL, free FA elutes with nearly equal absorbances at 277 and 318 nm. An incubation of FA with HRP and H_2O_2 in the absence of α-lactalbumin yielded 2 to 3 products eluting around 40 mL.

When FA was added to α-lactalbumin in the presence of both HRP and H_2O_2, free FA was absent. The rate of addition of FA to the incubation clearly affects the cross-linking. This is apparent from the size exclusion chromatograms in Figure 4 recorded after 4 h of incubation.

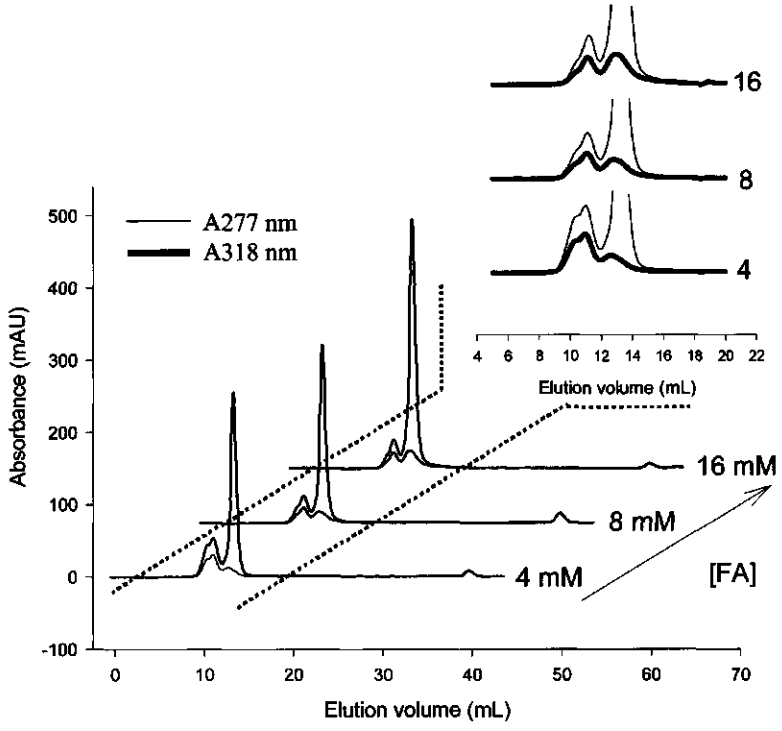

Figure 4: Size exclusion chromatograms of the incubations of α-lactalbumin with HRP and H_2O_2 under addition of 4 mM, 8 mM and 16 mM FA-aliquots

From the low intensity of the FA-peak around Ve = 40 mL, it is evident that in all incubations almost all FA becomes covalently bound to the monomeric or oligomeric protein. The lower elution pattern shows that with the slowest

addition of FA (4 mM), the protein still oligomerizes to some extent and that most of the absorption at 318 nm is associated with the oligomeric fraction with a significant higher ratio of A318/A277 than in the homo-incubation (cf. Figure 3a).

At higher addition rates of FA (Figure 4), the oligomerization is diminished and more absorption at 318 nm is associated with the monomeric protein. At the highest addition rate of FA (Figure 4, 16 mM), the 318 nm absorption associated with the monomer exceeds the 318 nm absorption associated with the oligomeric cross-linking products.

SDS-PAGE analysis of incubations of α-lactalbumin with FA

In the spectrophotometrically monitored incubations, SDS-PAGE was used to monitor the formation of new products after each addition of FA and subsequent return of HRP to its native state. In these incubations, the SDS-PAGE patterns show a gradually altered polymerization dependent on the concentration of the FA-aliquots added (Figures 5,6,7).

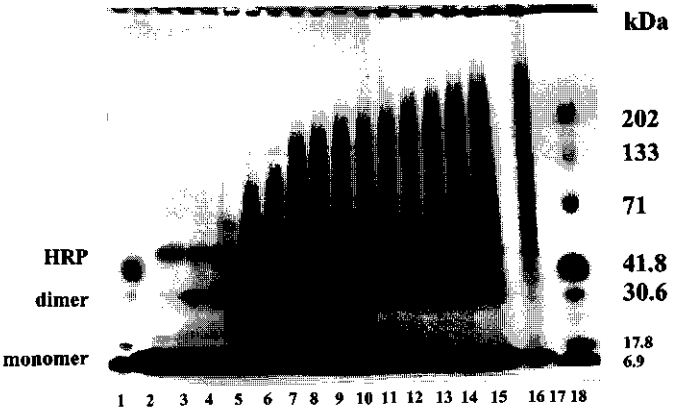

Figure 5: SDS-PAGE monitor of the incubation of α-lactalbumin with HRP and H_2O_2 after addition of 40 nmol FA-aliquots (lane 16 without β-mercaptoethanol prior to electrophoresis). Lane 2 (t=0) to lane 15 (t=4h)

The polymerization pattern during addition of 40 nmol FA-aliquots (Figure 5) shows a time dependence similar to that without addition of FA (Chapter IV), although no polymerization beyond 202 kDa occurs and somewhat more dimer is maintained. However, when more concentrated (120 nmol) aliquots of ferulic acid are added, a significant change in cross-linking is observed. Figure 6 shows that in this case the polymerization is more quantified and that more distinct oligomers -beyond dimer- are observed than after addition of 40 nmol FA-aliquots. The initially formed α-lactalbumin dimer again does not fully dissappear but now a probably trimeric protein that has never been observed in homo-incubations is present throughout the incubation. Still, extended polymerization is also observed.

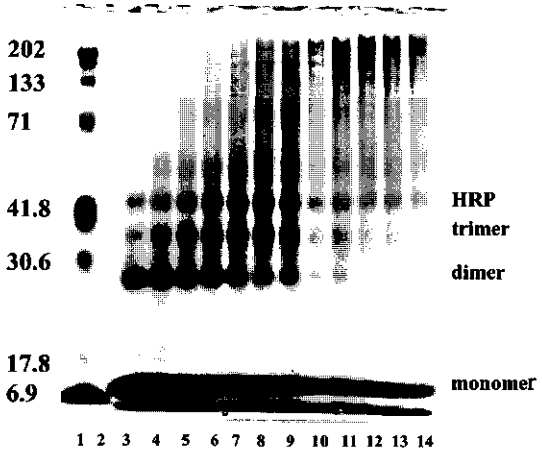

Figure 6: SDS-PAGE monitor of the incubation of α-lactalbumin with HRP and H_2O_2 after addition of 120 nmol FA-aliquots (t=10 min, lane 3 to t=2h lane 14)

A putative trimer of α-lactalbumin is also present when FA is added in 240 nmol aliquots (Figure 7). In this case, the dimer is fully maintained and hardly any polymerization of α-lactalbumin occurs.

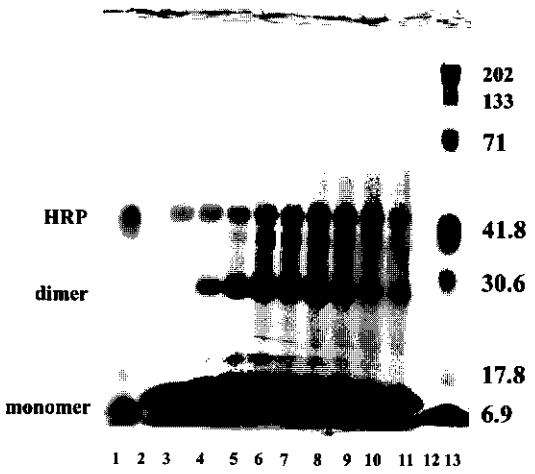

Figure 7: SDS-PAGE monitor of the incubation of α-lactalbumin with HRP and H_2O_2 after addition of 240 nmol FA-aliquots (t=0 lane 3, t=1.5 h lane 11)

Discussion

The cross-linking of α-lactalbumin with automated addition of HRP, H_2O_2 and buffer without FA, shows the time course of conversion from monomer to oligomer to polymer (Figure 2). Clearly, the monomer is not fully converted, which can be attributed to the fact that 30% of α-lactalbumin in the commercial preparation is not Ca^{2+}-depleted. Therefore, a population of holo-α-lactalbumin, which has no accessible tyrosines for cross-linking (Chapter IV), remains monomeric. The absorbance at 318 nm (Figure 3a) is caused by dityrosine bonds and the ratio of 318/277 nm is characteristic for dityrosine-polymerized α-lactalbumin. The period during which tyrosine cross-linking of α-lactalbumin takes place can be clearly seen in Figure 2 and this interval of about 250 min was therefore chosen to study the effect of added FA. To investigate whether any non-oxidative association of FA with α-lactalbumin occurs, aliquots of FA were added to α-lactalbumin in the presence of HRP but without H_2O_2. Figure 3b shows that without oxidative agent, polymeric protein products are absent and that the added FA is not associated with any protein at the end of the incubation. When H_2O_2 is added during the addition of FA, the protein polymerizes and elutes at the same volume as in the homo-incubation, with a slightly different shape, but a significant increase of absorption at 318 nm (Figure 4). Moreover, almost all FA becomes associated with the protein as can be concluded from the lack of free FA that elutes after size-exclusion (Figure 3b). The absorption at 318 nm in the polymeric fraction is maximally at least 60% of the absorption at 277 nm (Figure 4). From this drastic change in the ratio of the protein (277 nm) and ferulic acid (318 nm) absorption, it can be concluded that all ferulic acid added to the incubation is covalently bound to α-lactalbumin-oligomers and the monomer (Figure 3a). Significant more oligomerization takes place when the rate of FA-addition is relatively low (Figure 4, 4 mM trace). Due to the low incident concentrations of FA, available tyrosines in the protein can still combine with other tyrosines before they are blocked by coupling of FA. Such blocking by FA is progressively observed after addition of 8 and 16 mM aliquots during the incubation. When 16 mM FA-aliquots are added, the absorption at 318 nm in the monomeric fraction even exceeds that of the polymeric fraction.

A similar trend in FA incorporation and α-lactalbumin oligomerization was found in the spectrophotometrically monitored incubations during which FA-aliquots were added manually. Analysis of these incubations by SDS-PAGE (Figures 5-7) showed in detail that the addition of FA changed

the population of α-lactalbumin oligomers as compared to an incubation of α-lactalbumin without FA (Chapter IV). In the absence of FA, α-lactalbumin polymers with a molecular mass exceeding 200 kDa are formed. Addition of the lowest concentration of FA-aliquots (Figure 5) shows a similar oligomerization pattern, yet, no polymers with a molecular mass over 200 kDa are visible. This might be caused by blocking of all accessible tyrosines due to reaction with FA, after extended, but not maximal, polymerization (*16*). After addition of more concentrated FA-aliquots (Figure 6), more discrete oligomers of α-lactalbumin are clearly visible, yet polymerization to higher oligomers does take place. Possibly, the increased concentration of FA blocks one population of the protein molecules. That explains why a trimer is observed that was not found in any homo-incubation (Chapter IV). In all samples withdrawn from the incubation to which the most concentrated aliquots of ferulic acid were added, the dimer is present (Figure 7), whereas it disappears completely in homo-incubations of the apoprotein at 40°C (Chapter IV). After 1.5 h, despite the presence of active HRP, no further increase in absorbance at 318 nm (dityrosine) was observed. Additional aliquots of FA were still converted but no changes in the protein oligomerization pattern occurred (Figure 7, lanes 9-11), consistent with the lack of increase of the 318 nm absorbance. This is attributed to the finalization of the blocking of all tyrosines in the monomer, dimer and trace amount of trimer present at that moment. Whereas in the homo-incubation of the apoprotein at 40°C the dimer was concluded to be a reactive intermediate with a distorted structure with more readily available tyrosines, this dimer is obviously no reactive intermediate in the hetero-incubation.

We have shown here that the termination of the polymerization of α-lactalbumin is not merely by competition of FA for the HRP active site but permanent. The protein was rendered to a 'non-polymerizable' protein by addition of FA in that particular incident concentration. This would imply that *in vivo* an anti-oxidant like FA not only prevents oxidative damage by reducing oxidative species –the HRP active site in this case- but also renders protein free radicals to a form that can no longer react. Undesired polymerization of proteins causes severe damage in many brain and blood diseases, which is often attributed to oxidative damage (*1-7, 19-23*). Our studies with α-lactalbumin indicate that anti-oxidants like ferulic acid might not only react with harmful oxidative species, but also provide protection against oxidative damage of already oxidized proteins. Here we demonstrated that FA blocks the cross-linking of α-lactalbumin, *in vivo*, also

reactions with other free radical sites might be stopped. Figure 8 shows a tentative model on the HRP catalyzed polymerization of a tyrosine-containing globular protein in the presence and absence of FA. Figure 8 postulates that upon the availability of tyrosine on the surface of a globular protein in an oxidative environment two routes are open. Exposed tyrosines can either cross-link with other available tyrosines or be blocked by cross-linking with FA. In the latter case, the protein can not polymerize further.

In addition, the coupling of FA with α-lactalbumin is demonstrated, indicating the chemical feasibility of cross-linking globular tyrosine-containing proteins with FA-containing polysaccharides.

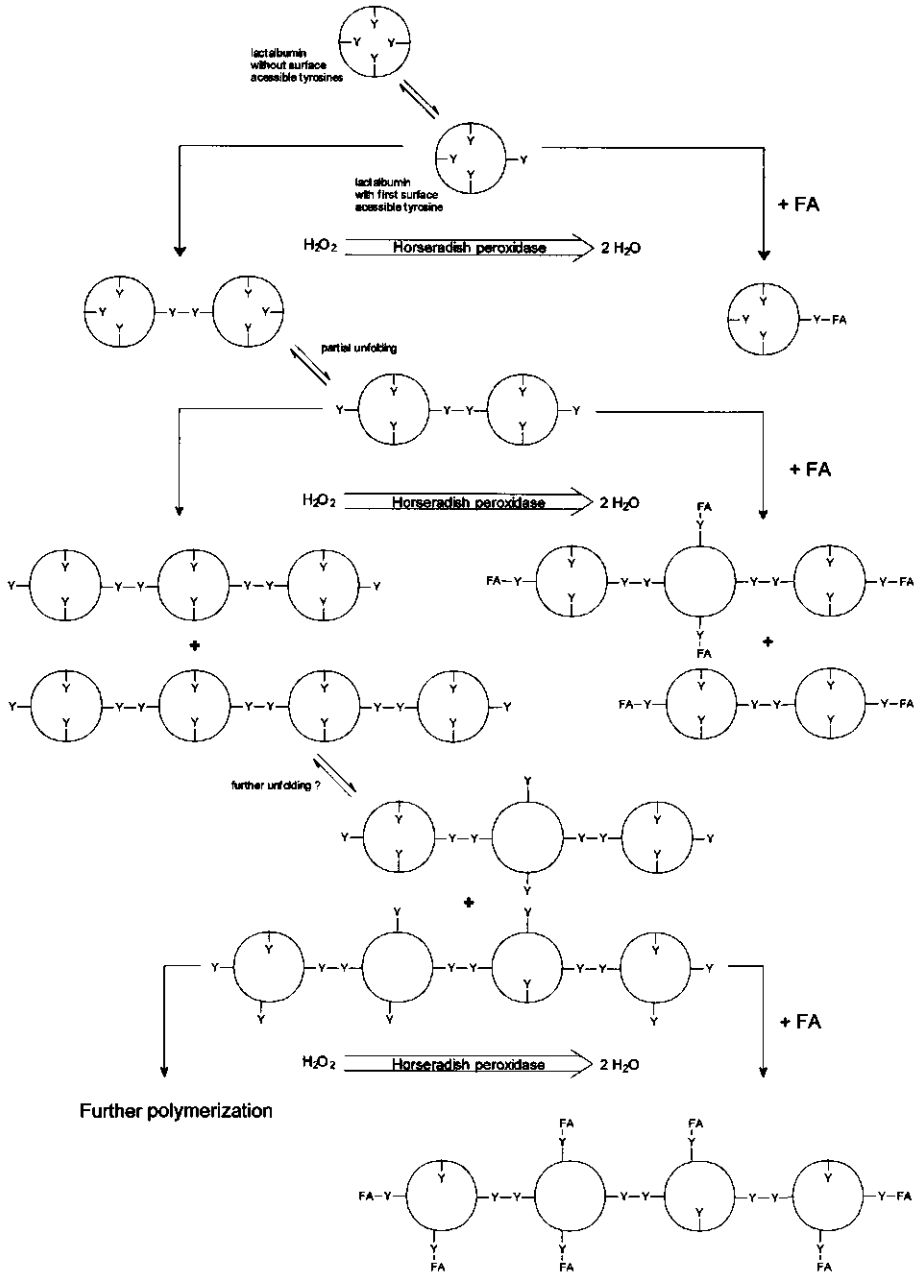

Figure 8: Tentative model of the role of FA in oxidative α-lactalbumin cross-linking

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Chapter VI

Horseradish peroxidase catalyzed cross-linking of feruloylated arabinoxylans with β-casein

Abstract

Heterologous conjugates of wheat arabinoxylan and β-casein were prepared via enzymatic cross-linking, using sequential addition of arabinoxylan to a mixture of β-casein, peroxidase and hydrogen peroxide. Maximal formation of adducts between the β-casein and feruloylated arabinoxylan was reached at high protein to arabinoxylan ratio, in combination with a low hydrogen peroxide concentration, long reaction time, and a molar substrate to enzyme ratio between 10^2 and 10^4. The protein-arabinoxylan adducts were separated from the arabinoxylan homopolymers by size exclusion and anion exchange chromatography. The molar ratio protein:arabinoxylan in the purified conjugates varied between 0.4 and 5.6. This is the first report on the large scale enzymatic preparation of heterologous protein:arabinoxylan conjugates.

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Introduction

Proteins and polysaccharides have found multiple applications in either the food and non-food industry. Functional properties of proteins of importance for industrial application include viscosity, oil and water binding, gelling, foaming, emulsifying properties, adhesive strength, colloid protective properties or a specific molecular size or shape [1]. The protein properties are largely influenced by environmental conditions, such as solvent nature, pH, temperature and ionic strength [2]. Polysaccharides are structure-forming macromolecules which have been used as thickening, gelling and suspending agents and as stabilizers in foams and emulsions.

Covalent coupling of proteins to polysaccharides, which maintains to a certain extent the structural integrity of the primary polymers, results in the formation of new macromolecular structures and may lead to improved or novel functional properties and increased resistance to heat, proteolytic attack and organic solvents. The properties of such protein-carbohydrate conjugates will probably depend on the size, structure and number of the attached protein and carbohydrate fragments.

Chemical coupling of proteins with polysaccharides has been reported. Usually, polysaccharides are coupled to proteins after random activation of functional groups along the internal part of the chain or after selective activation of their terminal reducing end group using a variety of activating agents [3]. Recently, the potential of the Maillard reaction for preparation of protein- polysaccharide conjugates has been investigated. Kim and coworkers [4] prepared bovine serum albumin (BSA) – galactomannan conjugates containing 2.5 to 7 moles of galactomannan per mole of protein. These conjugates showed higher stability and emulsifying properties than BSA alone. Conjugation of gelatin to chitosan catalysed by polyphenol oxidase has also been reported [5]. The authors described the formation of gel-like structures that differed mechanically from the gels that were obtained by cooling gelatin solutions, and that could be broken down by chitosinase.

As a new approach to prepare protein-polysaccharide conjugates, we investigated the coupling of proteins to polysaccharides catalyzed by the peroxidase/hydrogen peroxide system. Water-soluble wheat arabinoxylan containing ester-linked ferulic acid (FA, 3-methoxy-4-hydroxycinnamic acid) residues was used as the model polysaccharide. The milk proteins β-casein, a protein with an flexible, essentially random structure was used as model protein.

Horseradish peroxidase (EC 1.11.1.7, donor:hydrogen peroxide oxidoreductase) was selected as cross-linking biocatalyst since it is a versatile enzyme, which accepts a wide range of phenolic derivatives as hydrogen donor. *In vitro* peroxidase-mediated cross-linking via phenolic bridges has been reported both for proteins containing tyrosine residues [6-8] and carbohydrate polymers carrying phenolic residues such as cereal arabinoxylan [9-12] and beet pectin [13]. Formation of hetero-crosslinks between tyrosine moieties in a protein and phenolic residues in a polysaccharide by the action of peroxidase is therefore expected.

When a mixture of protein and arabinoxylan (AX) is incubated with peroxidase and hydrogen peroxide, several competitive enzymatic and chemical (e.g. radical) reactions can take place, resulting in the formation of a range of protein-protein, AX-AX and protein-AX conjugates (Figure 1).

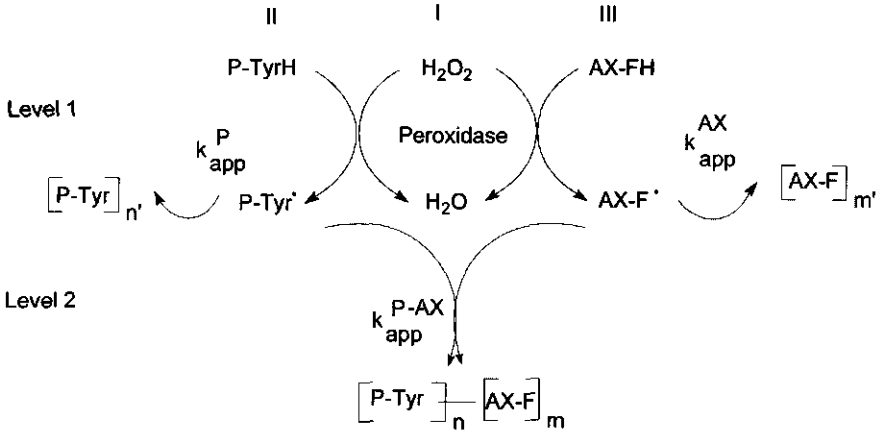

Figure 1. Schematic representation of the chemo-enzymatic cross-linking of proteins with polysaccharides. P-TyrH: protein with reactive tyrosine; AX-FH: arabinoxylan containing reactive ferulic acid residues; $(k^{P})_{app}$: apparent rate of enzymatic protein cross-linking; $(k^{AX})_{app}$: apparent rate of enzymatic AX cross-linking; $(k^{P\text{-}AX})_{app}$: apparent rate of enzymatic protein-AX cross-linking; n, n': number of protein units in protein-AX and protein-protein adducts, respectively; m, m': number of AX units in protein-AX and AX-AX adducts.

The relative contribution of each reaction is determined primarily by the difference in reactivity of the target groups with peroxidase. Also, the number and the accessibility of the reactive groups of each polymer control the reaction rate. Cross-linking of amino acids in a protein with functional moieties in a carbohydrate polymer requires exposed target groups and no or limited steric hindrance. Another factor that might affect cross-linking of the two macromolecules is their thermodynamic compatibility, which is related to the nature and intensity of the interaction as they approach each other [23, 24].

Cross-linking of arabinoxylan to proteins by oxidative enzymes has long been suggested [14-15], but never proven. Attempts of different research groups to cross-link proteins to arabinoxylans with either laccase or horseradish peroxidase [16] were unsuccessful.

Recently, we demonstrated the intermolecular cross-linking of a tyrosine containing peptide (GYG) with ferulic acid by peroxidase-mediated dehydropolymerization in a kinetic controlled reaction, and characterized the hetero-adducts formed under different reaction conditions [17, 18]. Moreover, we showed that cross-linking of both flexible and globular proteins by peroxidase-mediated oxidative dehydropolymerization in the presence of exogenous phenol is feasible [19]. In this study we report the synthesis of hetero-conjugates of arabinoxylan β-casein and α-lactalbumin, respectively, using peroxidase as catalyst for the oxidative cross-linking reaction and applying the kinetic-control concept developed in model studies [16, 17].

