Free Radical Research, December 2009; 43(12): 1167–1178

informa
healthcare

Modifications of amino acids during ferulic acid-mediated, laccase-catalysed cross-linking of peptides

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(Received 10 December 2008; in revised form date 19 July 2009)

Abstract

Mass spectral analysis demonstrated oligomerization of peptides that had been subjected to oxidation catalysed by *Trametes (Coriolus) versicolor* laccase. Peptide oligomerization occurred only when cysteines or tyrosines were present in the peptides. MS/MS confirmed the cross-linking in tyrosine-containing peptides to be located between tyrosine residues. Ferulic acid mediated oligomerization of cysteine-containing peptides, but prevented cross-linking of tyrosines when used in the same concentration as the peptides. This suggests an antioxidative effect of ferulic acid in relation to tyrosine oxidation, although incorporation of ferulic acid into peptide oligomers was found in some of the tyrosine-containing peptides. No other modifications to amino acid residues by laccase-catalysed oxidation were observed by mass spectroscopy. Thus, it is suggested that oxidative modifications of other amino acids observed in proteins oxidized by laccase are not major reaction products of laccase-catalysed oxidation.

Keywords: *Laccase, ferulic acid, protein cross-linking, peptide oligomerization, MALDI MS*

Introduction

Texture is a main quality feature in modern food production due to its importance for the functionality and sensory perception of foods. Proteins are the main contributors of the structural properties in many foods, which is why a controlled modification of proteins is a means of optimizing the functionality and sensory perception of foods. The formation of cross-links between proteins is a potential method for modifying the functional properties of proteins. In a review by Matheis and Whitaker [1] about potential enzymatic reactions inducing protein cross-linking, it is proposed that oxidative cross-linking by formation of di- or tri-tyrosine linkages or by linking intermediate benzoquinones to – SH or $–NH_3$ groups is possible by use of oxidoreductases. Using the laccase as the oxidoreductase, disulphide bonds were the only protein cross-link encountered [2,3] until Mattinen et al. [4] reported formation of laccase-induced dityrosine and isodityrosine cross-links.

Laccase is an 85 kDa copper-containing oxidoreductase (EC 1.10.3.2), found in plants and microorganisms. By reducing diatomic oxygen to water, laccase catalyses oxidation of phenolic and related compounds in one-electron reactions that involve radicals as intermediates. The phenolic amino acid tyrosine can be oxidized into tyrosyl radicals by laccase [4]. Tryptophan and cysteine have also been found to be oxidized by laccase [5,6].

Although some proteins have been successfully cross-linked by laccase [6], cross-linking has not been

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715760903247215

observed with many other proteins. The reactivity of laccase is negatively correlated with substrate size [7] and therefore several small redox-active substances including ferulic acid have been employed as mediators together with laccase for efficient oxidization of substrates that laccase does not oxidize directly. Several proteins have thus been cross-linked by laccase in the presence of a mediator [8]. Laccase-induced polymerization of tyrosine and tyrosine-containing tri-peptides has been shown to result in incorporation of ferulic acid into the polymer structure [4]. Ferulic acid is often abundant in plant-derived foods either in a free state or bound to carbohydrates and is approved for use as an antiox-idant in food [9]. In this respect, the fate of ferulic acid when used as a mediator of laccase-catalysed cross-linking of food components is of general interest.

Laccase-catalysed oxidation of proteins has been shown to result in oxidative modifications of several amino acids in addition to protein cross-linking involving tyrosines and cysteines: Oxidation of tryptophan into N-formylkynurenine and the carbonyl compound kynurenine, oxidation of histidine into 2-oxo-histidine and the formation of a methionine-erived carbonyl compound [10]. The aim of this study was to use small and simple peptides to get detailed information about the possible modifications to amino acids that may occur when food proteins undergo laccase-catalysed oxidation during possible modification of their texture. Attempts have been made to cross-link or oxidize a collection of six peptides, in which all 20 unmodified amino acids are includeed, by laccase and subsequently to analyse these peptides by two different MS techniques. In order to examine possible interactions between tyrosine and other amino acids, the tyrosine-containing tripeptide VYV was also included.