Materials and methods

Chemicals

Horseradish peroxidase (EC 1.11.1.7, Type VI, 250 U/mg) was obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Bovine β-casein [90 % β-casein based on weight, 95 % β-casein based on nitrogen (w/w)) was obtained from Eurial (Rennes, France), and contained mainly the genetic variants A^1 and A^2. Hydrogen peroxide (30 % solution, w/v) was from Merck (Darmstadt, Germany). Wheat arabinoxylan (arabinoxylan content: 97 % (w/w), containing approximately 0.2 % ferulic acid (w/w)) was from Megazyme (Bray, Ireland). All other reagents were of analytical grade.

Conjugation of arabinoxylan to proteins via peroxidase/hydrogen peroxide oxidative polymerisation

Oxidative crosslinking of protein with arabinoxylan with the peroxidase/hydrogen peroxide system was performed by adding 100 µl of horseradish peroxidase (1 mg/ml) and 100 µl of hydrogen peroxide (0.5 M) to 2.8 ml of 0.1 M sodium phosphate, pH 7.0, containing 1% (w/v) protein. The mixture was allowed to equilibrate at 25° C and subsequently 10 aliquots of 300 µl of arabinoxylan solution (1 %) were gradually added, during 1 hour, to the reaction mixture. The reaction mixture was maintained at 25°C for 4 hours and then the reaction was stopped by inactivation of the enzyme (2 minutes/ 100°C). The samples were frozen in liquid nitrogen and kept at -20°C until further analysis. In the blanks (control experiments without protein or arabinoxylan), the protein and or arabinoxylan solutions, respectively, were substituted by 0.1 M sodium phosphate, pH 7.0. All variations to the standard procedure are reported in the text.

Optimization of reaction conditions

To study the influence of reaction parameters on the cross-linking reaction, a fractional factorial design accommodating four variables, each one at two levels (-1/+1) was made. At the medium point (0), independent replicates were run to estimate the standard deviation. The variation between the central replicates reflects the variability of all design. After the runs, the response obtained (in this case the yield in high molecular mass adducts (HMW)) was submitted to the algorithm of Yates to calculate the effects of each parameter [20]. The response was used to calculate the coefficients of a first order equation, that shows the dependence of the product yield on all effects (parameters):

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 \qquad (1)$$

where y is the theoretical response (the value predicted by the model), x_I (i from 1 to 4) represents the factors, and b_i the coefficients determined by matrix calculation.

The reaction parameters whose influence on the product yield was studied are molar ratio protein to arabinoxylan (P/AX), molar ratio total substrate (protein and arabinoxylan) to enzyme (S/E), H_2O_2 concentration (HP) and reaction time (ζ). Table 1 gives the corresponding values, at each level.

Table 1. Parameters studied on the planned factorial design

Factor	Level		
	-1	0	+1
1. Molar ratio protein to arabinoxylan (P/AX)	1	3	5
2. Molar ratio substrate to enzyme (S/E)	10^2	$5x10^3$	10^4
3. Hydrogen peroxide concentration (mM), (HP)	1	5	10
4. Reaction time (h), (ζ)	1	2.5	4

The following conditions were maintained constant: temperature, T = 25° C; buffer molarity = 100 mM; pH = 7.0

Analytical size-exclusion chromatography

SE-HPLC analyses were carried out using a Waters HPLC system consisting of a Waters 600E solvent delivery/control system with a Waters 717 automatic sampler injector and a Waters 2487 Dual Wavelength Absorbance Detector. The column, a TSK-Gel G-2000 SW_{XL} (300x7.8 mm), was fitted with a matching guard column (TSK-Gel SW, 7.5x7.5 mm) and was maintained at 25 °C. Both columns were from TosoHaas (Tokyo, Japan). Before analysis, the samples were diluted with the eluent buffer and filtered through 0.45 um Orange Scientific Gyrodisc-PES 13 filters, and 20 µl of the supernatant was injected into the column. The components were eluted with 0.1 M sodium phosphate buffer pH 7.0 containing 0.3 M NaCl, at a flow rate of 0.25 ml/min, and detected at 214 nm, 280 nm and 320 nm. For β-casein samples, the eluent contained also 1 % (w/v) SDS, to minimize casein self-aggregation. The column was calibrated with standards of known molecular mass. The protein standards were α-lactalbumin (14.2 kDa), chymotrypsin (25.7 kDa), bovine serum albumin (66 kDa), aldolase (158 kDa) and catalase (232 kDa) . The size exclusion limit of the column was ≈ 300 kDa. The void volume of the column was experimentally determined with blue dextran (2000 kDa) and corresponded to R_t = 27.8 min. The relative abundance of reagent protein and reaction products in the reaction mixture was expressed as proportion (%) of the total peak area determined at 280 nm.

Anion exchange chromatography

Anion exchange chromatography was performed on a FPLC system AKTA explorer equipped with a UV-900 monitor and Frac-900 fraction collector (AmershamBiosciences, UK), using Q-Sepharose columns (0.7 x 2.5 cm for analytical runs and 2.6 x 40 cm for preparative runs, respectively). Samples were eluted with a buffer system consisting of 0.1 M Tris-HCl pH 7.5 and 0.1 M Tris-HCl pH 7.5, containing 1 M NaCl, with a linear salt gradient from 0 to 100%, at a flow rate of 1 ml/min. The fractions collected were dialyzed (cut-off membrane 15 kDa) and lyophilized prior to further characterization.

Preparative size-exclusion chromatography

Preparative size exclusion experiments were performed using a FPLC system AKTA explorer equipped with a monitor UV-900 and Frac-900 fraction collector on a column of Sephacryl S-300 HR (2.6 x 60 cm). Samples were eluted with 0.1 M sodium phosphate pH 7.0, at a flow rate of 1 ml/min. For samples containing β-casein, an eluent consisting of 0.1 M sodium phosphate pH 7.0, containing 1% (w/v) SDS and 0.15 M NaCl, was used, to minimize protein self-association. The column was calibrated with standards of known molecular mass. The protein standards were α-lactalbumin (14.2 kDa), chymotrypsin (25.7 kDa), bovine serum albumin (66 kDa), aldolase (158 kDa) and catalase (232 kDa). The fractionation range of the column was between 10 and 1500 kDa for globular proteins and 2 to 400 kDa for polysaccharides, respectively. Peak elution was monitored at 280 and 320 nm. The void volume of the column was determined with blue dextran (2000 kDa) and corresponds to 38 ml.

Characterization of protein-arabinoxylan conjugates by FT-IR spectroscopy and chemometry

IR spectra of protein, arabinoxylan and mixtures thereof, and of products isolated from crosslinking reactions were obtained on a Bio-Rad Fourier transform infrared spectrometer (FTS-60A) equipped with a MTC detector and ATR accessory, with a ZnSe ATR crystal. MID-IR spectra were recorded between 700 and 4000 cm^{-1} at a resolution of 2 cm^{-1} in the ATR mode, on thin films. 64 Interferograms were co-added for a high signal to noise ratio. The spectra were baseline corrected prior to further analysis. Three replicate spectra were measured for every sample and the mean spectrum was used for data analysis.

Partial least squares regression analysis (PLS) was applied to correlate the FT-IR spectroscopic data with the chemical composition of arabinoxylan-protein conjugates in terms of (i) moles of protein bound per mole arabinoxylan and (ii) % of arabinoxylan in the sample (w/w). A calibration set was built up from mixtures of arabinoxylan and casein, respectively, covering the concentration range 0-100% AX and a molar ratio [P]/[AX] between 0 and 20). The molar ratio protein to arabinoxylan was calculated using Mw = 24 kDa for β-casein and an average molecular mass of 300 kDa for the wheat arabinoxylan. The average molecular mass of arabinoxylan was determined from SEC-HPLC with RI detection (not shown). PLS models were constructed using the 2nd derivative of the spectra for the following spectral regions: 750-1800 cm^{-1} for [P]/[AX], 1250-1800 cm^{-1} for % protein and 750-1100 cm^{-1} for % AX, respectively. The PLS models were validated using the "full cross-validation" technique to ensure predictive validity, guarding against over-fitting. The predictive ability of the calibration models is described by the Root Mean Squares Error of Prediction (RMSEP) [20]. The PLS calibration models were used to determine the composition of each fraction isolated from the incubations of arabinoxylan with β-casein. The statistical software Unscrambler 6.1 (Camo A/S, Norway) was used for chemometric calculation. Figure 2 shows the calibration lines of the PLS models developed for the molar ratio protein to arabinoxylan ([P]/[AX] and AX concentration (%, w/w), respectively.

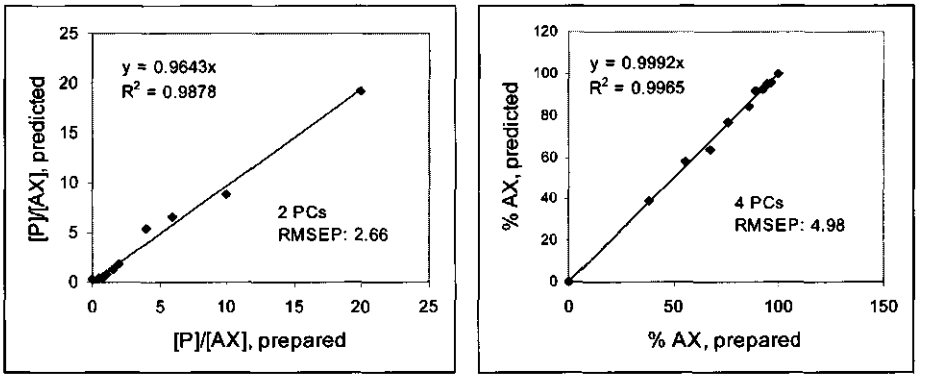

Figure 2. FT-IR based calibration models

Determination of the amount of arabinose and xylose

Pure AX, protein and the products isolated from the cross-linking reaction were hydrolyzed by stirring in 1 M H_2SO_4 for 2h at 100 °C. The hydrolysates were filtered through a Whatman GF/C glass fiber filter and neutralized with $BaCO_3$. Samples (10 µl) of the neutralized hydrolysates were analyzed for arabinose and xylose by using a HPLC system (Pharmacia LKB low-pressure mixer, HPLC pump 2248 and autosampler 2157) equipped with a Carbopack PA 1 column (250 x 4 mm, Dionex). The eluents consisted of Milli-Q water and 150 mM NaOH. Compounds were detected with a Dionex pulsed amperometric detector equipped with a gold working electrode. The applied potentials were set at E_1 = 0.1 V, E_2 = 0.6 V and E_3 = -0.6 V against an Ag/AgCl reference electrode. Pulse durations for the applied potentials were 500, 100 and 50 ms, respectively. Mannitol, added after hydrolysis of the samples, was used as internal standard. The sum of arabinose and xylose was used to estimate the amount of arabinoxylan in the isolated reaction products and the starting materials.

Results and discussion

Cross-linking of β-casein with arabinoxylan; optimisation of reaction conditions

The size exclusion chromatogram of a concentrated solution of arabinoxylan (0.4 mM) monitored at 280 nm, showed that the arabinoxylan consists of a mixture of low and high molecular mass species (insert, Figure 3). The main fraction has high molecular mass and elutes between 28 and 32 minutes. The minor fraction has a broad molecular mass distribution and elutes between 45 and 65 minutes. Both the high and the low molecular arabinoxylan fractions contain ferulic acid residues, as seen from the absorbance at 280 nm, and consequently can be involved in cross-linking reactions.

Figure 3 shows the analytical size exclusion chromatograms of incubations of β-casein with the peroxidase/hydrogen peroxide system with (1) and without (2) arabinoxylan. When β-casein was incubated with peroxidase and hydrogen peroxide, without arabinoxylan (trace 2) only the formation of a low molecular mass casein adduct was observed. Sequential addition of feruloylated arabinoxylan to a solution containing β-casein, peroxidase and hydrogen peroxide, and incubation at pH 7.0, results in the formation of higher-molecular mass protein - containing conjugates (trace 1). Two groups of new products were formed, one of relatively low molecular mass (LMW-AXP), eluting between 48 and 60 minutes, representing about 28 % of the mixture, and a fraction of high molecular mass (HMW-AXP). The high molecular mass products eluted with the void volume (elution between 28 and 32 min) and accounted for about 38 % of the mixture. These protein-containing adducts, absorbing at both 280 nm (characteristic for polymers containing aromatic residues) and 320 nm (specific for C-C aromatic linkages), are unambiguously identified as arabinoxylan-β-casein conjugates, since both arabinoxylan and AX-AX conjugates do not show any significant absorption at these wavelengths for the concentration used in the experiments.

Fractionation of the reaction mixture was achieved by sequential preparative size exclusion and ion exchange chromatography. Figure 4A shows the elution pattern of the reaction mixture on a Sephacryl-300 HR column.

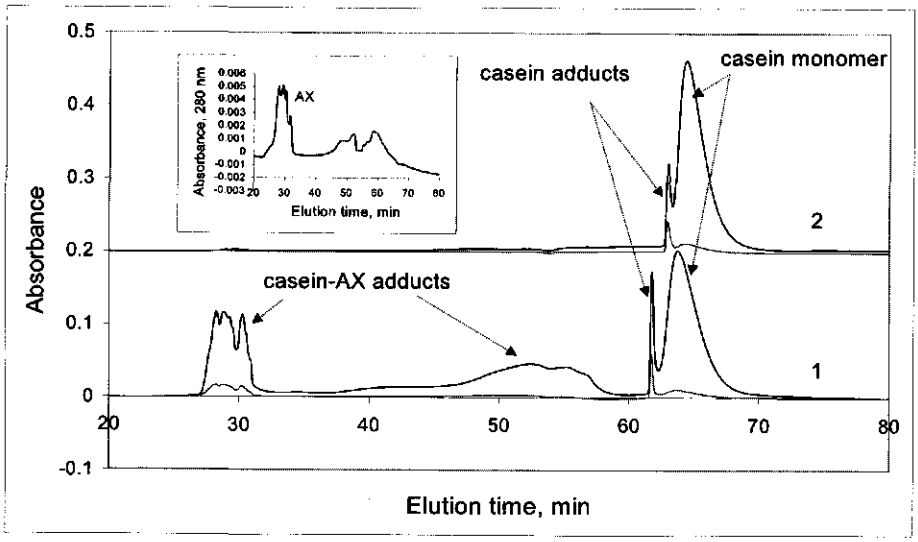

Figure 3. SE-HPLC of β-casein – arabinoxylan reaction at pH 7, 25 °C, 500nM HRP and 4 h; trace 1: 0.4 mM β-casein, 80 μM AX, 10 mM hydrogen peroxide, AX sequentially added; trace 2: 0.4 mM β-casein, 10 mM hydrogen peroxide; Black line: detection at 280 nm; dashed line; detection at 320 nm. The insert shows the elution pattern of a 0.4 mM arabinoxylan solution.

Five fractions (F1-F5) of different molecular mass were collected, as indicated on the chromatogram, and further fractionated on a Q-Sepharose column (Figure 4, B and C). Figure 4 B shows the anion exchange chromatogram of the high molecular mass fraction F-2 obtained from gel filtration. A first small fraction (F2-1) eluted at the void volume and consisted essentially of (cross-linked) arabinoxylan, as seen from the infrared spectrum (Figure 5, trace 1).

The first major fraction (F2-2), representing about 90 % of fraction F2, eluted with the running buffer and consisted of a population of arabinoxylan-casein conjugates with a variable, but relatively high content of protein, as determined from infrared analysis (Table 2). The last fraction (F2-3) eluted at 30 % salt gradient. Infrared spectral analysis revealed that this fraction contained an even higher amount of protein. Figure 4 C shows the anion exchange chromatogram of fraction F-4 isolated from size exclusion chromatography. The main product of this fraction eluted with the salt gradient (Ev = 26 ml, 50 % NaCl).

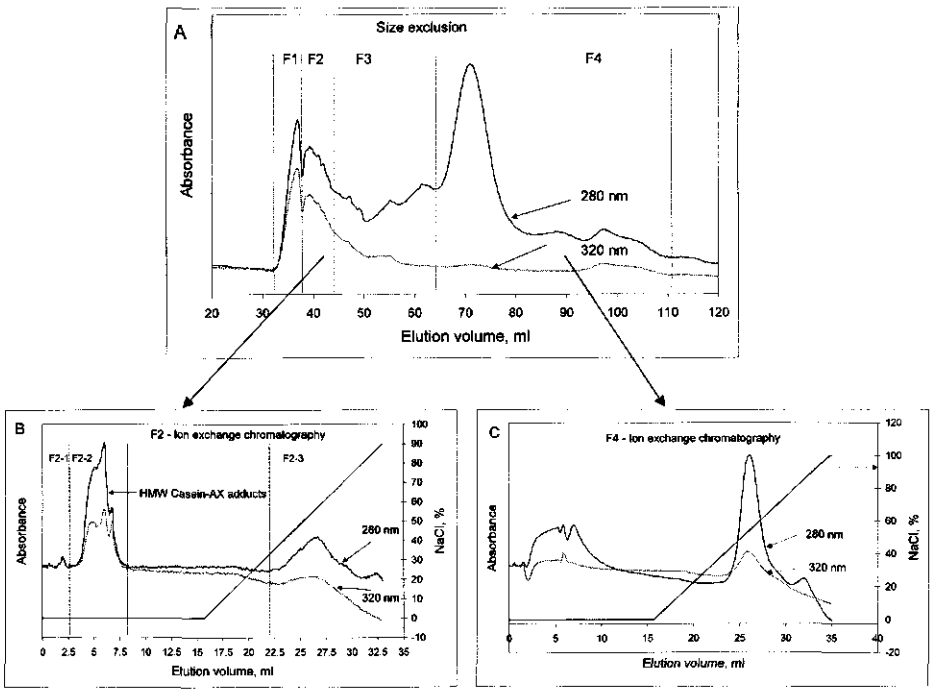

Figure 4. Fractionation of cross-linking products by (A) gel-filtration and (B,C) anion exchange chromatography. Crosslinking conditions: ([P]/[AX] = 10, [S]/[E] = 10^3, [H_2O_2]/[S] = 2, pH = 7.0, T = 25 °C, 24 h. Dark trace: absorbance at 280 nm; Dashed trace: absorbance at 320 nm; Black line: NaCl gradient. On the anion exchange column, β-casein shows three components, all eluting with the salt gradient: C-1: Ev = 20 ml, 20 % NaCl; C-2: Ev = 24 ml, 35 % NaCl, and C-3: Ev = 25.9 ml, 40 % NaCl (not shown).

The products separated by anion exchange chromatography were characterized by infrared spectroscopy and their chemical composition was determined by the PLS calibration method, as described in the Materials and Methods section.. The infrared spectra of the isolated products (Figure 5) show the specific vibrations for the amide I (1640 cm^{-1}) and amide II (1530 cm^{-1}) of the protein as well as for the COC (900-1220 cm^{-1}) in polysaccharides. Table 2 gives the composition of the products isolated from the anion exchange column as determined by the developed PLS calibration models.

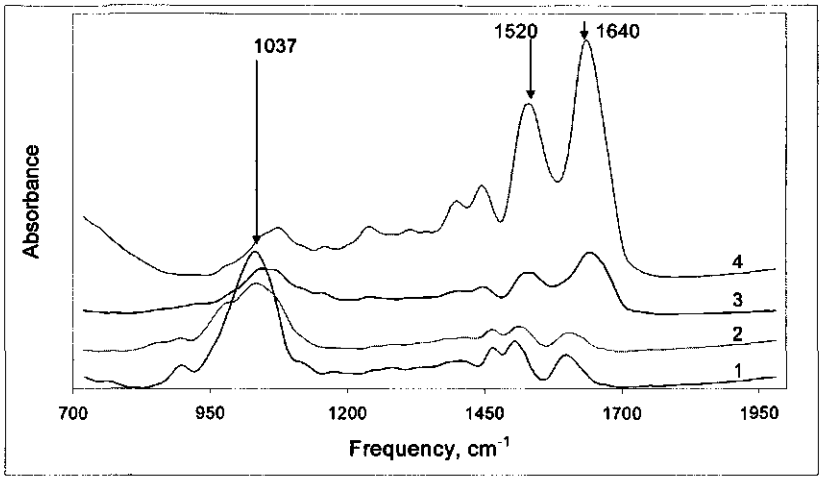

Figure 5. FT-IR spectra of products isolated from anion exchange chromatography. Trace 1: F2-1; trace 2: F2-2; trace 3: F2-3; trace 4: F4

Table 2. Composition of the protein:arabinoxylan cross-linking products isolated by sequential size exclusion and anion exchange chromatography

Product	[P]/[AX]	% β-casein	% AX
F2-1	0.1	0.8	99.6
F2-2	3.8	17.8	89.2
F2-3	14.8	56.4	40.4
F4	40	95	0

Within the experimental error of the method, we can conclude that F2-1 consists mainly of arabinoxylan and arabinoxylan homopolymers. This sample was uncharged, and could be well separated from the protein containing adducts. The products isolated from fraction F2-2 and F2-3 contained about 3.8 and 14.8 moles of protein per mole of arabinoxylan, respectively, indicative for different arabinoxylan-β-casein conjugates. These heteroconjugates were well separated from the homo-arabinoxylan species and from each other due to the difference in their charge density. The product isolated from fraction F4 of the preparative gel filtration column consisted mainly of β-casein.

These results clearly show the formation of arabinoxylan-β-casein hetero-conjugates by peroxidase mediated cross-linking. The feruloyl radicals generated by the peroxidase iron-oxo species react with the tyrosyl radicals generated on β-casein to form arabinoxylan-protein adducts. Since the protein substrate is in excess, the hetero-cross-linking reaction might be favored against homo-polymerization of the arabinoxylan.

Optimization of reaction conditions

Further experiments focused on the determination of optimal reaction parameters that allow tuning of the reaction towards the formation of protein-arabinoxylan hetero-adducts. Table 3 shows the yield in high-molecular mass hetero-adducts (HMW-AXP) for each experimental condition varied according to the experimental design. The relative abundance of different products in the mixture was determined from size-exclusion chromatograms and expressed as proportion (%) of the total peak area determined at 280 nm.

The effects of the variables on the yield of high-molecular mass arabinoxylan-casein adducts are given in equation 2:

$$\%\ \text{HMW-AXP} = 0.48(P/AX) + 5.4\times10^{-4}(S/E) - 1.54(HP) + 2.37\,\zeta + 17.62;\ R^2 = 0.81 \qquad (2)$$

This equation suggests that maximal formation of β-casein - arabinoxylan adducts will be reached at high protein to arabinoxylan ratio, in combination with a low hydrogen peroxide concentration, long reaction time, and a molar ration protein to enzyme between 10^2 and 10^4.

Table 2. Product yields (%) of levels –1/+1 and center replicates

Sample	[P]/[AX]	[S]/[E]	H_2O_2 (mM)	Time (h)	HMW-AXP (%)	LMW-AXP (%)	Unreacted casein (%)
S-1	1	100	1	1	18.2	42.5	39.3
S-2	5	100	1	4	38.0	28.0	30.3
S-3	1	10000	1	4	37.4	27.0	35.4
S-4	5	10000	1	1	27.0	18.0	33.1
S-5	1	100	10	4	13.1	44.6	42.3
S-6	5	100	10	1	12.5	23.0	43.7
S-7	1	10000	10	1	10.6	32.2	54.4
S-8	5	10000	10	4	8.1	20.0	44.9
S-9	2.5	5000	5	2.5	15.7	24.5	54.4
S-10	2.5	5000	5	2.5	17.8	35.8	40.8

Reaction conditions: 80 µmol arabinoxylan; pH = 7.0; T = 25°C

In the range of values studied, the response surface suggests that an increase in the molar ratio protein to arabinoxylan enhances the yield in hetero-adducts (no further data shown). Figure 6 shows the variation of the HMW-AXP product yield for an extended molar ratio, ranging from 1 to 25. High yields in high molecular mass hetero-adduct (HMW-AXP product) are obtained for a molar ratio protein to arabinoxylan ranging between 5 to 12.5, with an optimum at [P]/[AX] = 10. There is a good linear correlation ($R^2 = 0.96$) between the product yield and the ratio of the intensity of the peak absorbance at 320 nm (characteristic for phenolic cross-links) to that at 280 nm (specific for protein). This implies that the ratio (A_{320}/A_{280}) can be used for monitoring the cross-linking reaction and the quantification of polymeric adducts coupled through phenolic linkages.