Furthermore, it has also been the aim of this study to examine if and how ferulic acid becomes incorporated into the reaction products when used as a mediator. The influence and fate of ferulic acid, which is known to oligomerize and thereby form several different ferulic acid-dimers and trimers during oxidation [11,12], have been examined by performing all experiments in the presence and absence of ferulic acid, respectively, and by determining the amount of ferulic acid remaining after the laccase-catalysed oxidation of peptides.

Materials and methods

Chemicals

The substrate peptides used were; angiotensin II antipeptide (EGVYVHPV, $C_{42}H_{62}N_{10}O_{12}$, monoiso-topic Mw=898.455 Da), Val-Glu-Pro-Ile-Pro-Tyr (VEPIPY, $C_{35}H_{52}N_6O_{10}$, monoisotopic Mw=716.375 Da), influenza hemagglutinin (HA) peptide (YPYDVPDYA, $C_{53}H_{67}N_9O_{17}$, monoisotopic Mw=1101.466 Da), RanatachykininA (KPSPDRFYGLM, $C_{60}H_{92}N_{16}O_{15}S$, monoisotopic Mw=1308.665 Da), reduced Asn-Arg-Cys-Ser-Gln-Ser-Cys-Trp-Asn (NRCSQGSCWN, $C_{44}H_{67}N_{17}O_{16}S_2$, monoisotopic Mw=1153.439 Da) and Val-Tyr-Val (VYV, $C_{19}H_{29}N_3O_5$, monoisotopic Mw=379.211 Da), all coming from Sigma (St. Louis, MO). Pro-Thr-His-lle-Lys-Trp-Glu-Asp (PTHIKWGD, $C_{44}H_{64}N_{12}O_{12}$, monoisotopic Mw=952.477 Da) was from Bachem (Torrance, CA) and the mediator Ferulic acid ($HOC_6H_3(OCH_3)CHCH\text{-}CO_2H$, monoisotopic Mw=194.058 Da) was supplied by Fluka (Sigma-Aldrich, St. Louis, MO).

Laccase from *Trametes (Coriolus) versicolor* (Juelich Fine Chemicals GmbH, Julich, Germany) was a lyophilized powder with 1% enzyme and it had an enzyme activity measured to 300 U/g (1U=1 jimol of catechol/min).

The MALDI matrix alpha-cyano-4-hydroxycinnamic acid and acetonitrile were purchased from Fluka Chemie AG (Buchs, Switzerland). 2,5-Dihydroxybenzoic acid (DHB), angiotensin II, ACTH peptide 18–39 and somatostatin were bought from Sigma. Trifluoracetic acid (TFA) was from Merck (Merck KGaA, Darmstadt, Germany). All chemicals were of analytical grade.

Peptides and solutions were made up in a 50 mM phosphate buffer (pH 6.5, ionic strength: 0.16 M).

Enzymatic incubation

The peptides were made up in a concentration of 0.1 mM and exposed to 24 U/ml laccase in the absence and presence of 0.1 mM VYV and/or 0.1 mM ferulic acid, respectively. Samples were incubated for 24 h at room temperature in an open vessel, which allowed oxygen from the air to get in contact with the reaction volume. Ferulic acid concentrations of 0.05 mM and 0.3 mM were also included for analysis of remaining ferulic acid monomers and dimers.

Mass spectrometry

The substrate peptides and products were analysed by Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) and automatic liquid chromatography-electro spray ionization tandem mass spectrometry (LC-ESI MS/MS). A 2,5-DHB matrix was used for MALDI TOF MS analyses by dissolving the matrix in 1% (v/v) o-phosphoric acid:acetonitrile (1:1, v/v) to a final concentration of 20 g/L. A peptide solution, 0.5 µL, was mixed with an equal volume of DHB matrix solution, followed by deposition of 0.5 µL on the target plate. The samples were allowed to dry prior to acquisition of MS spectra. Mass spectra were calibrated by an external standard of angioten-sin II (Mr 1046.5423),

bombesin (1619.823), ACTH peptide 18–39 (2465.203) and somatostatin (3147.464). A mass spectrometric analysis was carried out on a MALDI-TOF MS REFLEX III (Bruker-Daltonics, Germany) equipped with delayed extraction technology. All mass spectra were acquired in positive reflectron mode and the monoisotopic peaks of the mass spectra were annotated using the Moverz software (Genomic Solutions, USA).