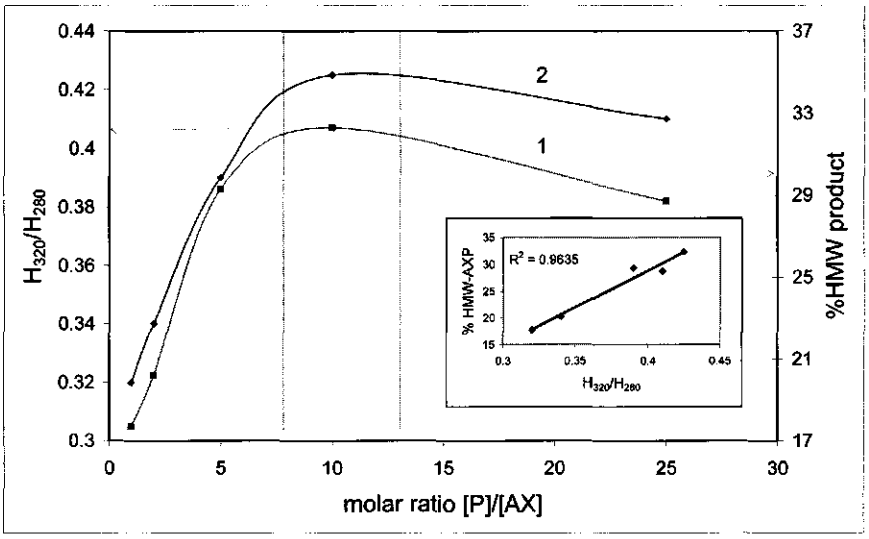

Figure 6. Determination of the optimal molar ratio protein/AX from SE-HPLC data. Reaction conditions: 40 µmol arabinoxylan, $[S]/[E] = 10^3$, $[H_2O_2]/[S] = 2$, pH = 7.0, T = 25 °C, 4 h. Trace 1: A_{320}/A_{280}; Trace 2: % HMW-AXP;

Preparative cross-linking reactions and characterization of products

Preparative (gram) scale cross-linking reactions were performed under the predicted optimal reaction conditions, to synthesize casein-arabinoxylan and arabinoxylan conjugates. For this purpose, the reaction time was extended to 24 h. This resulted in a significant increase of cross-linking, as shown by the ratio A_{320}/A_{280} determined from size exclusion chromatograms (Table 4). Fractionation of the reaction products of the large scale synthesis by size exclusion and ion exchange chromatography resulted in three major products: AE-1, eluting with the void volume of the anion exchanger; AE-2, eluting with the running buffer; and AE-3, eluting with the salt gradient. The products were characterized by FT-IR, and the amount of arabinoxylan was determined by HPLC, after hydrolysis of the samples. Products AE-1 and AE-2 were identified as arabinoxylan-β-casein adducts, containing 68.4% and 53.4 % arabinoxylan, respectively (Table 5). AE-3 mainly consisted of protein, and contained only 1.2% arabinoxylan. The arabinoxylan in this product had a higher arabinose to xylose ratio than the original wheat arabinoxylan used in the reaction.

Table 4. Influence of reaction time on the extent of cross-linking

Fraction	Elution volume, (ml)	H_{320}/H_{280}	
		4 h	24 h
F-1	30 - 38	0.53	0.64
F-2	38 - 42	0.42	0.52
F-3	42 - 70	0.04	0.25
F-4	70 - 80	0.08	0.08
F-5	> 80	0.2	0.1

Reaction conditions: ([P]/[AX] = 10, [S]/[E] = 10^3, $[H_2O_2]/[S]$ = 2, pH = 7.0, T = 25 °C

Table 5. Protein and carbohydrate composition of protein-polysaccharide conjugates isolated from incubation of arabinoxylan with β-casein

Sample*	FT-IR method		HPLC method			
	[P]/[AX]	% P	Arabinose (mg/mg)	Xylose (mg/mg)	Ara/Xyl	% AX
AX	0	0	0.28	0.64	0.44	91.7
AE-1	2.8	20.0	0.21	0.47	0.43	68.4
AE-2	5.6	22.6	0.16	0.37	0.43	53.4
AE-3	43.5	97.2	$4.8x10^{-2}$	$5.7x10^{-2}$	0.84	1.2

Reaction conditions: μmoles arabinoxylan, [P]/[AX] = 10, [S]/[E] = 10^3, $[H_2O_2]/[S]$ = 2, pH = 7.0, T = 25 °C, 24 h.

Conclusions

The results presented here clearly show that β-casein can be cross-linked with feruloylated arabinoxylan in a kinetically controlled reaction catalyzed by horseradish peroxidase. Optimal formation of β-casein:arabinoxylan adducts was reached at high protein to arabinoxylan ratio, in combination with a low hydrogen peroxide concentration, long reaction time, and a molar protein to enzyme ratio between 10^2 and 10^4. The purified protein-arabinoxylan adducts had a molar ratio protein:arabinoxylan between 0.4 and 5.6.

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Chapter VII

Catechol is cross-linked with peptidyl tyrosine and histidine by horseradish peroxidase *via* different reaction mechanisms

Abstract

To explore which amino acids in proteins can be coupled with catechols by peroxidase, catechol was incubated with peptides containing tyrosine, histidine, lysine or hydroxyproline in the presence of horseradish peroxidase and H_2O_2. Kinetically controlled incubation of the tripeptide GYG with catechol yielded hetero-oligomers of GYG and catechol, ranging from hetero-dimers to hetero-pentamers. Adducts of NAc-His-OMe with catechol ranged from hetero-dimers to hetero-tetramers. Covalent attachment of catechol to the side chain of the amino acids tyrosine and histidine was demonstrated by tandem MS of the adducts. Surprisingly, no adducts of lysine or hydroxyproline with catechol were found. It is shown that tyrosine can be coupled to catechol *via* straightforward oxidation by peroxidase. Two mechanisms for the cross-linking of catechol with proteins are proposed.

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Introduction

The formation of adducts of catechols with DNA (*1-6*) and proteins (*7-20*) is of great biological interest. Cross-linking of proteins contributes to the structural integrity in connective tissues. The cross-linking of neurofilament proteins by oxidized catechols has been suggested to cause Lew body formation in Parkinson's disease (*16*). Catechol mediated protein cross-linking has often been attributed to the formation of adducts between catechol and the protein lysine side chains solely. In model studies with mussel adhesive proteins, the lysine content of the proteins after cross-linking with oxidized L-DOPA, however, was unaltered (*9, 10*). Mussel adhesive proteins are rich in proline, glycine, serine, threonine, alanine, lysine and tyrosine but which of these amino acids participate in cross-linking oxidized catechols is still a matter of debate. Only recently, covalent cross-links between the imidazole nitrogens of histidine and the catechol moiety of *N*-acetyldopamine, *N*-beta-alanyldopamine and 3,4-dihydroxyphenylethanol in sclerotized insect cuticulum were reported (*14*). These catechol-histidine adducts were proposed to result from laccase mediated oxidation of the catechols. Three forms of protein-catechol interactions were recently suggested by Schweigert and coworkers, comprising direct lysine and cysteine modification with the catechol aromatic ring and catechol mediated radical combination of protein cysteines (*21*).

The cross-linking of tyrosine with catechol has never been suggested. The phenolic hydroxyl of tyrosine is not nucleophilic and cannot be coupled with electrophilic sites in oxidized catechols *via* a S_N2 mechanism. However, tyrosine can be alkylated *via* a radical mediated mechanism (*22*). Alkylation of tyrosine by catechols would therefore be feasible after one electron oxidation of the *ortho*-diphenol to the semiquinone radical, and subsequent radical combination with the tyrosine radical. As both free radicals can stem from peroxidase catalysis, combination might be feasible by kinetic control as we found previously for FA and GYG (*22, 23*).

Since in oxidative systems both singlefold and twofold oxidized catechols are present, two routes may lead to alkylation of amino acids, a radical mediated and a S_N2 mediated pathway. Catechol can also polymerize *via* either one of these routes. To test which sites in a protein might participate in cross-linking with oxidized catechols, we incubated GYG, *N*-acetyl-GYG, *N*-acetyl-tyrosine, N-acetyl-histidine, *N*-acetyl-hydroxyproline and *N*-acetyl-lysine with horseradish peroxidase and added catechol to these incubations in a kinetically

controlled fashion. Kinetic control is necessary to favour the hetero-coupling of the peptide/amino acid with the catechol over homo-polymerization of the catechol or homo-polymerization of the tyrosine-containing tripeptide.

Materials and methods

Chemicals

Gly-Gly-Gly (GGG), Gly-Tyr-Gly (GYG), *N*-acetylated Gly-Tyr-Gly (NAc-GYG), *N*-acetyl-tyrosine (NAcTyr) and the *N*-acetylated methyl esters of histidine (NAcHisOMe), hydroxyproline (NacHypOMe) and lysine (NAcLysOMe) were obtained from Bachem, Bubendorf, Swiss. Horseradish peroxidase (HRP) type VI-A, L-tyrosine and catechol were obtained from Sigma. Hydrogen peroxide was obtained from Merck. All other chemicals were of analytical grade.

General

Incubations were carried out at 25°C in a stirred 1 mL cuvette. A 25 μM HRP solution in 10 mM potassium phosphate buffer pH 7.0 was kept on ice after thawing from storage at -20°C. A 25 mM H_2O_2 solution was always prepared freshly and kept on ice protected from light before use. A 5 mM stock solution of catechol and 25 mM stock solutions of amino acids or peptides were prepared in 100 mM ammonium acetate, pH 6.7. For incubations at pH 8.0, 100 mM potassium phosphate/citric acid and for pH 9.0, 100 mM sodium carbonate were used.

For GYG and catechol an extinction coefficient 'of full conversion' and apparent values of V_{max}/K_M were determined in incubations containing 125 nM HRP and 250 μM H_2O_2. Spectral changes were monitored from 250-600 nm using a Hewlett-Packard 8453 A photodiode array spectrophotometer.

Incubations of catechol and GYG with HRP and H_2O_2

Catechol (20, 40, 60, 80, 100, 120 μM, respectively) was incubated with HRP and H_2O_2 and the changes in absorbance at 320 nm were used for the determination of initial reaction rates (*vide infra*). When the spectrum stopped changing, a 10 μL aliquot of H_2O_2 was added and the incubation was allowed to react until no further spectral changes occurred.

GYG (0.31, 0.63, 0.94, 1.25 and 2.5 mM, respectively) was incubated with HRP and H_2O_2 and the changes in absorbance at 318 nm were used for the determination of initial reaction rates (*vide infra*). When the spectrum stopped changing, a 10 μL aliquot of H_2O_2 was added and the incubation was allowed to react until no further spectral changes occurred.

Final absorbances at the end of the incubations were substracted from the initial absorbance and these absorbance differences were plotted against the initial concentration.

The slopes of both plots are the extinction coefficients of full conversion of both substrates. These extinction coefficients were used for determination of the initial rate of conversion for both substrates in the aforementioned incubations.

Kinetic control of the incubations of GYG with catechol was based on the kinetic parameters obtained for the separate conversions of GYG and catechol. Addition of 10 µL aliquots of 5 mM catechol to the GYG incubation was rationalized to result in equal populations of both radicals during the incubation. Whenever the increase in absorbance at 318 nm during the incubation stopped, the reaction was continued by the addition of another 10 µL aliquot of H_2O_2.

Incubations with NAc-GYG and GGG were carried out in a similar kinetically controlled fashion. Addition of H_2O_2 in the incubation of GGG was like in the incubation of GYG with catechol.

Incubations of *N*-acetylated amino acids and catechol with HRP and H_2O_2

Homo-incubations of NAcHisOMe, NAcLysOMe and NAcHypOMe were all performed in 1 mL incubation mixtures containing HRP, H_2O_2 and 25 mM of the amino acid. To the incubation mixture initially containing HRP and H_2O_2 and the amino acid, 10 µL aliquots of mM catechol were added. Aliquots of H_2O_2 were added whenever the absorbance did not change upon addition of catechol. Incubations were carried out at pH 7, 8 and 9.

LC-MS-MS analysis

For LC-MS analysis, 20 µl of the incubation-mixtures was separated on a 150 x 2.1 mm Alltima C18 column (Alltech, Breda, The Netherlands) running in 0.03% TFA (v/v) in water at a flow rate of 0.2 mL/min. Elution was performed with a linear gradient of 0-40 % acetonitrile in 0.03% TFA over a 50 min period. The eluate was monitored at 318 and 348 nm with a UV3000 detector (ThermoSeparations).

Mass spectrometric analysis (LCQ, ion trap, Finnigan MAT 95, San José, USA) was performed using electrospray ionization and detection in the positive mode with a capillary spray voltage of 2.5 kV and a capillary temperature of 200 °C. The instrument was controlled by Xcalibur software. The accuracy of the mass determinations is +/- 0.5 Da. After a full MS scan, ions with a mass to charge ratio within 10 m/z units of the most abundant ion were selected and subjected to collision induced dissociation (MS^2).

Results

In a first approach, the rates of the separate conversions of GYG and catechol by HRP and H_2O_2 were determined at different substrate concentrations. The extinction coefficients $\varepsilon_{fullconversion}(318)$, determined from the slope of the plots of the full conversion of GYG and catechol, were 0.24 AU/mM and 1.28 AU/mM respectively. The conversion rates of GYG and catechol, estimated from these ε values, are plotted in Figure 1.

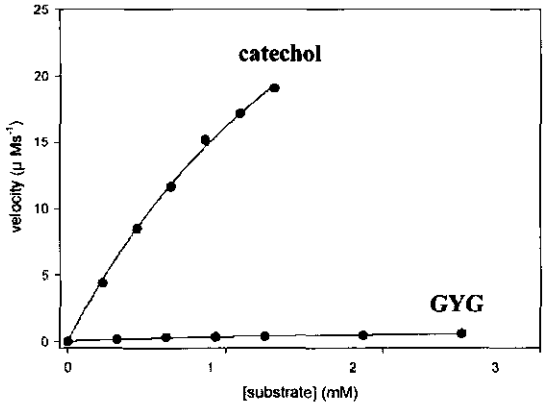

Figure 1 : Initial rates of the conversion of GYG and catechol incubated with HRP and H_2O_2

Based on the approximately 40-fold faster conversion of catechol as compared to GYG, catechol was added to the incubation of GYG with HRP and H_2O_2 in 10 μL aliquots of 5 mM. After each addition of H_2O_2, the absorbance at 318 nm, indicative for dityrosine formation, started to increase. Addition of each aliquot of catechol led to an instant increase of the absorption at 318 nm, followed by a less steep increase that finally flattened, untill the next aliquot of H_2O_2 was added. The period for the absorbtion at 318 nm to flatten increased during the incubation from 20 to 70 s. Immediate analysis of the incubation mixture by RP-HPLC-MS-MS showed a plethora of covalent hetero-adducts of GYG and catechol according to the m/z values of the ions detected (Figure 2).

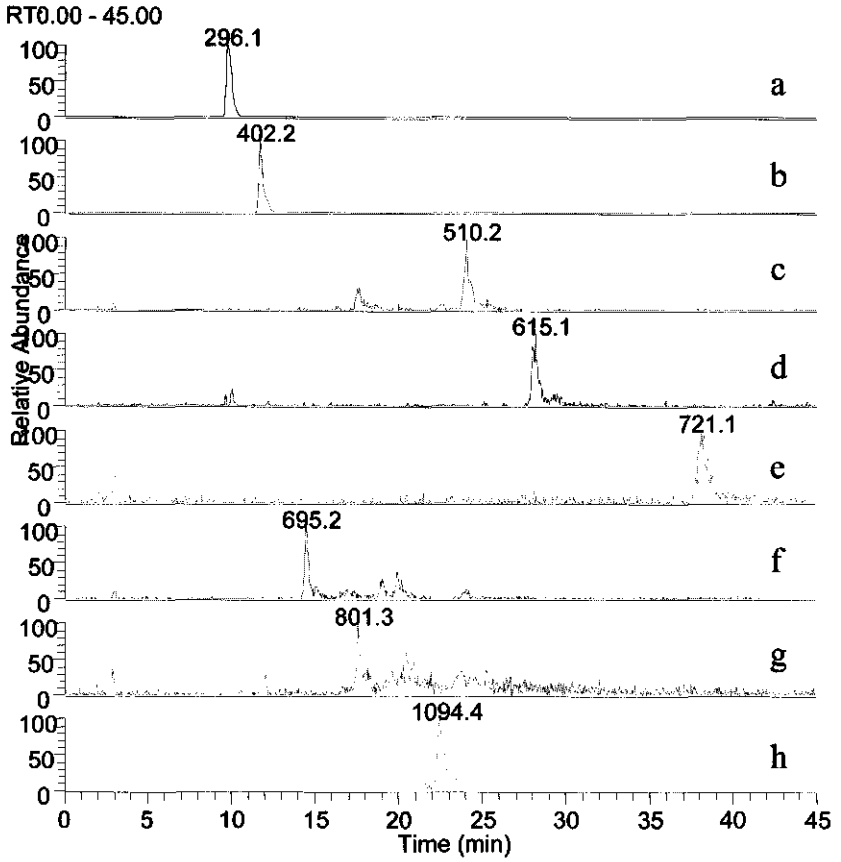

Figure 2 : RP-HPLC-MS of the incubation of GYG and catechol with HRP/ H_2O_2. The m/z-values are indicated at each peak, panel a shows the elution of GYG, panel b-h the elution of expected m/z species resulting from adduct formation of GYG with catechol

The selected ions of which the elution is shown in the chromatograms in Figure 2 are assigned to hetero-adducts of one GYG dehydrogenatively linked to 1, 2, 3 or 4 catechol moieties (panels b-e respectively), GYG-dimers or 2 bridged GYG linked to or by 1 and 2 catechols (Figure 2, panels f&g, respectively) and a trimer of 3 GYG and 2 catechols (Figure 2, panel h). All these species are singly charged as concluded from the first three ^{13}C isotopes in the zoom-scans of the detected compounds (data not shown) and therefore the molecular mass of the compounds is equal to the m/z-values. These compounds were all subjected to collision induced dissociation (CID). The resulting fragment ions for the m/z species of 296, 402 and 510 (Figure 2, panels a-c) are shown in Figure 3.

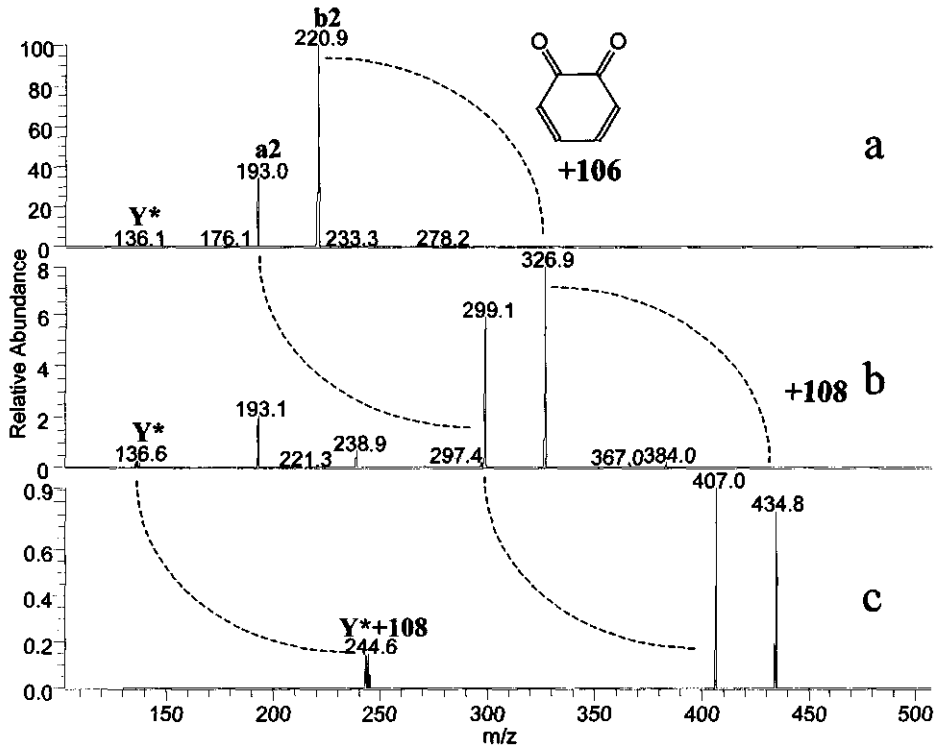

Figure 3 : CID of the m/z 296, 402 and 510 species, corresponding with masses of GYG (a), GYG coupled with 1 catechol (b) and GYG coupled with 2 catechols (C). Y*designates the internal fragment ion of GYG

Collision induced dissociation (CID) of GYG (Figure 3, panel a) results in fragment ions with a m/z of 193, 221, 239 (not visible) and 136 that correspond to the b2, a2, y2 ions of GYG and one internal fragment ion, the tyrosyl (y1 of the b2 or b1 of the y2). CID of the reaction product with m/z 402 (Figure 2, panel b) leads to the aforementioned fingerprint ions of GYG and furthermore to product ions with a m/z of 299 and 327 (Figure 3, panel b) that are assigned to the b2 and a2 ions of the modified peptide, comprising GYG with 1 catechol dehydrogenatively linked to the peptidyl tyrosine, after loss of the carboxyterminal glycine and the carboxyterminal glycine, respectively.

CID of the m/z 510 reaction product (Figure 2, panel c), that is assigned to a hetero-oligomer of GYG dehydrogenatively linked to 2 catechols of which two contain non-oxidized hydroxyls, results in the a2 ion of the modified peptide (m/z = 407) and b2 ion (m/z = 435) and an m/z 296 fragment ion corresponding to GYG. The fragment with m/z 244.6 is assigned to an internal fragment ion comprising dehydrogenatively linked tyrosine and catechol after loss of both the carboxy- and aminoterminal glycines from the precursor ion as well as 1

catechol. Few detectable fragment ions resulted upon CID of the higher molecular mass hetero-adduct ions.

The incubation of NAc-GYG with catechol led predominantly to the formation of NAc-GYG dimers (Figure 4, panel f) and trimers (Figure 4, panel g). One hetero-adduct comprising 1 NAc-GYG and 1 catechol dehydrogenatively coupled, was found (Figure 4, panel e). Incubations with NAc-Tyr resulted in a similar product pattern yielding only 1 hetero-adduct comprising the tyrosine and 1 catechol linked by dehydrogenation.

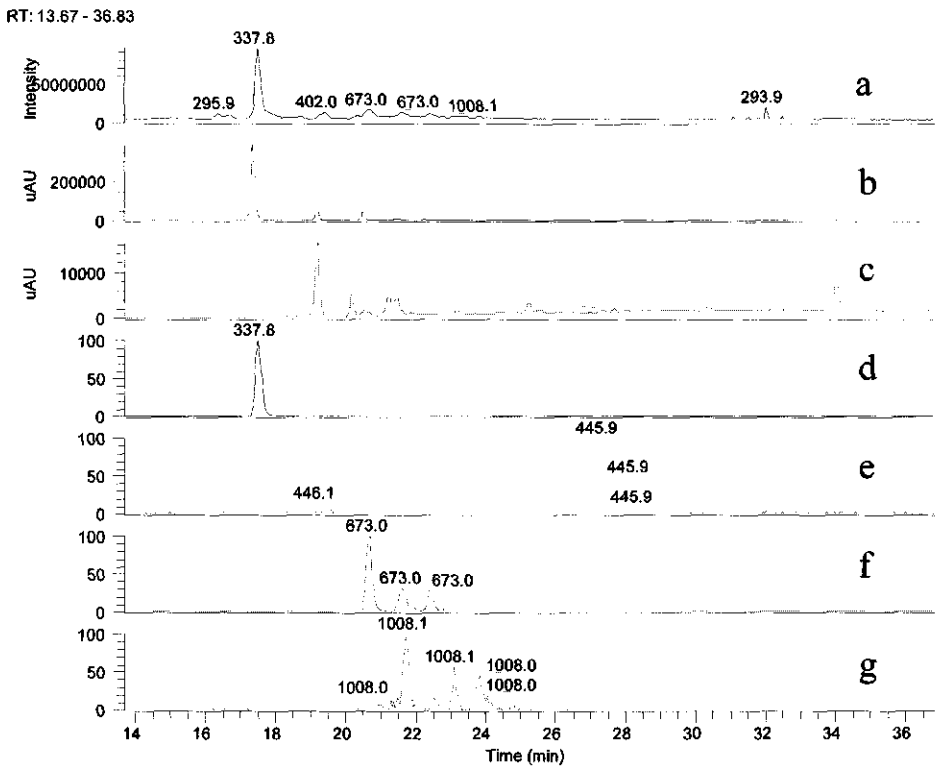

Figure 4 : RP-HPLC-MS of the incubation of NAc-GYG and catechol with HRP and H_2O_2. (a) TIC, (b) UV-277 nm (c) UV-318 nm (d) selected ion of NAc-GYG (e) selected ion of the expected m/z of NAc-GYG dehydrogenatively linked with 1 catechol (f) NAc-GYG dimers (g) NAc-GYG trimers

In the control incubation of GGG and catechol with HRP/ H_2O_2 no formation of hetero-adducts was observed.

Incubation of NAcHisOMe and catechol with HRP/ H_2O_2

The incubation of NacHisOMe with catechol resulted in the formation of of hetero-adducts of NAcHisOMe covalently linked to either 1 or 2 catechols as shown in the selected ion chromatograms (Figure 5).

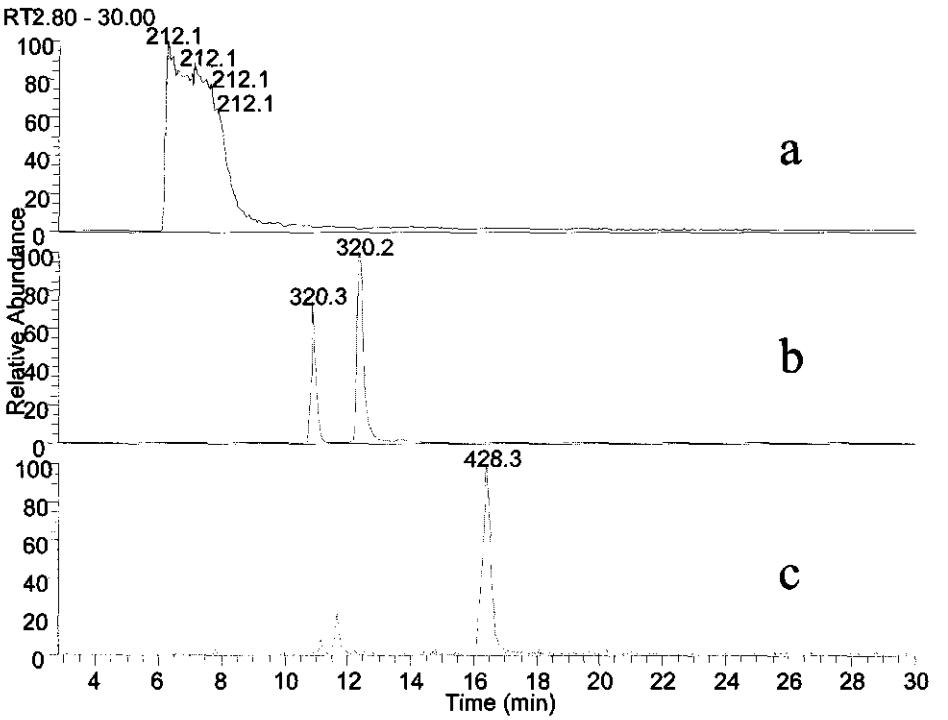

Figure 5 : RPHPLCMS of the incubation of NAcHisOMe. Selected ions are monitored (a) NAcHisOMe (b) the m/z expected for the heterodimer of NAcHisOMe coupled with catechol (c) NAcHisOMe coupled with 2 catechols

Collision induced dissociation (CID) of NAcHisOMe (Figure 6, panel a) results in fragment ions with a m/z of 180, 152, 170 and 110 (H*) that correspond to the b2, a2, y2 ions of NAcHisOMe and one internal fragment ion (y1 of the b2 or b1 of the y2), respectively. CID of the species with m/z 320 leads to product ions with a m/z of 288 and 261 (Figure 6, panel b) that are assigned to the b2 and a2 ions of NAcHisOMe with 1 catechol dehydrogenatively linked, after loss of the carboxyterminal glycine and carbon monoxide, respectively.

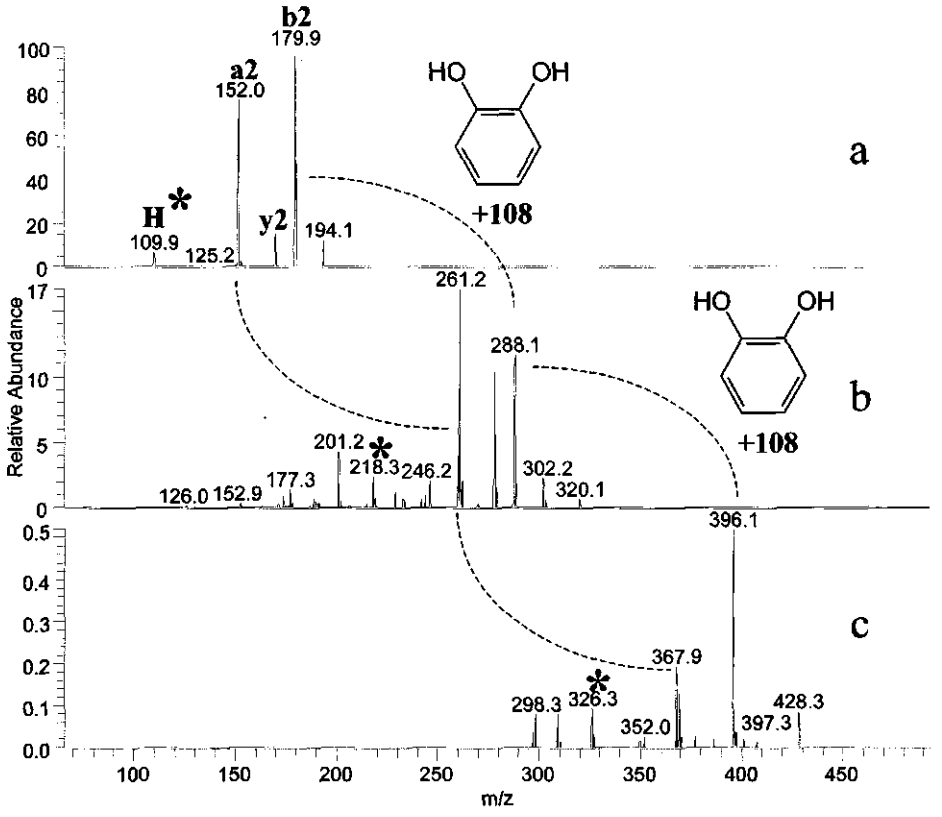

Figure 6 : CID of NAcHisOMe (a) and m/z species of 320 (b) and 428 (c) corresponding to NAcHisOMe linked with 1 and 2 catechols, respectively

The product ion with a m/z of 218.3 (*) corresponds to the internal fragment ion of the histidine, coupled with catechol. CID of the m/z 428 species (Figure 6, panel c), that is assigned to a hetero-trimer of NAcHisOMe coupled with 2 catechols of which two contain non-oxidized hydroxyls, results in the a2 ion of the modified peptide (m/z = 368) and b2 ion (m/z = 396). The fragment with m/z 326.3 is assigned to an ion comprising histidine and catechol after loss of both the carboxyterminal methoxy and aminoterminal acetyl from the precursor ion..

The incubations of NAcHypOMe and NAcLysOMe that were carried out similar to that of NAcHisOMe, did not lead to hetero-adducts as concluded from the RP-HPLC eluate of incubations of both NAc-Hyp and NAc-Lys with catechol. The unmodified *N*-acetylated amino acids were clearly present in the TIC (data not shown). In the UV-chromatogram, some new products were observed, which either were not detected by any mass, or by masses that could not be assigned to straightforward products of a Michael-type addition.

Discussion

Adduct formation of tyrosine and catechol

The conversion of catechol by HRP/H_2O_2 is an order of magnitude faster than the conversion of GYG. A hetero-incubation of equimolar amounts of both substrates would, therefore, result in catechol conversion solely. Based on apparent Michaelis-Menten parameters for both conversions, the hetero-incubation of GYG with catechol was performed by adding small aliquots of catechol to the GYG-containing incubation mixture. In this fashion, a continuous, approximately 40-fold, higher concentration of GYG compared to catechol was established. The retention times of RP-HPLC elution of all hetero-adducts of GYG and catechol (Figure 2) are consistent with the assignment based on their masses and fragmentation patterns. The higher the number of catechol moieties compared to GYG moieties in a hetero-adduct, the more hydrophobic the resulting hetero-oligomer and the expected higher retention time resulting is indeed seen both for the series of 1 GYG linked to 1 to 4 catechols and the series of 2 GYG linked to 1 or 2 catechols and 3 GYG linked to 2 catechols. Since catechol cannot be detected under the conditions used, no statements can be made about the relative abundancy of the products. It is possible that hetero-oligomers containing many catechols relative to GYG do not ionize, as well as the hetero-oligomers containing relatively a lot of GYG. The control incubation of GGG with addition of catechol clearly shows that the tyrosine in GYG is the target residue for alkylation with catechol. This is further evidenced by the results obtained with *N*-acetylated GYG, although only 1 hetero-adduct was found. A similar trend was observed in the incubations of tyrosine and NAc-Tyr. All acetylated amino acids/peptides rather oligomerized, that might be due to stacking of the acyl groups, favouring the homo-radical combination over the hetero-reaction with catechol. Furthermore it is noteworthy that clearly 3 isomeric dimers of NAc-GYG result from radical combination (Figure 4, panel f), which is in conflict with the generally assumed formation of two dityrosine dimers (Chapter I). This must stem from radical combination of the *para*-carbon of tyrosine, that is sterically hindered as compared to the hydroxyl oxygen or *ortho*-carbon atom.

Adduct formation of histidine and catechol

Histidine adducts with catechols have been detected in insect cuticulae (*13, 14*). The adducts we find here by *in vitro* peroxidase catalysis support the suggested formation of these adducts by laccase catalysis. To allow the reaction of the oxidized catechol with the histidine side chain, the only consideration was a continuous low incident concentration of catechol. Unlike

tyrosines that can polymerize to homo-oligomers, histidine is conjugated with catechol by nucleophilic attack and does not participate in a competing homo-oligomerization. Figure 5 shows that two isomeric hetero-dimers of NAcHisOMe with catechol are formed, indicating nearly equal probability of both imidazole nitrogens. The hetero-trimer of NAcHisOMe with 2 catechols consists of 1 isomer, in accordance with alkylation of each imidazole nitrogen *via* nucleophilic attack.

Mechanistic considerations

From the formation of hetero-adducts of catechol with both the nucleophilic amino acid histidine and the non-nucleophilic amino acid tyrosine it can be concluded that two mechanisms both mediated by peroxidase catalysis can lead to the modification of amino acid side chains. After one electron oxidation of catechol by the enzyme a radical is formed. If other radicals are simultaneously present in the incubation these can combine with the catechol radical. Therefore, kinetic control of the reaction is necessary. Otherwise, catechol would polymerize or be oxidized a second time, upon which the *ortho*-quinone is formed that cannot combine with a tyrosine radical. Since the phenolic hydroxyl of tyrosine is not nucleophilic enough to attack the semi- or *ortho*-quinone Michael acceptor, this linkage is concluded to proceed via a radical mediated mechanism.

The *ortho*-quinone, however, is a good electrophile and has four sites that are good Michael acceptors for nucleophilic attack. The adduct formation of catechol with histidine is, therefore, concluded to originate from Michael attack of the imidazole side chain.

Cross-linking of proteins with catechols

It has been assumed that tyrosines can only be involved in cross-linkng upon hydroxylation by tyrosinase and subsequent oxidation of the diphenol to an electrophilic *ortho*-quinone that can participate in adduct formation. The present results indicate that tyrosinase activity is not nescessarily required to involve the tyrosine side chains in the modification of these residues with catechol derivatives, since radical coupling can result in the cross-linking upon tyrosine oxidation by HRP solely. Carbon-oxygen cross-linkages were revealed by solid state NMR (*14*) but the formation of such linkage was not understood. We suggest that either the tyrosine hydroxyl oxygen or the catechol hydroxyl is involved in these carbon-oxygen linkages.

The reaction mechanism for the peroxidase mediated cross-linking of histidine and catechol is concluded to be a Michael-type addition of the nucleophilic amino acid side-chain to the catechol *ortho*-quinone. Tyrosine complexation with catechol is deduced to originate

from radical combination after one-electron oxidation of both peroxidase substrates by the enzyme.

Histidine adducts of DOPA derivatives have been demonstrated previously and a laccase mediated mechanism was proposed. Our results confirm the feasibility of the *in vivo* formation of these cross-links. Here we demonstated that the formation of the histidine-catechol adducts is accelerated by the presence of HRP but also occurs, albeit to a much lesser extent, in the absence of enzyme.

Lysine has often been suggested to be involved in the cross-linking of catechols to proteins and peptides. Our results indicate that such cross-linking cannot result from the oxidation of catechol moieties solely and that a conversion of the lysine side chain that is not catalyzed by peroxidase is required. For hydroxyproline, neither hetero-adducts were found in the incubation with catechol and peroxidase. Thus, the abundant presence of this amino acid in many structural cell wall proteins might rather be of conformational importance than as a nucleation site for attachment of catechol (spacers) to the proteins.

The results presented here confirm the involvement of histidine in cross-linking to proteins and indicate the involvement of tyrosine in the cross-linking of proteins to *ortho*-diphenols that are derived from catechol. We conclude that kinetic control of the peroxidase mediated oxidation of catechol and tyrosine can lead to adducts of catechol to the tyrosine side chain. Furthermore, we conclude that both the previously suggested histidine side chain can be modified with catechol. The implications for protein cross-linking mediated by catechol are outlined in Figure 7.

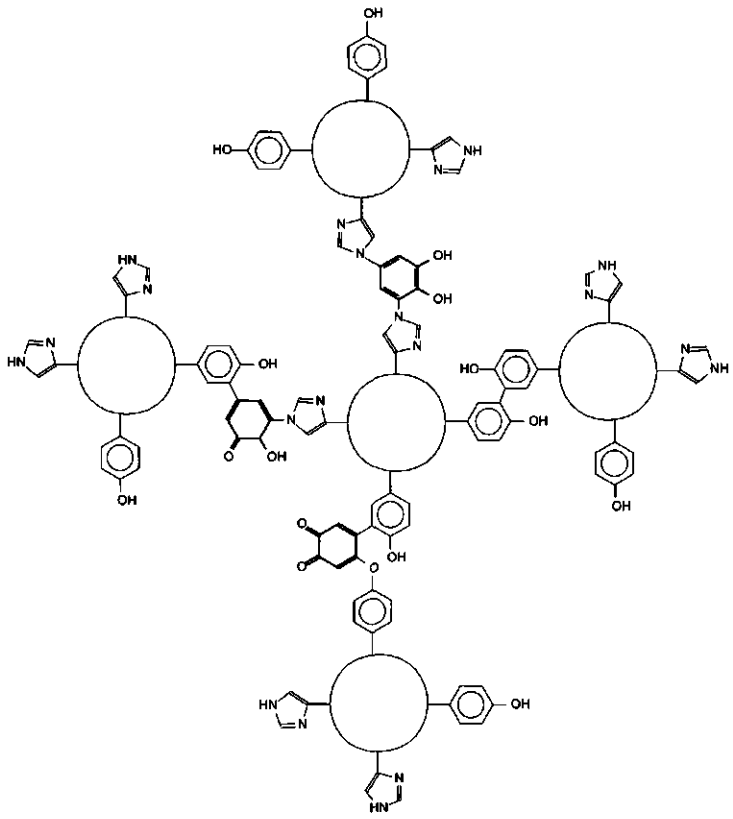

Figure 7 : Tentative model for the catechol mediated cross-linking of proteins by HRP

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Chapter VIII

Horseradish peroxidase catalyzed cross-linking of proteins in the presence of exogenous phenols

Abstract

Peroxidase-mediated cross-linking of β-casein, α-lactalbumin, and bovine serum albumin in the presence of hydrogen peroxide and a phenol as low molecular weight hydrogen donor has been studied as a model for the development of an efficient enzyme-catalyzed protein cross-linking technique. A range of mono-, di-, and polyphenols with different ring substitution pattern served as the second enzyme substrate. As products of the enzymatic oxidative cross-linking reaction, heterologous protein dimers and polymers were identified by SE-HPLC and SDS-PAGE. All three proteins were cross-linked, but only β-casein was efficiently converted into high molecular weight polymers. Cross-linking of the globular proteins α-lactalbumin and bovine serum albumin mainly resulted in the formation of oligomers. The product pattern and product yield of the cross-linking of β-casein varied with the nature of exogenous phenol. *Ortho*- and *para*-diphenols were more efficient as cross-linking agents than monophenolic acids. Possible mechanisms for the peroxidase-mediated cross-linking of proteins in the presence of phenol mediators are discussed.

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Introduction

Plant peroxidases (EC 1.11.1.7, donor:hydrogen peroxide oxidoreductases) are heme-containing enzymes that are able to catalyze the oxidation of a wide range of compounds like phenols, arylamines, halides and thiols with the simultaneous conversion of hydrogen peroxide to water. Phenolic residues in plant proteins or plant cell-wall material, such as tyrosine in extensin and hydroxycinnamates in suberin and pectin, are also modified by peroxidases in this oxidative dehydrogenation reaction [1-7].

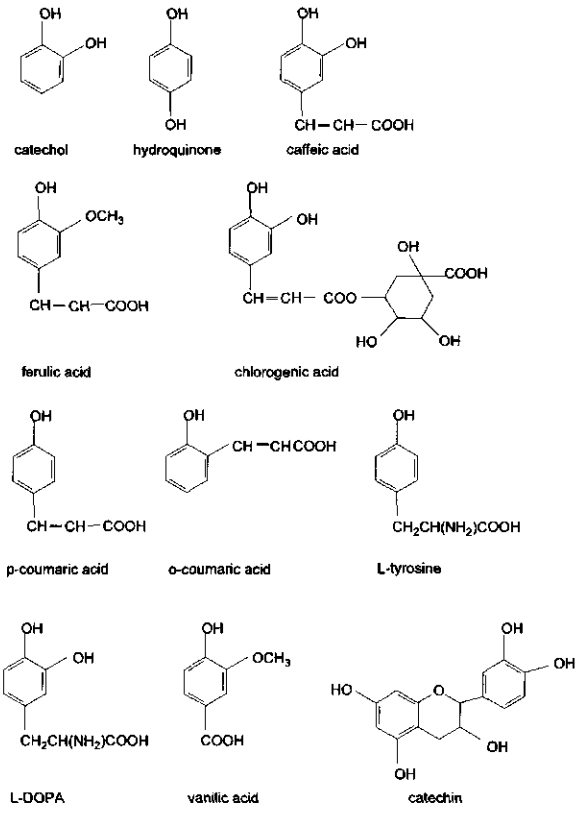
In vitro oxidative dehydrogenation reactions catalysed by peroxidase are studied in relation to lignification (31-32), cross-linking of cell wall biopolymers (1-2), and for synthesis of new chemicals (33-34). One of the peroxidase-catalysed reactions with high potential applications in biochemistry, medicine, biotechnology and food and non-food industry is protein cross-linking. Cross-linking results in the modification of the native structure of proteins, which can lead to significant changes in chemical and functional properties. Cross-linking can increase the thermal stability of proteins, improve their resistance towards organic solvents and proteolytic attack, and changes their rheological properties [8, 11].

Several studies have demonstrated that oxidation of proteins and peptides with peroxidase and hydrogen peroxide results in the formation of covalent intra- and intermolecular dityrosine bonds [12-15]. This type of cross-linking is primarily encountered in fibrous, structural proteins where it improves the mechanical properties (rigidity, elasticity) of the cross-linked polymers and causes insolubility [29]. Inspired by nature, insoluble gels and films were obtained by peroxidase-mediated oxidative coupling of soluble collagens [30] and soy protein isolates [9].

Peroxidase-mediated cross-linking of proteins in the presence of hydrogen peroxide and exogenous low molecular weight phenols as hydrogen donor has also been reported [19-21]. In these studies, formation of various oligomers and polymers of casein, lysozyme and soybean protein was demonstrated using SDS-PAGE and size exclusion chromatography. Stahmann and coworkers (1977) [19] showed that cytochrome, β-lactoglobulin, bovine serum albumin and ovalbumin formed covalent and non-covalent protein polymers.

Although it has been established that the peroxidase-mediated oxidation of proteins with a phenolic mediator can result in the formation of covalent cross-links, little is known about the effect of (i) protein conformation and (ii) structure and reactivity of the phenol mediator on the

efficiency of crosslinking, the type of products formed and the mechanism that governs the cross-linking reaction. Therefore, we addressed in the present study the peroxidase/phenol-mediated oxidative coupling of β-casein, a protein with a flexible open structure, and compared the results with data for the cross-linking of two globular proteins, α-lactalbumin and bovine serum albumin. With all three proteins, the formation of protein-protein adducts was investigated for a range of mono-, di-, and polyphenols with different ring substitution pattern (Scheme 1). Characterisation of these reactions provided valuable insight into the reactivity of phenolic mediators in protein cross-linking and yielded useful information for potential applications.



Scheme 1. Phenolic derivatives used in the cross-linking experiments.

Materials and methods

Chemicals

Horseradish peroxidase (EC 1.11.1.7, RZ > 3.0, Type VI, 250 U/mg), α-lactalbumin (calcium depleted), bovine serum albumin (fraction V), protein standards and all phenols used, with exception of L-tyrosine, were obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Bovine β-casein (90 % β-casein based on weight, 95 % β-casein based on nitrogen (w/w)) was obtained from Eurial (Rennes, France) and contained mainly the genetic variants A^1 and A^2. Hydrogen peroxide (30 % solution) and L-tyrosine were from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Oxidative cross-linking of proteins with peroxidase/hydrogen peroxide/phenol

Reactions were performed at 20°C under stirring in a volume of 1 ml. In all experiments, the enzyme-substrate ratio (w/w) was maintained at 10^{-3}, as it was previously determined [21]. Hydrogen peroxide was added in excess as related to the phenolic substrate, but the concentration was always below 10 mM to avoid enzyme inactivation and undesired protein degradation. The standard model system consisted of 10 mg/ml protein dissolved in 100 mM citrate-phosphate buffer, pH 6, and a phenol, added to give a molar ratio of phenol to protein tyrosine of 2:1. The incubation mixture also contained 10 mM hydrogen peroxide and 0.2 μM peroxidase. Five 10 μl aliquots of a 45 mM phenol solution were added to the mixture during 30 minutes. Aliquots were withdrawn at different reaction times and analyzed by SDS-PAGE to follow the progress of the reaction. After 2 hours of incubation, aliquots of the reaction mixture were diluted with the corresponding buffers for SDS-PAGE and SE-HPLC, respectively, and either analyzed immediately or frozen in liquid nitrogen and stored at -20 °C until further analysis. All experiments were performed at least in duplicate. Variations to the standard procedure are reported in the Results section.