Automated LC-ESI MS/MS was performed using a hybrid QTOF mass spectrometer (MicroTOFQ; Bruker Daltronics, Bremen, Germany) and an Ultimate nano-HPLC system (Dionex/LC Packings, The Netherlands) mounted with a vented-column setup [13]. Reversed phase columns (pre-column 2 cm, 75 [im id; separation column 12 cm, 50 μm internal diameter) were packed in-house with Repro-Sil-Pur C18-AQ 3 μm resin (Dr Maisch GmbH, Ammer-Buch-Entringen, Germany) using a high-pressure vessel (Proxeon Biosystems, Odense, Denmark). Aliquots of the peptides corresponding to 1 μL were injected onto the pre-column with a flow rate of 3 μL/ min and subsequently eluted at 175 nL/min using a 30 min gradient of 5–40% acetonitrile in 0.6% acetic acid and 0.005% heptaflurobutyric acid. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition selecting the three most abundant precursor ions. The tandem MS data were deconvoluted and deisotoped by Dataanalysis software (Bruker Daltronics, Bremen, Germany) prior to automated database searching and manual data mining.

Tandem MS spectra were analysed by automatic database searching using the VEMS software [14,15] (www.yass.sdu.dk) and in-house Mascot search engine version 2.2 [16] (www.matrixsciences.com) using a database comprising the amino acid sequences of the substrate peptides. For all automated searches the parameters were: Enzyme: no enzyme; Partial modification (potentially modified residue(s)): addition of ferulic acid $+C_{10}H_8O_4$ (PFWYDE), addition of ferulic acid by decarboxyla-tion + $C_9H_8O_2$ (PFWYDE), addition of diferulic acid $C_{20}H_{16}O_8$ (PFWYDE), addition of diferulic acid by decarboxylation $C_{19}H_{16}O_6$ (PFWYDE), addition of decarboxylated diferulic acid $C_{19}H_{14}O_6$ (PFWYDE), multidecarboxylated diferulic acid $C_{18}H_{14}O_4$ (PFWYDE), formylkynurenine $+O_2$ (W), kynurenine $-C+O$ (W), methionin carbonyl $+O$ or $+O_2$ (M) and histidin carbonyl $+O$ (H).

Peptide tolerance was 0.05 Da for both internally and externally calibrated spectra. All identifications were manually validated. Extracted ion chromato-grams were generated using Data analysis 3.4 (Bruker Daltronics, Bremen, Germany). In MS/MS spectra single- and double-charged mass peaks are denoted 1+ and 2+ , respectively.

Determination of ferulic acid monomers and dimers

The method used to determine ferulic acid and dimers of ferulic acids was based on the HPLC-method described by Andreasen et al. [17]. Before analysis, the samples were freeze-dried and redis-solved in methanol/water (80:20, v/v), filtered through a 0.45 μm Minisart SPR 25 filter (Bie & Berntsen, Rødovre, Denmark) and transferred directly into 4 mL HPLC vials. The samples were analysed by analytical HPLC (Shimadzu, Kyoto, Japan) using a photodiode array (PDA) detector (La Chrom, L-7450, Merck, Darmstadt, Germany) operating at 280 nm and 320 nm. Separations were performed at 35°C on a reverse phase C18 column (Purospher STAR-RP-18 endcapped, 5 μm, 250 × 4.6 mm, Phenomenex, Allerød, Denmark) by gradient elution with solvent A: 0.5% TFA in water, and solvent B: 100% methanol. The elution profile was: 0–25 min: 5% B, 25–30 min: 30% B, 60 min: 50% B; 70 min: 90% B, 100–110 min: 100% B. The flow rate was 1 mL/min and the injection volume was 20 μL. Ferulic acid was identified by spiking with an authentic ferulic acid standard and from spectral data. The dimers of the ferulic acids were tentatively identified by their spectral data. Ferulic acid and the dimers were quantified using an external standard curve of ferulic acid, constructed by concentrations from 2–20 μg/ml. The standard curve showed linearity over the tested concentrations with a correlation coefficient of $r^2 = 0.997$.