Size exclusion chromatography

SE-HPLC analysis was carried out at room temerature using a Waters HPLC system consisting of a Waters 600E solvent delivery/control system with a Waters 717 automatic sampler injector and a Waters 2487 Dual Wavelength Absorbance Detector. The column, a TSK-Gel G-2000 SW_{XL} (300x7.8 mm), was fitted with a matching guard column (TSK-Gel SW, 7.5x7.5 mm). Both columns were from TosoHaas (Tokyo, Japan). Before analysis, samples were diluted with the running buffer and filtered through 0.45 µm Orange Scientific Gyrodisc-PES 13 filters. Then, 20 µl of the supernatant was injected into the column. The components were eluted with 0.1 M sodium phosphate buffer pH 7.0, containing 0.3 M NaCl, at a flow rate of 0.25 ml/min, and detected at 214 nm, 280 nm and 320 nm. For β-casein samples, the eluent contained also 1 % SDS, to minimize casein self-aggregation. The reagent proteins were identified by analysis of untreated blanks. The gel filtration column was calibrated with standards of known molecular mass. Standards were α-lactalbumin (14.2 kDa), chymotrypsin (25.7 kDa), bovine serum albumin (66 kDa), aldolase (158 kDa), catalase (232 kDa) and blue dextran (2000 kDa). The size exclusion limit of the column was ≈ 300 kDa. The relative abundance of reagent protein and reaction products in the reaction mixture was expressed in percentage of the total peak area determined at 280 nm.

Gel electrophoresis

SDS-PAGE was performed on a Pharmacia Phast system using 5-20 % gradient gels. Pharmacia HMW and LMW kits for SDS-PAGE were used as standards. Samples were diluted to an appropriate concentration in 10 mM Tris-HCl buffer pH 8, containing 2.0 % SDS and 5 % ß-mercaptoethanol. Samples were heated at 100 °C for 5 minutes in SDS buffer before loading onto the gels. Gels were stained with Coomassie Blue R-250.

Results and discussion

Effect of secondary and tertiary structure of proteins on cross-linking

β-Casein: Treatment of β-casein with peroxidase and hydrogen peroxide in the presence of catechol as mediator, at pH 7, resulted in almost complete conversion of the protein into high molecular weight polymers (Figure 1). The polymeric casein-adducts eluted with the void volume. About 80 % of the casein was transformed in polymer adducts, as determined from the percentage of the area of the eluting peaks (trace 2, in Figure 1). In control experiments performed under identical conditions, but without catechol, no significant conversion of β-casein occurred (trace 1, Figure 1).

The insert in Figure 1 shows the SE-HPLC elution profile of the incubation mixtures with detection at 320 nm. The protein fraction, ascribed to polymeric β-casein adducts, has high absorption at 320 nm (trace 2, insert Figure 1) whereas the unmodified β-casein has no absorption at this wavelength (trace 1, insert Figure 1). Since the absorption at 320 nm is indicative for intermolecular C-C linkages of aromatic residues, this supports that the high molecular weight protein fraction corresponds to covalently cross-linked protein and not to non-covalently linked casein aggregates.

The formation of covalently-linked adducts of β-casein was verified by SDS-PAGE (Figure 2). Most of the β-casein protein material did not penetrate into the gel (lanes 3 to 6) as a result of the peroxidase-catechol induced cross-linking reaction. In contrast, treatment of β-casein with either catechol-hydrogen peroxide (lane 9) or peroxidase-hydrogen peroxide (lane 10) did not result in polymer formation.

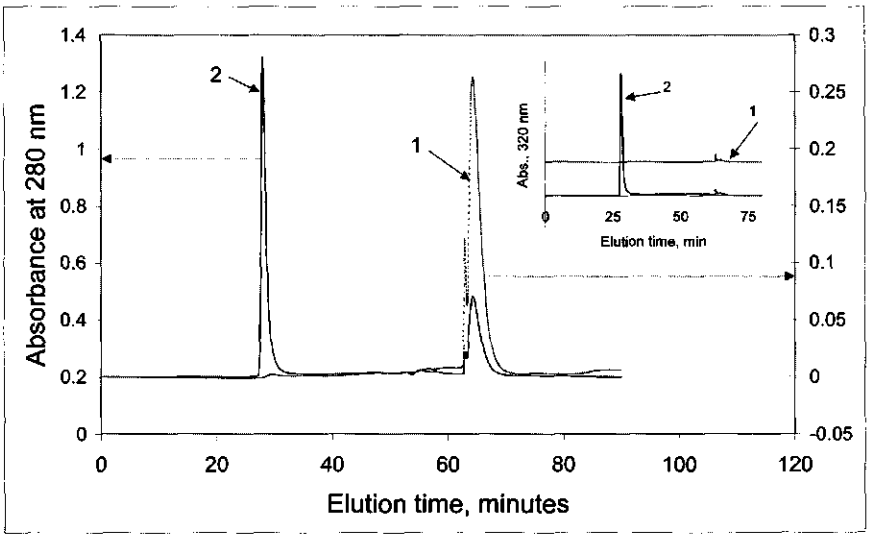


Figure 1. SE-HPLC of 1 % β-casein (0.4 mM), incubated with 0.2 μM peroxidase and 10 mM hydrogen peroxide: (1) during 2 h without catechol, (2) during 2 h with 2.75 mM catechol. The insert shows the elution patterns of the same samples, detected at 320 nm.

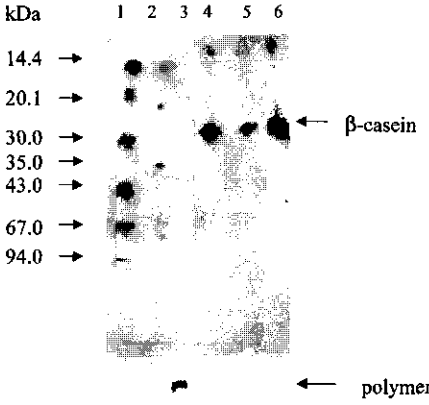


Figure 2. SDS-PAGE gel of a 1 % solution of β-casein, at pH 7.0 with 10 mM hydrogen peroxide, and 0.2 μM peroxidase, 2 h at 20°C: (lane 3) with 2.7 mM catechol; (lane 4) β-casein blank; (lane 5) with 2.7 mM catechol, without peroxidase; (lane 6) without catechol. Lanes 1 are 2 are protein standards.

Figure 3 shows the effect of the pH on the amount and type of products formed during incubation of β-casein with the peroxidase – hydrogen peroxide - catechol system. Since β-casein is insoluble in acidic solutions, the experiments were performed at pH values ranging from pH 5.5 - 9.0. Almost complete polymerisation of β-casein into high molecular weight adducts was observed between pH 5.5 and pH 7.0. In this pH range the activity of horseradish peroxidase is high and therefore, the cross-linking reaction is controlled by the very open and flexible conformation [25] of the β-casein target protein.

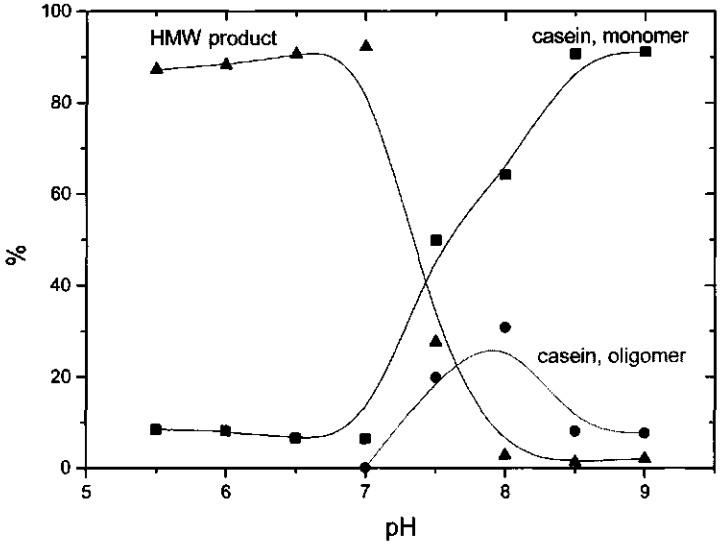


Figure 3. Influence of pH on cross-linking of β-casein with horseradish peroxidase/hydrogen peroxide/catechol. Incubation conditions: 10 mg/ml β-casein, 0.2 μM peroxidase, 2.7 mM catechol, 10 mM hydrogen peroxide, 2 hours at 20 °C. The following buffers were used: 100 mM citrate-phosphate for the pH range between pH 5.5 and 7, and 100 mM borate above pH 7.0. The relative percentage of protein species in the reaction mixture was determined from SE-HPLC data, based on the estimation of peak area.

Polymerization of β-casein by the peroxidase/hydrogen peroxide/catechol system was strongly inhibited above pH 7. At pH 8, mainly protein oligomers were formed. Above pH 8.5, nearly no cross-linking was observed due to enzyme inactivation.

Bovine serum albumin: Treatment of bovine serum albumin (BSA) with peroxidase/hydrogen peroxide and catechol as mediator, at pH 7.0, resulted in the formation of dimers (elution time = 32.5 min; n = 2.1), trimers (elution time = 30.8 min; n = 2.7) and tetramers (elution time = 28.6 min; n = 3.8) (Figure 4, trace 3). The degree of polymerization, n, was calculated from the chromatographic data. No significant cross-linking was observed when BSA was incubated with peroxidase/hydrogen peroxide in the absence of catechol (Figure 4, trace 2).

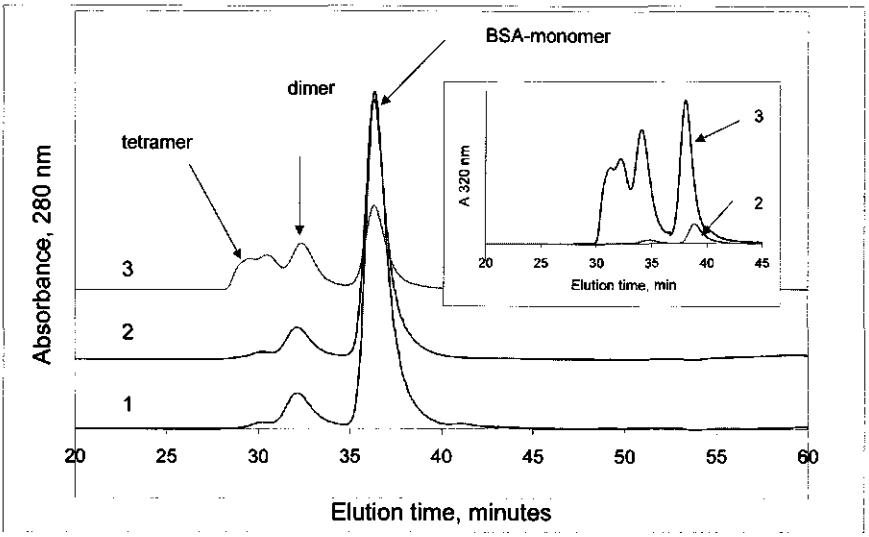


Figure 4. SE-HPEC of 1 % BSA (0.15 mM), at pH 7, with 0.2 μM peroxidase and 10 mM hydrogen peroxide, after 2 h at 20 °C: (1) BSA blank, (2) after 2 h without catechol, (3) after 2 h with 2.75 mM catechol. The insert shows the elution profile of samples (2) and (3), with detection at 320 nm.

The insert of Figure 4 depicts the SE-HPLC elution profiles of the peroxidase-treated BSA samples with detection at 320 nm. All peaks corresponding to BSA adducts formed during the reaction with catechol (trace 3) showed significant absorption at this wavelength, suggesting that the cross-linking occurs through the formation of intermolecular C-C aromatic linkages. However, the peak corresponding to monomeric BSA also showed significant absorption at 320 nm, indicative for the formation of intramolecular aromatic linkages. This suggests that the generated catechol radicals not only react with exposed tyrosyl residues of BSA, but also with more buried tyrosine moieties. These long-lived tyrosyl radicals might also react with each other and generate dityrosines (C-C coupling) or isotyrosines (C-O coupling). A similar mechanism has

been proposed by Ostdal et al. [18] for the role of free tyrosine as radical-transfer mediator between peroxidase and serum albumin. In this study, the formation of long-lived tyrosyl radicals was substantiated from EPR studies.

From Figure 4 (insert, trace 2) it can be seen that treatment of BSA with peroxidase/ hydrogen peroxide in the absence of catechol also induced the formation of some intra-molecular C-C cross-links. This suggests the formation of BSA-derived tyrosyl radicals and is in agreement with the results of Ostdal et al. ([18]. These authors suggested that the protein tyrosyl radicals are formed through a protein-to-protein radical transfer mechanism. Similar mechanisms have been reported for radical transfer from other heme proteins upon activation with hydrogen peroxide [16-18].

The cross-linking of BSA by the peroxidase/hydrogen peroxide/catechol system was also performed at pH 4.5. Figure 5 shows the SE-HPLC elution profiles of BSA incubations at pH 7 and 4.5, respectively. Lowering the pH to 4.5 resulted in an increase of BSA conversion (70 %) and a change in product profile. At this pH value, the reaction product consisted of approximately 30 % high molecular weight species, 10 % trimer and 20 % dimer. This significant increase of reactivity of BSA at low pH is most probably a result of the conformational isomerisation of the protein. At pH < 4.3, BSA adopts a more open conformation through unfolding of domain III [26-28]. As a consequence, the accessibility of reactive amino acid moieties increases and results in more cross-linking and higher molecular weight products. For example, Cys-34, the only free thiol in BSA, is shielded in the native protein, but exposed upon unfolding. This strong nucleophile may react with quinoid intermediates generated from catechol radicals, inducing the formation of higher molecular weight BSA adducts. In this respect, unfolded BSA behaves similar to the flexible β-casein. More cross-linking may also result from higher activity of HRP at pH 4.5.

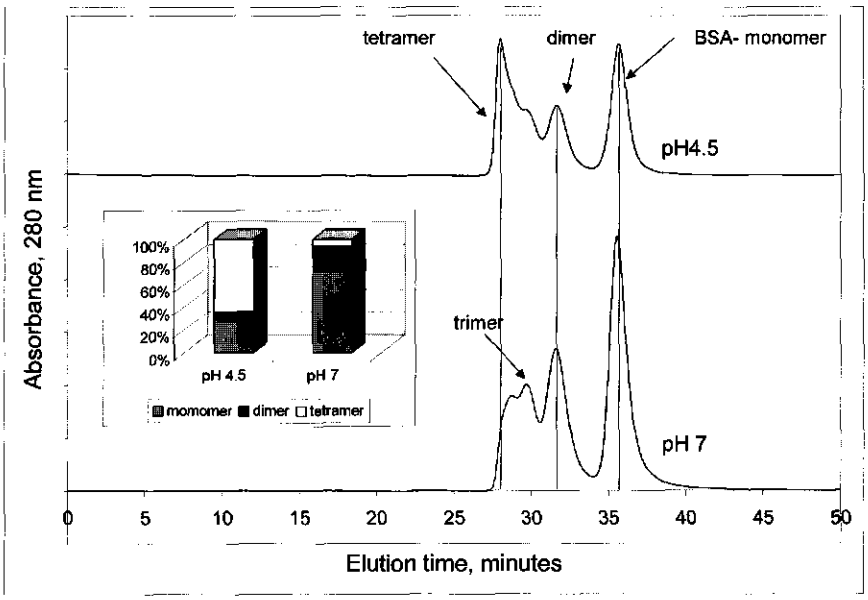


Figure 5. Effect of pH on the crosslinking of BSA with peroxidase/hydrogen peroxide/ catechol. Reaction conditions: 1 % BSA, 0.2 μM peroxidase, 10 mM hydrogen peroxide and 6.5 mM catechol, in 100 mM citrate – phosphate buffer of pH 4.5 and pH 7.0, respectively. Reaction time: 2 h at 20 °C.

α-Lactalbumin: The reaction between α-lactalbumin and the peroxidase/hydrogen peroxide/catechol system was studied at pH 7.0. SE-HPLC product analysis (Figure 6, trace 3) revealed that under the conditions applied, the reaction mixture contained about 60 % unreacted protein (M_r = 14.4 kDa). The major product eluted at 41.9 minutes, corresponding to a molecular mass of approximately 33.7 kDa (n = 2.3). Small amounts (less than 5 %) of a higher molecular weight product were observed. This product eluted at 39.1 minutes, and corresponds to a tetramer (M_r = 57.3 kDa; n = 4). Besides from the cross-linked adducts, the monomeric α-lactalbumin also showed significant absorption at 320 nm (insert Figure 6, trace 3). This indicates the formation of inter- and intramolecular aromatic cross-links, formed according to a similar mechanism as discussed for BSA.

Treatment of α-lactalbumin with peroxidase/hydrogen peroxide without catechol did not significantly change the monomeric state of the protein (Figure 6, trace 2). Moreover, under these conditions no significant intramolecular aromatic cross-links were formed (insert Figure 6, trace 2).

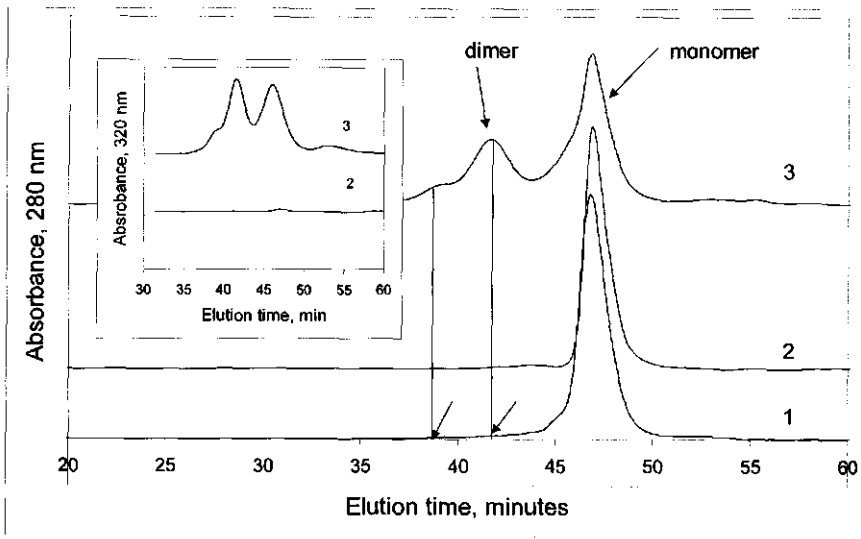


Figure 6. SE-HPEC of 1 % α-lactalbumin (0.7 mM), at pH 7, with 0.2 μM peroxidase and 10 mM hydrogen peroxide after 2 h at 20 °C: (1) α-lactalbumin blank, (2) after 2 h without catechol, (3) after 2 h with 2.75 mM catechol. The insert shows the elution profile of samples (2) and (3), with detection at 320 nm.

Effect of the concentration of catechol mediator on protein cross-linking.

The peroxidase-catalysed oxidative coupling of β-casein and BSA was studied in more detail by varying the concentration of catechol mediator between 0.1 and 4.5 mM. The products obtained were characterised by SDS-PAGE and SE-HPLC. Table 1 and 2 show the composition of products formed during the incubation of β-casein and BSA, respectively, as function of the molar ratio between the added catechol mediator and the tyrosine residues of each protein,

considering 4 tyrosine residues per mole of β-casein [25] and 21 tyrosine residues per mole of BSA [29].

Table 1. Effect of catechol concentration on the peroxidase-mediated oxidation of β-casein.

Molar ratio catechol/tyrosine	Protein species in reaction mixture (%)		
	Unreacted casein	Oligomers	Polymers
0.05	58.5	14.4	10.5
0.5	30.6	2.1	62.5
1.1	16.9	1.0	77.4
1.6	4.3	-	93.4
2.2	3.4	-	92.2
2.7	3.5	-	92.7

Reaction conditions: 1 % β-casein, 0.2 μM peroxidase, 10 mM hydrogen peroxide, pH 7.0; Reaction time: 2 h at 20°C.

Table 1 shows that an excess of catechol is required to achieve high protein conversion and formation of high molecular weight casein adducts. At a molar ratio catechol/tyrosine = 1.6, which corresponds to a molar ratio catechol/protein of 6.4, the reaction product consists of 93 % polymeric casein. At low catechol concentration, oligomers of casein are preferentially formed, indicating that the cross-linking reaction follows a sequential mechanism.

It should be mentioned here that only part of the catechol radicals initially formed reacts with the casein and induces protein cross-linking. Catechol radicals can also participate in secondary radical reactions to generate phenolic-type polymers. Formation of low molecular weight phenolic polymers has been experimentally observed in the size exclusion chromatograms of incubation mixtures, at long elution times (not shown).

Table 2 shows the effect of catechol concentration on the peroxidase-mediated cross-linking of BSA. When BSA was treated with increasing amounts of catechol at pH 7.0, up to a molar ratio

catechol/tyrosine of 1.5, increasing amounts of BSA dimers were obtained. In contrast, the formation of tetramers was optimal at a molar catechol/tyrosine ratio of 0.9. This suggests that the peroxidase-mediated cross-linking of native BSA with a phenol mediator occurs preferentially by dimerisation of the exposed tyrosyl radicals of the protein.

At pH 4.5, with partially unfolded BSA, elevation of catechol concentration stimulated the formation of BSA tetramers. This strongly suggests that under these conditions, more tyrosine residues and other potentially reactive amino acid residues are exposed and involved in the cross-linking reaction.

Table 2. Effect of catechol concentration on the peroxidase-mediated oxidation of β-casein

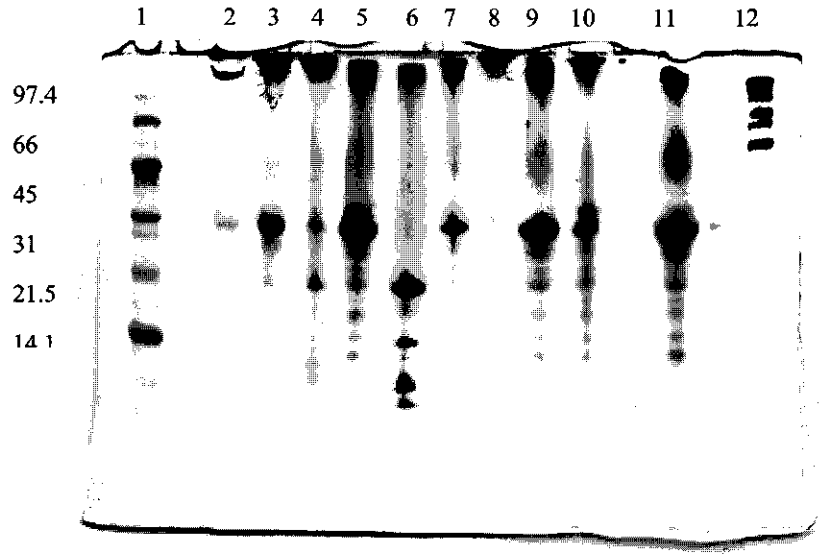
Molar ratio	Treatment at pH 7.0			Treatment at pH 4.5			
	monomer	dimer	tetramer	monomer	dimer	trimer	tetramer
0.03	86.6	11.2	0.9	66.5	12.9	8.4	3.0
0.3	81.2	14.8	2.4	54.9	17.3	11.6	8.7
0.6	76.2	17.0	5.1	44.7	20.3	13.3	17.8
0.9	68.5	18.4	11.4	33.5	22.9	11.9	32.6
1.2	67.5	21.0	6.5	26.3	23.2	-	54.2
1.5	68.0	21.3	5.6	19.1	19.9	-	66.9

Reaction conditions: 1 % BSA, 0.2 μM peroxidase, 10 mM hydrogen peroxide. Reaction time: 2 h at 20°C

Effect of phenol structure and reactivity on protein cross-linking.

To investigate the effect of the structure and reactivity of phenolic mediators on the peroxidase-hydrogen peroxide mediated cross-linking of proteins, a range of mono-, di- and polyphenols with different ring substitution was used. The structural formulas of the selected phenolic cross-linking mediators, which are all substrates for peroxidase, are depicted in Scheme 1. With the exception of catechol and hydroquinone, all these compounds are naturally occurring in plants and food products.

The protein cross-linking reactions with the different phenolic mediators were performed at pH 7.0. At this pH, the phenols are mainly present in their non-dissociated form (the pK_a of the phenolic hydroxyl group is about 10; the pK_a of the phenolic hydroxyl in cinnamic acid derivatives is between 7.4-7.6, due to the electron withdrawing effect of the unsaturated propenoic acid moiety). This is significant since (a) the reaction of the phenolic mediators with the peroxidase-hydrogen peroxide system follows the general route with two one-electron transfer steps for compound I and compound II formation (ref), and (b) the secondary reactions of phenolates are avoided. The phenolic mediators were used in a concentration of 2.7 mM, which



corresponds to the optimal ratio catechol-tyrosine experimentally determined. Figure 7 shows the SDS-PAGE gels of casein mixtures obtained upon 2 hours of incubation with peroxidase/hydrogen peroxide/ free phenol.

Figure 7. SDS-PAGE of a 1 % solution of β-casein, incubated at pH 7.0 with 0.2 μM peroxidase, 10 mM hydrogen peroxide and 2.7 mM phenol mediator for 2 h at 20°C: (lane 2) *o*-coumaric; (lane 3) caffeic acid; (lane 4) L-DOPA; (lane 5) vanillic acid; (lane 6) catechin; (lane 7) ferulic acid; (lane 8) hydroquinone; (lane 9) chlorogenic acid; (lane 10) hydroquinone; (lane 11) *p*-coumaric acid. Lane 1 contains the protein standards.

As shown above before for catechol, treatment of β-casein with caffeic acid, and hydroquinone resulted in the exclusive formation of polymeric casein adducts, which do not penetrate into the gel (lanes 3, 8). In the reactions with the other phenols, casein dimers were formed, sometimes together with casein polymers.

Interestingly, the protein bands corresponding to the non-polymerised β-casein showed an apparent molecular mass of approximately 30 kDa, corresponding to an increase in molecular mass of approximately 6 kDa with respect to untreated β-casein (cf. Figure 2, lanes 4-6). This suggests that during incubation with the peroxidase/hydrogen peroxide/phenol system, β-casein also participates in other reactions than protein-protein cross-linking. The most plausible reaction is the interaction of casein radicals with the homo-phenolic polymer radicals formed by polymerisation of free phenoxyl radicals in solution. Such hetero-coupling reactions between oligomeric phenoxyl radicals and a tyrosine-containing tripeptide have been reported by Oudgenoeg et al. [22, 23].

The amount of polymeric β-casein products formed was quantified from SE-HPLC data. Figure 8 shows the percentage of high molecular weight casein adducts formed by peroxidase-mediated oxidative coupling in the presence of the different phenolic co-substrates.

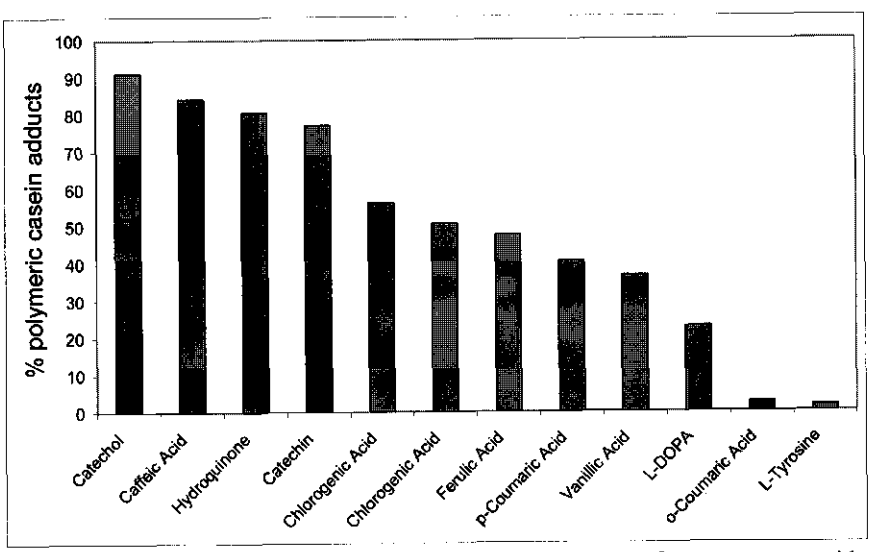


Figure 8. Efficiency of phenolic mediators in cross-linking of β-casein by peroxidase/hydrogen peroxide/phenol mediated oxidation. Reaction conditions: 1 % β-casein in 100 mM citrate-

phosphate pH 7.0, incubated with 0.2 μM peroxidase, 10 mM hydrogen peroxide and 2.7 mM phenol mediator for 2 h at 20°C.

Catechol, hydroquinone and substituted catechol derivatives (caffeic acid and catechin) were the most efficient cross-linking agents. *para*-Substituted monophenolic acids were less reactive. *o*-Coumaric acid represents a particular case. With this compound, less than 10 % conversion of β-casein into a polymeric protein adduct was obtained. This behaviour can be explained by the particular *ortho*-structure of the molecule, where the phenolic hydrogen forms an intramolecular hydrogen bond with the *ortho*-carboxyl group and consequently cannot be involved in the peroxidase-catalysed chain reaction.

The results show that there is a significant difference in the extent of casein polymerisation with catechol-type mediators as compared with mono-phenolic derivatives, such as ferulic acid. This difference can be explained by the assumption that several pathways are involved in the cross-linking reaction. The first step of the reaction is the generation of free phenoxyl radicals by one-electron transfer between the low molecular weight phenols and the active compound I and compound II of peroxidase. The free phenoxyl radicals can then further react with the phenolic moiety of protein tyrosines to generate tyrosine-derived phenoxyl radicals and eventually protein-protein adducts through C-C and C-O dityrosine cross-links. Phenoxyl radicals generated from both monophenols and *ortho*- and *para*-diphenols can react according to this pathway.

However, the phenoxyl radicals of *ortho*- and *para*-diphenols can also participate in disproportionation reactions and generate semiquinones or quinones. These quinone species can react further with other amino acid residues containing sulfur, oxygen, nitrogen or carbon nucleophilic groups, such as cysteine, methionine, serine, histidine, and tryptophan via a Michael-type addition, to form higher protein adducts. These quinone reactions may account for the extra 30 to 40 % conversion of the casein in polymer adducts.

To confirm this assumption, crosslinking of β-casein with catechol was performed in the presence of ascorbic acid (vitamin C), a reductive agent that continuously reduces *o*-quinones possibly formed during the peroxidase-catalysed reaction with catechol. Figure 9 compares the SE-HPLC profiles of reaction mixtures with low and high ascorbic acid content. At molar ratio [ascorbic acid]/[catechol] lower than 1, total conversion of casein into polymeric adducts was obtained. Increasing the molar ratio to 4 resulted in a decrease of the amount of polymeric

products formed. At high excess of ascorbic acid, the conversion of casein into high molecular weight adducts was about 30 %, in the same range as found for monophenolic cross-linkers.

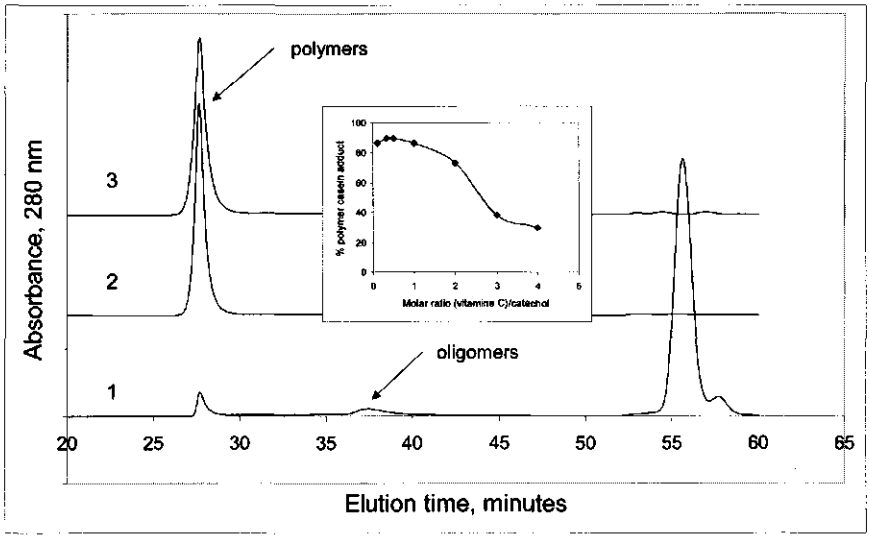


Figure 9. Effect of vitamin C on β-casein cross-linking. Reaction conditions: 1 % β-casein in 100 mM citrate-phosphate pH 7.0 incubated with 0.2 μM peroxidase, 10 mM hydrogen peroxide, 2.7 mM catechol, and 0.27 mM (trace 3), 2.7 mM (trace 2) and 10.8 mM (trace 1) ascorbic acid for 2 h at 20°C. The insert shows the yield of polymer casein adducts as function of the molar ratio [ascorbic acid]/[catechol].

Based on these results, it is concluded that cross-linking of β-casein by peroxidase/hydrogen peroxide in the presence of a catechol mediator occurs via both a radical-chain polymerisation reaction induced by the free phenoxyl radicals and a Michael-type addition of nucleophilic amino acid residues to the *o*-quinones formed.

The efficiency of ferulic acid, a monophenolic mediator, towards cross-linking of globular protein was investigated for native BSA and α-lactalbumin. Protein products were analysed by SE-HPLC and the results were compared with those obtained for incubations with catechol (Table 3). Similar product patterns and product yields were obtained for the cross-linking of BSA and α-lactalbumin, regardless of the phenolic mediator used. This suggests that cross-linking of globular proteins by the peroxidase/hydrogen peroxide/phenol sytem proceeds mainly via the radical chain mechanism.

Table 3. Cross-linking of BSA and α-lactalbumin by peroxidase/hydrogen peroxide/ferulic acid.

Mediator	BSA		α-lactalbumin
	% Dimer	% Tetramer	Dimer, %
Ferulic acid	20.0	8.2	44.3
Catechol	18.4	11.4	40.5

Reaction conditions: 1 % protein solution in 100 mM citrate-phosphate pH 7.0 incubated with 0.2 μM peroxidase, 10 mM hydrogen peroxide and 2.7 mM phenolic mediator for 2 h at 20°C.

Conclusions

Protein cross-linking initiated by the peroxidase/hydrogen peroxide/phenol system is an efficient process, resulting in the formation of protein adducts. The course of the reaction is primarily determined by the conformation of the substrate protein and the chemical properties of the phenolic mediator. Catechol derivatives are the most efficient phenolic protein cross-linking agents. The peroxidase-mediated reaction of flexible proteins with these diphenols results in the formation of protein polymers as well as intramolecular protein modifications. Cross-linking of globular proteins mainly results in the formation of oligomers. These factors should be considered when designing a process for modification of proteins for industrial application.

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Chapter IX

General discussion

1. From peptide model studies to protein-polysaccharide cross-linking

In the research described in this thesis, the possibility of *in vitro* cross-linking proteins with polysaccharides *via* endogenous or exogenous phenols by horseradish peroxidase (HRP) was explored. In order to investigate the feasibility of cross-linking *via* endogenous phenols, a first challenge (Chapters II & III) was the demonstration of covalent bond formation between tyrosine (Tyr) and ferulic acid (FA). The suggestion of such bond formation was often made since 1978 (*1*) (Chapter 1), nevertheless a Tyr-FA complex has never been isolated from biological sources, nor synthesized *in vitro*. Chapter II describes the unequivocal demonstration of the peroxidase catalyzed formation of a covalent bond between FA and a peptidyl tyrosine via a range of products found. Results presented showed that kinetic control of the addition of reactants is essential to achieve heterocoupling. The findings from the model studies were successfully applied in cross-linking of α-lactalbumin, cross-linking of α-lactalbumin with FA, and cross-linking of β-casein with arabinoxylan (Chapters IV-VI).

In order to investigate the feasibility of cross-linking *via* exogenous phenols, the first challenge (Chapter VII) was the demonstration of covalent bond formation between catechol and peptide amino acid side chains. Adducts between histidine and catechols from biological sources were reported recently (*2, 3*), but were never synthesized by peroxidase catalysis *in vitro*. Protein cross-linking in the presence of catechols has often been attributed to reaction with lysine residues. From our model studies, no evidence for this reaction was obtained. However, the peroxidase catalyzed formation of a covalent bond between FA and a peptidyl tyrosine, serine and histidine was unequivocally demonstrated (Chapter VII). The findings from these model studies were

successfully applied in cross-linking of proteins by use of catechol (Chapter VIII).

The products identified in the model studies with the tripeptide GYG and FA could be demonstrated merely due to the availability of mass spectrometry. As can be seen in Chapters II and III, no good separation of the products was obtained by RP-HPLC. Considering the range of products that can be formed and the small differences in hydrophobicity between these products, this is not inexplicable. The peptide oligomers that are formed range from monomers to nonamers and these are coupled to either one or two FA moieties. Furthermore, a range of adducts of GYG and 1 to 9 ferulic acids after 1 or 2 decarboxylations can be present as well, as described in Chapter III. On top of this, each of these products has many different regio-isomers. Consequently, the amount of possible products doubles for each additional tyrosine in an oligomeric tyrosine product and more than doubles for each additional FA. After coupling, FA can rearrange to other forms and also ring closures in FA dimers can occur. A simple calculation predicts over a 1000 products for these incubations. Even when 2D chromatography would be used to separate the products, collection of useful NMR data would be hampered by impurities.

2. Upscaling of cross-linking of GYG with FA

An effort was made to purify the products and, therefore, a large scale synthesis was prepared. This large scale synthesis could, clearly, not be performed in a 1 mL cuvet akin to the incubations leading to the synthesis of GYG oligomers coupled with 1 or 2 FA molecules (Chapter II). As a consequence, the scaled-up incubation could not be monitored spectrophotometrically as was done for the previous incubations. A fractional factorial design approach was therefore applied. Conditions for a large scale synthesis were optimized with respect to the concentration of GYG, FA and HRP and furthermore pH and temperature. A major drawback of this method is the fact that 'extreme' conditions are chosen to determine the effect of the different parameters, under the presumption that the chemistry of the reaction remains the same. Herewith, false response factors are measured when the response to variation of one parameter induces a different type of chemistry. Furthermore, the lack of direct spectroscopic monitoring prohibited feedback information on the amount of dityrosine formation and the efficiency of conversion of FA during the large scale incubation. As a consequence, the products obtained deviated drastically from those obtained during the small scale incubation.

Hardly any of the GYG oligomers, as found after the incubations that are described in Chapter II, were obtained using this approach. Instead, a range of products comprising one GYG and a range of coupled ferulic acids resulted. The mechanism of GYG-oligomer formation terminated by FA-coupling as described in Chapter II, (Figure 6), was no longer valid. Instead, FA was allowed to react with itself due to a sufficiently high incident concentration over a prolonged incubation period. In that same year a revolution in FA-chemistry, the demonstration of FA-trimers, was reported by Ward et al. (*4*) that provided an explanation for the unexpected range of products as identified by their masses after the scaled-up incubation. The identification of these products is described in Chapter III.

3. The cinnamic switch

Based on the oligomerization of FA on the template of the tyrosine in GYG, a hypothesis was postulated about the role of FA in plant cell wall growth regulation. This hypothesis was called "The cinnamic swith" (Figure 1) and is based on several known features about plant cell wall composition and might explain a missing link in the regulation of plant cell wall synthesis.

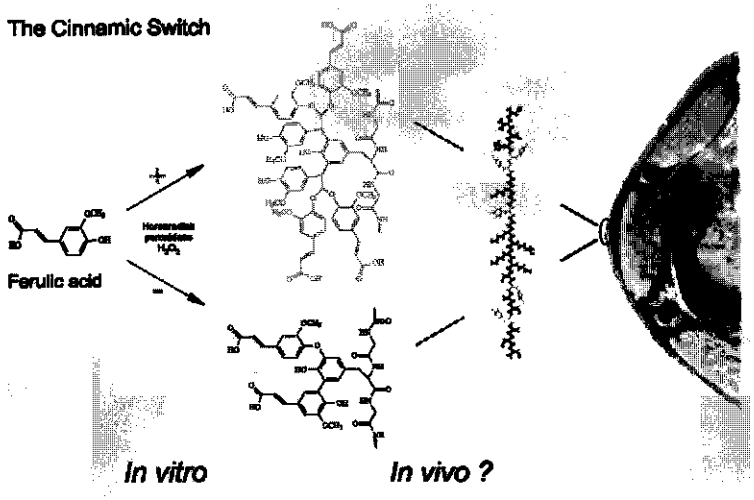

Figure 1 : The cinnamic switch

Since the decarboxylation after dehydrogenative dimerization was found to be crucial to switch between blocking or enhancing polymerization this phenomenon was called the *cinnamic* switch because it might also apply to other cinnamic acid derivatives. It is still unknown, how lignin deposition is spatially regulated in the cell. Proteins can be targeted by signal sequences but simple phenols do not have such a targeting possibility. If, however, lignin deposition is intermediated by the initial oligomerization of FA on tyrosines from targeted plant cell wall proteins, this spatial organization would be explained. On top of

that, it can be speculated that FA would be its own antagonist in lignin deposition by blocking tyrosines at low concentration (Chapter II) or allowing polymerization of lignin and increasing radical nucleation sites in high concentration (Chapter III).

4. Role of protein conformation during cross-linking

After the meticulous exploration of FA and tyrosine coupling, studies with an actual protein were started. The protein chosen was α-lactalbumin for several reasons besides the industrial availability of α-lactalbumin from bovine whey. First, bovine α-lactalbumin contains four of the target amino acid residues, tyrosine. Second, a lot is known about α-lactalbumin conformation. The crystal structure of α-lactalbumin has revealed that each tyrosine, at positions 18, 36, 50 and 103, is differently exposed on the surface of the protein (1HFZ SwissProt). Tyrosine accessibility of α-lactalbumin in solution has been investigated by various researchers with contradictory results, as discussed in Chapter IV.

In the studies described in Chapter IV, homocoupling of α-lactalbumin appeared to be far from clear-cut. The 'native' holo-protein does not participate in cross-linking at all and the apo-protein oligomerizes only moderately at room temperature. The expected dimer was not observed in these incubations (Chapter IV). At elevated temperatures a dimer was observed but always polymerized further. An interesting feature about this further polymerization is that this could not be possible from any maintained conformation of α-lactalbumin that is known from conformational studies at either low pH or in organic solvent. Polymerization beyond a degree of polymerization of 16 must involve at least two exposed tyrosines, but probably more. Therefore, it is hypothesized that the coupling reaction induces conformational changes in the resulting dimer as compared to the initial monomer. This hypothesis is now increasingly substantiated in literature on the pathogenisis of protein aggregation diseases (*5,*

6). Since α-lactalbumin is not a protein that has a role in the structural integrity of any tissue and was found to be cross-linked under certain conditions, these studies might contribute to the explanation of the formation of such aggregates. For an industrial application, the extended polymerization means that the modification of proteins is enhanced by the modification reaction. Another finding was that an elevated temperature is required for the modification reaction. A restriction using HRP is, therefore, the enzyme stability/activity.

Literature reports on the enzymatic modification of amino acid side chains in α-lactalbumin deal with lysines and glutamines but not tyrosine. Modification of lysines or glutamines in α-lactalbumin with a Ca^{2+}-independent bacterial transglutaminase did not occur in the presence of Ca^{2+} at 30°C, whilst in the absence of Ca^{2+} lysines 13, 16, 108 and 114 and glutamines 39 and 43 were modified. At pH 6 at 50°C, however, lysines 13, 16, 108 and 114 in holo-α-lactalbumin were modified but no glutamines. Under these conditions, the apoprotein was modified at Lys 5, 13, 16, 108 and 114 and Gln 39, 43, 54, 65 and 117 (*7*).

Deamidation of α-lactalbumin by protein-glutaminase at 42°C appeared to be strongly dependent on the presence of Ca^{2+} (*8*). In the presence of Ca^{2+}, the degree of deamidation was 20% after 4 h and 55% after 24 hours whilst in the absence of Ca^{2+}, 61% deamidation was reached after 4 hours and 66% after 24 hours. An interesting cooperativity was observed for the deamidation of α-lactalbumin; once the first glutamine is deamidated, the newly formed carboxyl group enhances the unfolding of the protein via electrostatic repulsion, thereby increasing the possibility of modification of other glutamines. This finding somewhat resembles our hypothesis that polymerization of α-lactalbumin is stimulated after the first cross-link is formed.

Both the homo-cross-linking of two proteinyl tyrosines (inter- or intra-molecularly) and the radical combination of proteinyl tyrosines with other

phenols is increasingly investigated. In Chapters IV and V of this thesis the tyr-tyr radical combination and the hetero-combination of tyrosine with a different phenol radical, FA, are studied, respectively. The homo-cross-linking of tyrosines in globular proteins is especially interesting in relation to the conformational state of the protein. In biology such cross-linking can be highly undesired, whilst for industrial processing of globular proteins this can be desired for the formation of films, or to improve the texture of food systems. Localization of reactive tyrosines can provide an explanation for *in vivo* cross-linking of proteins after oxidative damage and can enable cross-linking of industrially relevant globular proteins.

5. Towards the identification of modified tyrosines in α-lactalbumin

The identification of the tyrosines participating in the radical combinations at different α-lactalbumin oligomers is of great interest. As in the identification of the products from incubation with model compunds (Chapters II and III), mass spectrometry is the method of choice. Proteolytic digestion of proteins followed by mass spectrometry has become a standard tool in protein sequencing. In the identification of cross-linked moieties after disulfide cross-linking of protein, mass spectrometry has also successfully been applied (*9-17*). Also, the identification of post-translational modifications like phosphorylation are routinely performed with MS. Yet, several difficulties are met in the identification of cross-linking products (homo- and hetero) of α-lactalbumin.

First, software for the particular purpose of identifying peptides in a digest of cross-linked and modified peptides is not commercially available. The software we designed can calculate masses of peptides with charges ranging from 1^+ to 5^+. Furthermore, the peptides that are calculated can have missed cuts ranging from 1 to 3 missed cleavages. Finally, these peptides can comprise cross-linked peptides resulting from cross-links of all 4 tyrosines in α-lactalbumin. The

software was designed in reverse order as compared to the process of cross-linking and subsequent digestion. First, a database is created of all possible peptides resulting from digestion of the protein sequence that is submitted. Then, all tyrosine containing peptides resulting from this are *in silico* cross-linked. Also peptides containing 2 tyrosines, if cleavages are missed or if tyrosine are close in the primary sequence, are cross-linked *in silico* and allowed to polymerize *via* both tyrosines. Finally, all peptides resulting from this are charged and yield the output as seen on screen. Ultimately, all possible peptides that contain tyrosine can be modified with 1 or 2 FA molecules after dehydrogenative coupling. Therefore this in-house designed sotware was named *MADCoMP*: Mass Analysis Digested Cross-linked or Modified Proteins.

Second, as is apparent from Chapters II-V, peroxidase mediated cross-linking is a far from clean reaction and a plethora of products results from both homo- and hetero-incubations. In the ideal case that only one type of dimer is formed, still other difficulties are met with respect to the quantity of identifyable dimerized peptides cut from the α-lactalbumin dimer. Since two peptides must be cleaved at 4 sites from the dimer, a great number of possible dimerized peptides can result. If on each cleavage site 1 cleavage is missed already 16 different peptides result from the same tyr-tyr cross-link, after missing 3 cleavage sites even 81. The yield of dimer has therefore to be maximized in order to enable detection above the noise-level. So for identification of the tyrosine responsible for the dimerization reaction, purification of the dimer was required. But as learned from the homo-incubations (Chapter IV), the dimer is an intermediate product in extended polymerization so prolonged incubations are undesired to obtain dimer on a 'large scale'. Therefore, incubations were carried out with an increasing amount of monomer and a fixed amount of added H_2O_2. In this fashion, an optimum can be found between the formation of undesired higher oligomers at low concentrations and the formation of M* (modified monomer, Chapter IV) at high concentrations, that statistically favour

monomer+monomer combinations, due to the continuous excess of monomer as compared to dimer or trimer. From these incubations the optimal production method of dimer was determined and applied to preparative SDS-PAGE. The dimer band was then excised from the gel and the dimer was brought into solution by electro-elution. Subsequently, the dimer was incubated with pepsin or endoproteinase Glu-C. Analysis of the digests using LCMS or MALDI-TOF led not to the assigment of modified tyrosines. Also unpurified mixtures from hetero-incubations of α-lactalbumin with FA were digested with pepsin and endoproteinase Glu-C and analyzed by RP-HPLC-MS-MS. Furthermore, an incubation of α-lactalbumin modified with FAAE was digested and analyzed by MS. FAAE was chosen in order to utilize the characteristic loss of the arabinose group after collission induced dissociation as a mass label for the modified tyrosine containing peptide from α-lactalbumin. However, no assigment of modified tyrosines was achieved.

5. Use of catechol as exogenous phenol

Another approach to cross-link biopolymers is the use of diphenols as exogenous phenols. Herewith the number of possible reactive sites in a protein can be increased. In the specific case of catechol, not only radical combination reactions but also nucleophilic attacks can contribute to the cross-linking reaction. In Chapters VII and VIII, studies with catechol are described. Again, like in the studies with FA, studies with model peptides preceded the incubations with actual proteins. In this case catechol was incubated with peptides containing possible reactive amino acid side chains. The finding that serine can react with catechol has never been reported. Again, this finding may provide explanations regarding plant cell wall architecture since many tandem repeats in cell wall proteins are extremely rich in serine. Also the reaction of catechol with tyrosine has not been reported before and might play a role in plant cell wall architecture as well. The involvement of tyrosine in cross-linking

mediated by catechols has always been attributed to catalysis by tyrosinase. From our findings (Chapter VII) it can be concluded that also peroxidases can induce Tyr-catechol cross-linking. Catechol adducts with histidine have been isolated from insect cuticulae but have never been reported after *in vitro* synthesis. The most surprising finding, however, was that no adducts of catechol with lysine could be synthesized. Any protein cross-linking by use of catechol has generally been attributed to nucleophilic attack by lysine. Since we could very well synthesize and analyze catechol adducts with tyrosine and histidine, it is not likely that lysine can be cross-linked with catechol. Therefore, ascriptions of lysine as the responsible moiety for catechol mediated protein cross-linking might be reconsidered. Nonetheless, from the model studies in Chapter VII it is clear that catechol introduces a lot of 'cross-linking potential' to peroxidase in increasing the number of potential reactive sites in the protein.

Incubations of β-casein, α-lactalbumin and BSA with HRP, hydrogen peroxide and catechol revealed that the extent of cross-linking is increased as compared to incubations in the absence of catechol (Chapter VIII). Furthermore, *ortho*- and *para*-diphenols were demonstrated to be more efficient in enhancing protein polymerization than monophenolic acids. All three proteins were cross-linked, but only β-casein was efficiently converted into high molecular weight polymers. Cross-linking of the globular proteins α-lactalbumin and bovine serum albumin mainly resulted in the formation of oligomers, illustrating again that the conformation of the protein plays a crucial role in the degree of cross-linking.

Chapter VI describes the preparation of heterologous conjugates between β-casein and feruloylated arabinoxylan. These studies nicely demonstrate that the peroxidase mediated cross-linking of proteins and polysaccharides can be optimized using strict kinetic control.

Recommendations for further research

Highly interesting studies could be performed by using all permutations of replaced tyrosines in α-lactalbumin in exactly the same incubations as described in Chapter IV and V of this thesis. Such studies would allow the identification of the tyrosine that starts the cross-link formation if this is indeed one particular tyrosine. Concommitantly, information would be obtained about the conditions minimally required to allow this tyrosine to react. Furthermore, replacing the second reactive tyrosine could, if this is indeed only the second tyrosine to react, confirm the postulated theory of further unfolding and concommitant polymerization of α-lactalbumin. If no dimerization occurs after replacing the first tyrosine and only dimerization occurs upon knocking out the second tyrosine but the proteins with four tyrosines polymerizes as found in Chapter IV, this is validated.

For a facile identification of cross-linked peptides in α-lactalbumin by mass spectrometry by straightforward proteolytic digestion and no separation, a labeled form of α-lactalbumin would be of great benefit. This label could be an esterification of all lysines e.g. with either deuterated methanol or non-deuterated methanol. If an incubation is carried out with 50% of labelled α-lactalbumin and 50% unlabelled α-lactalbumin, all cross-linked peptides will yield triplets separated by the mass of the label in the mass spectrum. A prerequisite for this reaction is that the reactivity of tyrosine is not significantly altered by the labeling reaction. Also proteolytic digestion would need further elaboration since cleavage sites might be altered by the label.

Growing plants fed with a well defined range of FA would be interesting for many reasons. FA was proven to be incorporated into lignin. A concentration effect of the incorporation on itself would be interesting therefore as well. Furthermore, the cinnamic switch, i.e. whether lignin growth is blocked or

enhanced before and after a critical concentration of FA could be tested (Chapter III).

Industrial relevance

This project was started exploring conditions yielding protein-polysaccharide cross-linking with a long term industrial objective, as to improve gelling, foam stabilizing and emulsifying properties of food proteins by attachment of polysaccharides to these proteins. FA is esterified to certain industrial available polysaccharides, wheat arabinoxylans and sugar beet pectins, and tyrosine is present in most industrial relevant proteins. It was demonstrated unambiguously that FA and tyrosine can be cross-linked (Chapter II, III). The method required to cross-link two substrates (phenol containing biopolymers) by peroxidase has been patented and is currently further investigated for commercialization. The industrial reality differs from the model studies described in Chapters II-V in various ways. The use of FA or the arabinose ester of FA as a model for FA containing polysaccharides merely represents the chemical properties of FA; steric factors are not included in such model. The probability for hetero-complex formation increases with the number of reactive sites in the biopolymer. This applies both for the polysaccharide and the protein. For an industrial application it should also be taken into account that the concentrations of peroxidase used to obtain reasonable speed in the cross-linking of α-lactalbumin are rather high. A ratio of enzyme to proteins of 1:100 was necessary to stay within reasonable reaction times for the system of α-lactalbumin that had no impurities at all and was sufficiently concentrated. Therefore, for application in food products only the production of an ingredient of cross-linked proteins and polysaccharides might be realistic. Still, as shown in Chapter VI, for that purpose, carefully chosen incubation conditions must be applied and kinetic control of the incubation is a first prerequisite.

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Summary

Peroxidases catalyze the one electron oxidation of phenolic substrates. In biology, the oxidized phenols react to products that contribute to the functioning of both plants and animals. Examples of these reactions involving tyrosine and ferulic acid are described in **Chapter I.** In particular, the radical combination mediated coupling of tyrosines in proteins or ferulic acids esterified to polysaccharides is outlined. These homo-cross-linking reactions of proteins with proteins and polysaccharides with polysaccharides have been reported after *in vitro* use of peroxidases as well (**Chapter I**). The challenge in the research described in this thesis was the development of a method to establish the hetero-cross-linking reaction of proteins with polysaccharides. A first prerequisite for the cross-linking of proteinyl tyrosine and polysaccharide ferulic acid was the chemical feasibility of the coupling of tyrosine with ferulic acid and a method to favour this hetero-coupling over the homo-reaction of tyrosine or ferulic acid.

Chapter II describes the method that leads to hetero-coupling of a peptidyl tyrosine and ferulic acid, that was unambiguously demonstrated by LC-MS-MS analysis. The tyrosine-containing peptide Gly-Tyr-Gly (GYG) was oxidatively cross-linked by horseradish peroxidase (HRP) in the presence of hydrogen peroxide. As products, covalently coupled di- to pentamers of the peptide were identified. Kinetic studies of conversion rates of either the peptide or the ferulic acid revealed conditions that allowed formation of heteroadducts of GYG and ferulic acid. To a GYG-containing incubation mixture was ferulic acid added in small aliquots, therewith keeping the molar ratio of the substrates favorable for heterocross- linking. This resulted in a predominant product consisting of two ferulic acid molecules dehydrogenatively linked to a single peptide and, furthermore, a range of dimers to pentamers consisting of two ferulic acids linked to peptide oligomers. Also, mono- and dimers of the peptide were linked to one molecule of ferulic acid. FA was concluded to block the polymerization of tyrosines by coupling to the terminal tyrosines in a oligomerizing peptide chain. Further investigation of the reaction under altered conditions led to a deviating range of products of dehydrogenatively coupled GYG and FA.

Chapter III describes how the kinetically controlled incubation of FA and GYG with HRP and H_2O_2 yielded a range of new cross-linking products. Two series of hetero-oligomers of FA dehydrogenatively linked to the peptidyl tyrosine were characterized by (tandem) mass spectrometry. One series comprises singlefold decarboxylated tetra- to heptamers of dehydrogenatively linked FA-moieties coupled with one GYG. A second series ranges from twofold decarboxylated tetra- to nonamers of dehydrogenatively linked FA-moieties coupled with the peptide. Two mechanisms for the formation of the FA-Tyr oligomers and their possible implications for the regulation of plant cell wall

tissue growth are presented. After these model studies with GYG, tyrosine-coupling of the milk protein α-lactalbumin was explored.

Chapter IV describes the results of incubations of α-lactalbumin with HRP and H_2O_2. Here, the role of protein conformation on tyrosine-tyrosine coupling and protein cross-linking was investigated. In the absence of the protein conformation stabilizing Ca^{2+}, the rate of dityrosine formation was strongly dependent on temperature. The presence of Ca^{2+} inhibited the increase in rate of dityrosine formation at higher temperatures. Incubations of α-lactalbumin in urea confirmed the direct relation between protein conformation and extent of oligomerization. Higher oligomers were only obtained at denaturing urea concentrations. Prolonged incubation of apo-α-lactalbumin at 40 °C in the absence of Ca^{2+} resulted in large covalently linked protein polymers. It is proposed that a distortion of α-lactalbumin conformation is induced by the initial coupling of two structured monomers, rendering the other tyrosines available for extended cross-linking. These findings were subsequently applied in order to cross-link FA with α-lactalbumin.

Chapter V describes the influence of the presence of FA on the HRP catalyzed oligomerization of α-lactalbumin. Ferulic acid (FA) is abundantly present in plants and indisputably plays a role in plant cell wall architecture *via* oxidative polymerization. After kinetically controlled addition of FA, all FA became coupled with α-lactalbumin or α-lactalbumin-oligomers formed during the incubation. The degree of polymerization of α-lactalbumin was reciprocal to the incident concentration of free FA; in the presence of high concentrations of FA, mainly α-lactalbumin dimers were formed. In the presence of low concentrations of FA, polymerization of α-lactalbumin proceeded to a large extent like in the absence of FA. We suggest a role of FA to prevent oxidative protein polymerization not by scavenging radicals solely, but also by blocking sites for further polymerization. A tentative model for the conversion of polymerizable α-lactalbumin to non-polymerizable α-lactalbumin by FA is presented. The next step was the HRP-mediated cross-linking of FA-containing polysaccharides with proteins.

Chapter VI describes the cross-linking of protein with polysaccharide. Heterologous conjugates of wheat arabinoxylan and β-casein were prepared via enzymatic cross-linking, using sequential addition of arabinoxylan to a mixture of β-casein, HRP and H_2O_2. Maximal formation of adducts between the β-casein and feruloylated arabinoxylan was reached at high protein to arabinoxylan ratio, in combination with a low H_2O_2 concentration, long reaction time, and a molar protein to enzyme ratio between 10^2 and 10^4. The protein-arabinoxylan adducts were separated from the arabinoxylan homopolymers by size exclusion and anion exchange chromatography. The molar ratio protein:arabinoxylan in the purified conjugates varied between 0.4 and 5.6. This is the first report on

the large scale enzymatic preparation of heterologous protein:arabinoxylan conjugates. With these studies the utilization of endogenous phenols, FA and tyrosine, was finalized. Further studies were dedicated to the use of exogenous phenols.

Chapter VII describes the possibility of HRP catalyzed formation of adducts between catechol and a number of amino acids. To explore which amino acids in proteins can be coupled with catechols by peroxidase, catechol was incubated with peptides containing tyrosine, histidine, lysine or hydroxyproline in the presence of HRP and H_2O_2. In the kinetically controlled incubation of GYG with catechol, hetero-oligomers of GYG with catechol ranging from hetero-dimers to hetero-*pentamers* were formed. Adducts of NAc-His-OMe with catechol ranged from hetero-dimers to hetero-tetramers. Covalent attachment of catechol to the side chain of the amino acids tyrosine and histidine was revealed by tandem MS of the adducts. Surprisingly, no adducts of lysine or hydroxyproline with catechol were found. It is shown that tyrosine can be coupled to catechol *via* straightforward oxidation by peroxidase. Two mechanisms for the cross-linking of catechol with proteins are proposed. These findings were subsequently applied in peroxidase catalyzed cross-linking of proteins *via* catechol and other phenols.

Chapter VIII describes the HRP-mediated cross-linking of β-casein, α-lactalbumin, and bovine serum albumin in the presence of H_2O_2 and a phenol as low molecular weight hydrogen donor. A range of mono-, di-, and polyphenols with different ring substitution pattern served as the second enzyme substrate. As products of the enzymatic oxidative cross-linking reaction, heterologous protein dimers and polymers were identified by SE-HPLC and SDS-PAGE. All three proteins were cross-linked, but only β-casein was efficiently converted into high molecular weight polymers. Cross-linking of the globular proteins α-lactalbumin and bovine serum albumin mainly resulted in the formation of oligomers. The product pattern and product yield of the cross-linking of β-casein varied with the nature of exogenous phenol. *Ortho-* and *para*-diphenols were more efficient as cross-linking agents than monophenolic acids. Possible mechanisms for the peroxidase-mediated cross-linking of proteins in the presence of phenol mediators are discussed.

In **Chapter IX** the results of the thesis are discussed in terms of the effect of protein conformation during cross-linking. A comparison is made with the cross-linking of α-lactalbumin by transglutaminase. These data substantiate the hypothesis proposed that polymerization of α-lactalbumin is stimulated after the first cross-link is formed. Also the role of exogenous phenols in cross-linking is discussed.

Next, the attempts made to identify the modified tyrosines in α-lactabumin using LC-MS-MS and MALDI-TOF are presented. In particular, the development of software identifying the modified

tyrosine in a protein digest is described and a discussion on the number of products possibly being formed is given.

Finally, the earlier mentioned possible implications of the formation of the FA-Tyr oligomers for the regulation of plant cell wall tissue growth is further discussed and visualised.

Samenvatting

Peroxidases katalyseren de 1-electron oxidatie van phenolische substraten. In de natuur reageren deze geoxideerde phenolen tot verbindingen die bijdragen aan het functioneren van zowel planten als dieren. Voorbeelden van dit soort reacties met betrekking tot tyrosine en ferulazuur zijn beschreven in **hoofdstuk I**. Met name de radicaalcombinatie van tyrosine in eiwitten of die van ferulazuur veresterd aan polysacchariden wordt uiteengezet. Ook de homo-koppelingen van eiwitten met eiwitten of polysacchariden met polysacchariden na *in vitro* gebruik van peroxidasen worden uiteengezet (Hoofdstuk I). De uitdaging in het onderzoek beschreven in dit proefschrift lag in de ontwikkeling van een methode om hetero-koppeling van eiwitten met polysacchariden mogelijk te maken. Een eerste voorwaarde voor de koppeling van een tyrosine in een eiwit met een ferulazuur dat veresterd is aan een polysaccharide is de chemische mogelijkheid van koppeling van tyrosine met ferulazuur en een methode om deze hetero-koppeling te laten domineren ten opzichte van de homo-koppeling van tyrosine of ferulazuur.

Hoofdstuk II beschrijft de methode die leidt tot hetero-koppeling van een tyrosine-bevattend peptide en ferulazuur, wat ondubbelzinnig is aangetoond met LC-MS-MS- analyse. Het tyrosine bevattende eiwit Gly-Tyr-Gly (GYG) werd oxidatief gekoppeld door mierikswortelperoxidase (HRP) in de aanwezigheid van H_2O_2. Als produkten werden covalent gekoppelde di- tot pentameren van het peptide geidentificeerd. Uit kinetische studies aan de omzettingssnelheid van zowel het peptide als ferulazuur konden omstandigheden worden afgeleid waaronder hetero-addukten van GYG en ferulazuur gevormd konden worden. Aan een incubatiemengsel dat GYG bevatte werd ferulazuur in kleine porties toegevoegd, waardoor de molaire verhouding van beide substraten dusdanig was dat hetero-koppeling mogelijk was. Dit leidde tot een hoofdproduct bestaande in twee ferulazuren die dehydrogenatief gekoppeld waren met één peptide en verder een reeks van dimeer tot pentameer GYG gekoppeld met twee ferulazuurmolekulen. Ook mono- en dimeer GYG was gekoppeld met 1 ferulazuur molecuul. De polymerisatie werd geconcludeerd gestopt te worden door het blokkeren van terminale tyrosines van een oligomeriserende peptide keten door koppeling met ferulazuur. Verder onderzoek aan deze reactie leidde tot een afwijkende reeks producten bestaande uit dehydrogenatief gekoppelde GYG en FA.

Hoofdstuk III beschrijft hoe de kinetisch gecontroleerde incubatie van FA en GYG met HRP en H_2O_2 een reeks nieuwe, door cross-linking ontstane, produkten opleverde. Twee series hetero-oligomeren bestaande in dehydrogenatief gekoppeld ferulazuur gebonden aan de tyrosine zijketen van GYG werden gekarakteriseerd met (tandem) massaspectrometrie. Één serie omvatte enkelvoudig gedecarboxyleerde tetra- tot heptameren van dehydrogenatief gekoppelde ferulazuren gekoppeld aan één GYG. De tweede serie omvatte tweevoudig gedecarboxyleerde tetra- tot nonameren van

dehydrogenatief gekoppelde ferulazuren gekoppeld met één peptide. Twee mechanismen voor de vorming van FA-Tyr oligomeren en de mogelijke implicaties hiervan voor plantcelwandgroei regulering worden gepresenteerd. Na deze model-studies met GYG, werd tyrosine koppeling van het melkeiwit α-lactalbumin onderzocht.

Hoofdstuk IV beschrijft de resultaten van incubaties van α-lactalbumin met HRP en H_2O_2. Hierin is de rol van eiwitconformatie in tyr-tyr- koppeling en eiwitverknoping onderzocht. In afwezigheid van het eiwitconformatie stabiliserende Ca^{2+}, bleek de snelheid van dityrosine vorming sterk afhankelijk van de temperatuur. In aanwezigheid van Ca^{2+} nam de snelheid van dityrosinevorming bij hogere temperaturen niet toe. Incubaties van α-lactalbumin in ureum bevestigden de directe relatie tussen eiwitconfonformatie en de mate van polymerisatie. Hogere oligomeren werden uitsluitend verkregen bij denaturerende concentraties ureum. Voortgezette incubaties van α-lactalbumin bij 40°C in de afwezigheid van Ca^{2+} leidden tot hoogmoleculaire covalent gebondden eiwit polymeren. Er wordt gesuggereerd dat een verstoring van de conformatie van α-lactalbumin wordt geinduceerd door koppeling van de hooggestructureerde monomeren, waardoor andere tyrosines beschikkbaar komen voor verdere verknoping. Deze bevindingen werden vervolgens toegepast om FA te koppelen aan α-lactalbumin.

Hoofdstuk V beschrijft de invloed van de aanwezigheid van FA op de HRP gekatalyseerde oligomerisatie van α-lactalbumin. FA is in overvloed aanwezig in planten en speelt ontegenzeggelijk een rol in de architectuur van de plantencelwand via oxidatieve polymerisatie. Na kinetisch gecontroleerde toevoeging van FA werd alle FA gekoppeld met α-lactalbumin of oligomeren van α-lactalbumin die tijdens de incubatie werden gevormd. De graad van polymerisatie van α-lactalbumin was omgekeerd evenredig met de momentane concentratie vrij FA.; in aanwezigheid van lage concentraties FA werden voornamelijk α-lactalbumin dimeren gevormd. In aanwezigheid van lage concentraties van FA verliep de polymerisatie van α-lactalbumin goeddeels als in afwezigheid van FA. We suggereren dat FA oxidatieva eiwitpolymerisatie voorkomt door niet alleen radicalen weg te vangen maar ook door reactieve groepen in het eiwit te blokkeren. Een hypothetisch model voor het modificeren van polymeriseerbaar α-lactalbumin tot niet polymeriseerbaar α-lactalbumin met FA wordt gepresenteerd. De volgende stap was het gebruik van FA-bevattende polysaccharides met eiwit.

Hoofdstuk VI beschrijft de verknoping van eiwit met polysaccharide. Heterologe conjugaten van arabinoxylaan en β-casein werden verkregen door enzymatische verknoping, gebruikmakend van sequentiele toevoeging van arabinoxylaan aan een mengsel van β-casein, HRP en H_2O_2. Maximale vorming van adducten tussen β-casein en geferuloyleerd arabinoxylaan werd bereikt bij een hoge verhouding eiwit : arabinoxylaan, in combinatie met een lage concentratie H_2O_2, lange reactie tijden

en een molaire verhouding eiwit:enzym tussen 10^2 en 10^4. De eiwit-arabinoxylaan adducten werden gescheiden van de arabinoxylaan homopolymeren met behulp van gelfiltratie en anion-wisseling chromatografie. De molaire verhouding eiwit:arabinoxylaan in de gezuiverde conjugaten varieerde tussen 0.4 en 5.6. Dit is de eerste melding van het op grote schaal enzymatisch verkrijgen van eiwit-arabinoxylaan conjugaten. Met deze studies werd het gebruik van endogene phenolen, FA en tyrosine, afgerond. Verder onderzoek betrof het gebruik van exogene phenolen.

Hoofdstuk VII beschrijft de mogelijkheid van de door HRP gekatalyseerde vorming van adducten tussen catechol en een aantal aminozuren. Om te ontdekken welke aminozuren in eiwitten gekoppeld kunnen worden met catechol door peroxidase, werd catechol geincubeerd met peptiden die tyrosine, histidine, lysine of hydroxyproline bevatten, in aanwezigheid van HRP en H_2O_2. In de kinetisch gecontroleerde incubatie van GYG met catechol, werden hetero-oligomeren van GYG met catechol varierend van hetero-dimeren tot hetero-pentameren gevormd. Adducten van N-Ac-His-OMe met catechol varieerden van hetero-dimeren to hetero-tetrameren. Covalente binding van catechol met de aminozuurzijketens van tyrosine en histidine werd bewezen met tandem MS van de adducten. Merkwaardig genoeg, konden geen adducten van lysine of hydroxyproline met catechol worden aangetoond. Er wordt getoond dat tyrosine met catechol kan worden gekoppeld via rechtstreekse oxidatie door peroxidase. Twee mechanismen voor het verknopen van catechol met eiwitten worden voorgesteld. Deze bevindingen werden vervolgens toegepast in peroxidase gekatalyseerd verknopen van eiwitten via catechol en ander phenolen.

Hoofdstuk VIII beschrijft het met HRP verknopen van β-casein, α-lactalbumin, en BSA. In aanwezigheid van H_2O_2 en een phenol als LMW waterstof-donor. Een serie mono- di- en polyphenolen met verschillende substitutie op de aromaat dienden als tweede substraat. Heterologe eiwit dimeren en polymeren werden mbv SE-HPLC en SDS-PAGE geidentificeerd als reactieproduct. Alledrie de eiwitten werden verknoopt, uitsluitend β-caseine werd efficient omgezet in hoog moleculaire polymeren. Verknoping van de globulaire eiwitten α-lactalbumin en BSA leidde voornamelijk tot de vorming van oligomeren. De aard van de produkten en de opbrengst van het verknopen van β-casein was sterk afhankelijk van het soort exogeen phenol. *Ortho-* en *para-*diphenolen leidden tot een efficientere verknoping dan monophenolen. Mogelijke mehanismen voor het met peroxidase verknopen van eiwitten in aanwezigheid van phenolen worden bediscussieerd.

In **hoofdstuk IX** worden de resultaten van dit proefschrift bediscussieerd met betrekking tot eiwitconformatie tijdens de verknoping. Een vergelijking wordt getrokken met het effect van α-lactalbumin verknoping met transglutaminase. Deze bevindingen ondersteunen de hypothese dat verdere polymerisatie van α-lactalbumin mogelijk wordt gemaakt nadat de eerste verknoping heeft

plaatsgevonden. Ook de rol van exogene phenolen bij verknopingsreacties wordt bediscussieerd. Vervolgens worden de pogingen gepresenteerd die zijn ondernomen om gemodificeerde tyrosines in α-lactalbumin te identificeren mbv LC-MS-MS en MALDI-TOF. In het bijzonder wordt de ontwikkeling van software om gemodificeerde petides in een digest te identificeren beschreven en de hoeveelheid mogelijk gevormde produkten wordt bediscussieerd. Ten slotte wordt de eerder besproken rol van de vorming van FA-Tyr oligomeren in de regulatie van plantcelwandgroei verder belicht en gevisualiseerd.

Résumé

Les peroxydases catalysent l'oxydation mono-électronique de substrats phénoliques. En biologie, les phénols oxydés réagissent aux produits contribuant au développement des plantes comme des animaux.

Le **premier chapitre** donne des exemples de telles réactions impliquant la tyrosine et l'acide férulique. C'est notamment le couplage par combinaison radicalaire assistée de la tyrosine en protéines ou l'estérification de l'acide férulique en polysaccharides qui fait l'objet de l'étude. Ces réactions de réticulation entre protéines ou entre polysaccharides ont également été décrites après l'utilisation in-vitro de peroxydases. La recherche exposée dans cette thèse a pour principal objectif le développement d'une méthode permettant à la réaction de réticulation entre protéines et des polysaccharides de se produire. Une condition préalable à l'obtention d'une réaction de réticulation entre une tyrosine protéinique et un acide férulique d'origine polysaccharidique s'appuie sur la possibilité de couplage entre la tyrosine (Tyr) et l'acide férulique (AF) ainsi qu'une méthode favorisant ce couplage par rapport à l'homo-réaction Tyr-Tyr ou AF-AF.

Le **chapitre II** décrit la méthode conduisant au couplage d'une tyrosine peptidique avec l'AF, validée par le biais d'analyses CL-SM-SM. Le peptide à base de tyrosine Gly-Tyr-Gly (GYG) fut réticulé par voie oxydante via la HRP (horseradish peroxidase) en présence de peroxyde d'hydrogène. Des di- à pentamères couplés au peptide par liaison covalente ont été identifiés comme produits de réaction. Les études cinétiques des taux de conversion tant du peptide que de l'AF ont révélé les conditions permettant la formation d'adduits entre GYG et l'AF. De petites quantités identiques d'acide férulique ont été ajoutées à un mélange à base de GYG mis en incubation, tout en gardant une proportion molaire en substrats favorisant la réaction de réticulation. Le produit majoritairement formé est composé de deux molécules d'acide férulique liées par réaction de déshydrogénation à un peptide ainsi que d'un ensemble de di- à pentamères formés de deux molécules d'AF liées à des oligomères peptidiques. Par ailleurs, des monomères et dimères du peptide se sont liés à une molécule d'AF. Cela a permis de montrer que l'AF empêche la polymérisation des tyrosines par association avec les tyrosines terminales de la chaîne oligopeptidique. Une étude plus approfondie de la réaction sous des conditions modifiées a permis d'identifier un ensemble de produits secondaires résultant du couplage par réaction de déshydrogénation entre GYG et FA.

Le **chapitre III** montre que le contrôle cinétique de l'incubation de FA et de GYG avec la HRP et H_2O_2 conduit à de nouveaux produits de réticulation. Deux séries d'hétéro-oligomères d'AF liés par réaction de déshydrogénation à la tyrosine ont été caractérisées par spectrométrie de masse en mode tandem. Une série était composée d'espèces tétra- à heptamères mono-décarboxylées, liées par réaction de déshydrogénation à l'AF et couplées à un GYG tandis que la deuxième série comportait

des structures tétra- à nonamères doublement décarboxylées, liées par réaction de déshydrogénation à l'AF et couplées à un GYG. Deux mécanismes expliquant la formation d'oligomères AF-Tyr et leur possible implication dans la régulation de la croissance des parois cellulaires des plantes sont proposés. A la suite de ces études de modèles basés sur le GYG, le couplage de la protéine α-lactalbumine du lait avec la tyrosine fut étudié.

Dans le **Chapitre IV** sont exposés les résultats relatifs aux incubations de l'α–lactalbumine avec HRP et H_2O_2. Le rôle de la conformation de la protéine sur le couplage entre tyrosines et sur la réticulation de la tyrosine avec la protéine fut étudié. En l'absence du cation Ca^{2+}, stabilisant la conformation de la protéine, le taux de formation du dimère Tyr-Tyr apparaît très dépendante de la température. En revanche, la présence du cation Ca^{2+} inhibe l'augmentation de la vitesse de formation du couple Tyr-Tyr à des températures plus élevées. Les incubations de α- lactalbumine en présence d'urée ont confirmé qu'il existe une relation directe entre la conformation de la protéine et l'importance de l'oligomérisation. Seules des concentrations dénaturées d'urée ont permis d'obtenir des oligomères de poids moléculaires plus élevés. L'incubation prolongée d'apo-α-lactalbumine à 40°C en absence de Ca^{2+} a conduit à des polymères de protéines. Il a été formulé l'hypothèse selon laquelle une modification de la conformation de l'α–lactalbumine serait une conséquence du couplage initial de deux monomères structurés, rendant les autres tyrosines disponibles pour d'autres réactions de réticulation. Ces résultats furent utilisés par la suite afin d'obtenir la réticulation de l'AF avec l'α–lactalbumine.

Le **chapitre V** décrit l'influence de la présence d'AF sur l'oligomérisation de l'α–lactalbumine catalysée par HRP. L'AF est omniprésent dans les plantes et joue incontestablement un rôle dans l'architecture des parois cellulaires des plantes via par le biais de la polymérisation oxydante. Après l'addition cinétiquement contrôlée d'AF, toutes les molécules d'AF se sont liées a l'α–lactalbumine ou aux oligomères d'α-lactalbumine formés lors de l'incubation. Le degré de polymérisation de l'α–lactalbumine est inversement proportionnel à la concentration en AF non liés ; en présence de concentrations élevées d'AF, des dimères de lα–lactalbumine ont été majoritairement formés. En présence de faibles concentrations d'AF ou en l'absence d'AF, c'est surtout la polymérisation de α–lactalbumine qui est obeservée. Ainsi, nous avons suggéré l'implication de l'AF dans le blocage de la polymérisation oxydante des protéines, non seulement par la neutralisation des radicaux mais aussi par le blocage des sites réactifs où se produit la polymérisation. Un modèle a été proposé pour tenter d'expliquer la conversion de l'α–lactalbumine polymérisable en α–lactalbumine non polymérisable par l'AF. L'étape suivante a consisté à utiliser des polysaccharides contenant de l'AF avec des protéines.

Le **chapitre VI** décrit la réticulation de protéines avec des polysaccharides. Des espèces hétéro-conjuguées d'arabinoxylane de blé et de β-caséine furent préparées via une réticulation enzymatique par addition séquentielle d'arabinoxylane à un mélange de β-caséine, de HRP et de H_2O_2. Une quantité maximale d'adduits entre la β-caséine et l'arabinoxylane a été atteinte en utilisant une proportion de protéines de haut poids moléculaire/arabinoxylane élevée, combinée à une faible concentration de H_2O_2, un temps de réaction important, et une proportion molaire protéine/enzyme comprise entre et 10^2 et 10^4. Les adduits protéine-arabinoxylane furent séparés des homopolymères d'arabinoxylane par les chromatographies d'exclusion de taille et l'échange d'anion. Le rapport molaire protéines/arabinoxylane dans les combinaisons purifiées varie entre 0,4 et 5,6. Ceci est la première étude concernant la réparation enzymatique à grande échelle de combinaisons protéine/arabinoxylane. A la suite de cette étude, l'utilisation de phénols endogènes, AF et tyrosine a pu être mise au point. Des études additionnelles relatives à l'utilisation de phénols exogènes furent également entreprises.

Le **chapitre VII** décrit la possible formation, catalysée par l'HRP, d'adduits entre le catéchol et divers acides amines. Dans l'optique d'explorer quels acides aminés de protéines peuvent être couplés à des catéchols par le biais de peroxydase, du catéchol fut incubé avec des peptides contenant de la tyrosine, de l'histidine, de la lysine ou de l'hydroxyproline en présence d'HRP et H_2O_2. Dans l'incubation cinétiquement contrôlée de GYG avec du catéchol, des hétéro-oligomères de GYG/catéchols, allant d'hétéro-dimères à hétéro-pentamères, furent formés. Des adduits de NAc-His-OMe associés à du catéchol allant d'hétéro-dimères a hétéro-tétramères furent formés. La liaison covalente de catéchol à la chaîne pendante de tyrosine et histidine a été mise en évidence par l'analyse par SM tandem des adduits. Contre toute attente, aucun adduit lysine/catéchol ou hydroxyproline/catéchol ne fut décelé. Il a été montré que la tyrosine se lie au catéchol par une oxydation directe due à la peroxydase. Deux mécanismes expliquant la réticulation du catéchol aux protéines ont été proposés. Ces résultats ont été par la suite appliqués à la réticulation entre protéines via le catéchol et d'autres phénols, catalysée par la peroxydase.

Le **chapitre VIII** décrit la réticulation de la β-caséine, de l'α-lactalbumine, et de sérum bovin assistée par HRP et en présence de H_2O_2 et d'un phénol intervenant comme un donneur d'hydrogène de bas poids moléculaire. Un ensemble de mono-, di- et polyphénols diversement substitués ont été utilisés comme substrat pour la deuxième enzyme. Les trois protéines ont été réticulées, mais seule la β-caséine fut efficacement convertie en polymères de haut poids moléculaire. La réticulation des protéines globulaires d'α-lactalbumine et de sérum bovin a principalement conduit à la formation d'oligomères. Le type de substitution et le rendement des produits issus de la réticulation de la β-caséine varient en fonction de la nature du phénol exogène. Les Ortho- et para-diphénols sont plus

efficaces comme agents de réticulation que les mono phénols acides. Les mécanismes possibles décrivant la réticulation de protéines, assistée par la peroxydase et en présence de phénol, sont discutés.

Les résultats de ce travail de thèse sont exposés dans le **chapitre IX** en termes d'effet de la conformation de protéines durant la réticulation. Une comparaison avec les effets de la conformation de l'α-lactalbumine sur sa réticulation avec la transglutaminase a été entreprise. Ces données confortent l'hypothèse selon laquelle la polymérisation de l'α-lactalbumine est stimulée après la formation de la réticulation. Le rôle de phénols exogènes dans la réticulation est également traité. Puis les tentatives entreprises pour identifier les tyrosines modifiées présentes dans l'α-lactalbumine via les techniques CL-SM-SM et MALDI-TOF sont présentées. En particulier, il est décrit le développement d'un logiciel permettant d'identifier une tyrosine modifiée dans une protéine digérée et une discussion s'ensuit sur le nombre de produits de réaction possibles. Enfin, sont discutées les implications de la formation d'oligomères AF-Tyr intervenant dans la régulation de la croissance de la paroi cellulaire des plantes, précédemment exposée.

Nawoord

De resultaten beschreven in dit proefschrift zijn tot stand gekomen met medewerking van velen, zowel binnen het projectteam als daarbuiten. Op deze plaats worden deze mensen erkend voor hun bijdrage en wordt deze kort besproken.

De promotoren: Colja Laane & Fons Voragen

Fons Voragen begroette met groot enthousiasme elke ontwikkeling in het onderzoek en voorzag deze van een historisch kader en een mogelijke toekomst. Het gezamenlijk voorbereiden van de internationaal gehouden presentaties was altijd een goed reflectief moment.

Colja Laane was als hoogleraar biochemie zeer nauw betrokken bij het project. Tijdens elke tweewekelijkse vergadering in het eerste jaar werd al gauw duidelijk wie er goed knopen door kon hakken maar ook na zijn overgang naar DSM werd geen driemaandelijkse of halfjaarlijkse IOP bijeenkomst overgeslagen. Dit garandeerde dat er bij elke vergadering minimaal 1 persoon aanwezig was die de notulen had gelezen en wist waar de grote lijn van het project lag. En mede dankzij email en mobiele telefonie kon ook vanuit Delft/Tokyo/Basel/ of de TGV voortdurend worden besproken wat de vorderingen waren.

(Ex)-Copromotoren : Harry Gruppen, Willem van Berkel, Riet Hilhorst

Riet Hilhorst stond aan de wieg van dit project en is als projectleider medeverantwoordelijk voor de voortvarende start van het onderzoek en was ook dagelijks betrokken bij de vorderingen van experiment tot experiment. Initiatieven werden altijd vooraf uitgebreid geevalueerd en achteraf werden de daaruit voortvloeiende resultaten gretig verwelkomd. Door deze intensieve begeleiding stond het project prima op de rails toen Riet de universiteit verliet.

Willem van Berkel nam vervolgens de projectleiding over van dit project waarvan hij niet aan de wieg stond maar zeker wel de rijping vorm gaf. Willems begeleiding begon telkens zodra er resultaten lagen. Vooral het gezamenlijk schrijven aan tweede versies van manuscripten werd optimaal gestalte gegeven; achteraf lag er een gestroomlijnd opgebouwd verhaal en ondertussen werd goed duidelijk wat de beperkingen van sommige resultaten waren en hoe die aangevuld zouden kunnen worden met andere experimenten.

Harry Gruppen was als enige van de tot dusver genoemde personen continu vanuit dezelfde hoedanigheid betrokken bij het project. Gedurende de gehele periode was het dan ook Harry die uit alle plannen en resultaten snel de kern destilleerde en daar zeer bruikbare adviezen aan verbond. Harry was de meest constante factor en wist bij het voltooien van dit proefschrift de weg naar Biochemie goed te vinden.

(Dagelijkse) begeleiding : Sander Piersma, Carmen Boeriu

Sander Piersma was voortdurend betrokken bij het uitvoeren van experimenten en de nodige aanwijzingen daarbij. Vanaf de eerste incubaties op de HP -en de eindeloze uitleg van de gemeten kinetiek daarna- tot de Åkta en, uiteraard, de LC-MS aan toe. Achter elk massapiekje in dit proefschrift gaat een dag staren naar een leeg scherm van de, destijds nieuwe, LC-MS schuil. Dankzij Sander werd dit vaak gevuld met nuttige adviezen mbt het project, een goed gesprek ter afwisseling daarop en verschenen er veelal uiteindelijk nog piekjes op het scherm ook. Bij het vertrek van Sander na 3 jaar was al een hoop bereikt maar verloor het project een hoop inbedding in de laatste ontwikkelingen op gebied van vele omringende disciplines. Carmen Boeriu was prominent aanwezig in het begin van het project, vooral in de organisch-chemische discussies over de mogelijke chemie achter die merkwaardige patronen in de produkten.

De studenten: Eef Dirksen & Ralph Meulepas

De leukste tijd van het project was die met twee, destijds in de dop verblijvende, jonge wetenschappers. Zowel wetenschappelijk als moreel waren zij een grote versterking voor het project. Een werkdag op onze gedeelde kamer liet zich makkelijk meten met een cafebezoek. Deze lijn hebben we dan ook doorgezet na hun afstuderen maar ook kwamen beiden juist terug om te werken op vrije dagen, om te helpen bij aanvullende proeven. Het succes van de op deze manier ingevulde samenwerking is terug te lezen in de drie gezamenlijke hoofstukken/artikelen die hieruit voort zijn gekomen.

De resultaten beschreven in dit proefschrift zijn mede tot stand gekomen dankzij de medewerking van een groot aantal medewerkers van WUR die niet binnen het project werkzaam waren, wier rol ik waar deze hun functieomschrijving in ruime mate overstijgt bij deze noem.

Leden van Grote verdiensten: Marcel Meinders, Huub Haaker

Marcel Meinders mag hierbij als lichtend voorbeeld worden genoemd en heeft tijdens dit onderzoek bewezen dat het wel degelijk nut heeft een hoop onderzoekers niet ieder hun eigen hut op de heide te geven maar tezamen in 1 gebouw te huisvesten. Het begon in 1999

toen de eerste reeksen adducten van FA&GYG met MS werden geidentificeerd dat het progamma Gidical™Marcel Meinders de massa's van deze adducten met elke mogelijke samensteling van FA en GYG en elk denkbare ladingstoestand met 1 druk op de knop op het scherm –ontworpen in gewenste kleur met tailor made interface- deed verschijnen, of ingevoerde massa's direct toekende. Dit bespaarde niet alleen enorm veel tijd maar leidde ook wel eens tot toekenningen die anders wellicht over het hoofd zouden zijn gezien. Of, waar Ralph Meulepas zeker van mee kan praten, bespaarde eindeloos rekenen aan massa's die je wel ziet maar absoluut niet toe te kennen zijn. Generaties studenten zijn groot geworden met Gidical, of het nu incubaties van FA met GYG of catechol met allerhande peptides betrof, op de markt heb ik het nog steeds niet kunnen ontdekken. Dat waren de model studies, het echte werk met eiwitten vond pas 3 jaar later plaats en massa's van op 4 tot 16 verschillende plaatsen geknipte peptides die via 123 verschillende soorten cross-links kunnen zijn gekoppeld uitrekenen is nog iets complexer. Wederom bood de gang naar Marcel Meinders uitkomst, ditmaal het progamma M.A.D.C.o.M.P., wederom naar persoonlijke wensen ontworpen; al dan niet dehydrogenatieve modificatie van welk aminozuur -natuurlijk of synthetisch- dan ook, in elk denkbaar eiwit, met een ander eiwit, FA of FA-bevattende oligosacchariden van elke graad van polymerisatie, in willekeurige mate van volledigheid geknipt met een enzym naar keuze. Dat er uiteindelijk geen gemodifieerd tyrosine is geidentificeerd heeft hier zeker niet aan gelegen.

Huub Haaker heeft een doorslaggevende rol gespeeld bij het automatiseren van de incubaties van α-lactalbumin met of zonder ferulazuur. De rapid-mix bleek na vele dagen gezamenlijk progammeren het apparaaat bij uitstek om langzaam enzym/co-substraat en substraat reproduceerbaar toe te voegen onder verschillende condities. Dankzij een half jaar incuberen en bemonsteren ontstond een goed beeld van de polymerisatie van α-lactalbumin, dat geleid heeft tot de hoofdstukken IV en V uit dit proefschrift.

Walter van Dongen en Ben van den Broek hebben veel inspanningen geleverd 'het dimeer' te isoleren, Ben met preparatieve SDS-PAGE, Walter met electroelutie. Uiteindelijk had ik dat dimeer ook zuiver in 'handen', ook aan hen heeft het niet gelegen.

Zonder specifiek te worden waren er nog vele andere medewerkers die het predikaat *mede* zeker waarmaakten wat betreft mijn werkzaamheden op 'de Dreijen' :

Pieter Walstra, Jan-Willem Borst, Mark Hink, Jolanda van den Boomgaard, Ivonne Rietjens, Willy van den Berg, Mamoudou, Ton Visser, Adrie Westphal, Mirjam Kabel, Gert-Jan van Alebeek, Laura van Egmond, Andre Stork, Hans Wassink, Yves, Hans Ippel, Erwin de Beet, Marielle, Tiny van Boeckel, Boudewijn, Henk Schols, Annemie Emons, Ans Soffers, Rob Hamer, Harmen de Jongh en Harold Bult

Verder hebben een aantal collega's die niet direct betrokken waren bij mijn werkzaamheden wel bijgedragen aan een prettige werksfeer, waarbij aangetekend dient te worden dat dat evengoed kan gelden voor voornoemde collega's die inhoudelijk meer betrokken waren: Hans Wassink, Bas Kuipers, Graca, Jean-Paul, Kerensa, Sjuuls, Peter Immerzeel, Peter Wierenga, Morten, Marianne Renkema, Sacco, Jan, Catriona, Harold Bult, Martina Duyvis, Gerrit Beldman, Jolan de Groot. Aagje, Casper, Jacques, Gerrit van Koningsveld and most of all: Fran

Annemarije Pronk en Olivier Haillant zijn verantwoordelijk voor de Franse samenvatting, die in korte periode onder hoge tijdsdruk is geproduceerd.

Ewald spieker heeft het idee achter dit onderzoek kunnen verwerken in de illustratie op de voorkant dankzij de vele 'besprekingen' in het atelier aan de groenburgwal.

Roeland Oevering heeft een prachtige etage aan de Bloemgracht gefaciliteerd alwaar in alle rust de niet gepubliceerde resultaten voor dit proefschrift zijn geschreven (H4,5).

Richard Blaauw was tijdens dit onderzoek, zoals ik alle jaren daarvoor al van hem gewend was, altijd bereid zich te verdiepen in de diverse onderwerpen. Vanaf m'n eerste artikel in Wageningen (zie acknowledgement H2) tot en met het laatst ingestuurde artikel (co-auteur R. Blaauw) werd alles doorgenomen naar goede Amsterdamse traditie.

Curriculum vitae

De auteur van dit proefschrift is geboren te Amsterdam op 15-05-1973. Het eindexamen Gymnasiun werd behaald aan het Gymnasium Bredanum in 1991. De studie scheikunde werd gevolgd aan de Universiteit van Amsterdam, in 1999 werd het doctoraal behaald in de richting Organische Scheikunde alsmede Biochemie. Promotieonderzoek werd uitgevoerd aan Wageningen Universiteit in het Centrum voor Eiwittechnologie bij de vakgroepen Biochemie en Levensmiddelenchemie. In 2003 was de auteur gedeeltelijk als adviseur in dienst van TNO voeding, betrokken bij het ontwikkelen van het patent dat uit het promotieonderzoek is voortgekomen. In 2004 verdedigt de auteur dit proefschrift.

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Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid
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patents

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