Dityrosine measurements

Dityrosine was detected by using acid hydrolysis of the cross-linked proteins followed by high performance liquid chromatography (HPLC) separation and identification according to the method by Østdal et al. [18]. The samples were added HCl to a concentration of 6 M flushed with argon, hydrolysed overnight (105°C) and subsequently neutralized with 6 M NaOH. A hydrolysed sample (20 μl) was injected onto an HPLC column (Microsorb 100–5 C–18, 250 × 4.6, Varian, Walnut Creek, CA), equilibrated with 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55), with a flow of 1 ml/min. Chromato-graphic separation was performed on an HPLC system consisting of a Varian 9012 HPLC pump connected to a Varian 9100 autosampler and a Varian 9075 fluorescence detector (ex. 283 nm, em. 410 nm) (Varian Chromatographic Systems, Walnut Creek, CA). Dityrosine was quantified using a standard curve made from a dityrosine standard prepared according to Nomura et al. [19].

Results

Mass changes in five different peptides oxidized by laccase were characterized by MALDI-TOF-MS. Analysis

of the observed y-ion series [20] in MALDI-TOF-MS/MS spectra was used to gain insight into the position of possible modification in the peptide chain.

The unmodified peptide KPSPDRFYGLM was observed as a mass peak at 1309.8 Da, corresponding to the mass of the peptide plus 1.0078 Da (the mass of the charge carrying hydrogen) (Figure 1). Mass peaks corresponding to the mentioned adducts in which a number of H^+ -ions have been exchanged with Na^+ from the buffer can be seen in these and all subsequent spectra. Together with the unmodified peptide, less prominent mass peaks at 1066.6 and 1179.7 Da were also seen (Figure 1A). These masses correspond to the peptide fragments KPSPDRFYG and KPSDRFYGL, respectively, hence impurities in the sample. Dimers of the peptide (2616.5 Da) can be detected after incubation with laccase (Figure 1B), together with hetero dimers between the peptide and the peptide fragments. What appears to be a dimer is also observed without laccase (Figure 1A) and after incubation with laccase in the presence of ferulic acid and VYV (Figure 1D), but the mass, which is 2 Da higher than in a dimer, shows that no bonds have formed between the two peptides. Also a more pronounced exchange of H^+ -ions with Na^+ from the buffer indicates that this peptide cluster has stayed in the solution longer than the dimer. In the presence of ferulic acid, where no dimerisation was observed (Figure 1C), a mass peak at 1844.1 Da is observed, corresponding to the addition of three ferulic acid residues to the peptide KPSPDRFYGLM, where one of the additions is formed by decarboxylation. An identical adduct is also seen when VYV is present together with ferulic acid (Figure 1D) as well as after addition of an ferulic acid dimer (1694.0 Da). When VYV is present in the absence of ferulic acid during incubation with laccase, a mass peak is observed at 1687.6 Da, corresponding to the addition of VYV to the peptide (Figure 1E).

MS/MS analysis of the 1844.1 Da mass peak assigned as the peptide fragment KPSPDRFYGLM with three ferulic acid residues added, of which one is decarboxylated (Figure 2), shows that the added ferulic acid residues are cleaved from the peptide prior to any fragmentation of the peptide, whereby the location of the addition cannot be determined. It is evident though that the decarboxylated ferulic acid residue is the last to be cleaved from the peptide, which indicates that the ferulic acids are most likely added as a trimer [12] with the decarboxylated residue bonded to the peptide. In the MS/MS spectra of the 1694.0 Da mass peak, the two ferulic acid residues are cleaved from the peptide in fragments prior to any fragmentation of the peptide, as illustrated in Figure 3. The locations of the formed peptide cross-links are found by MS/MS to be located between the tyrosine residues, and MS/MS also confirms the assignment of the 1066.6 and 1179.7 Da mass peaks as peptide fragments (spectranot shown).

The unmodified peptide EGVYVHPV is observed as a mass peak at 899.6 Da, together with a mass peak (881.6 Da) corresponding to the dehydrated peptide (Figure 4A). Di- and trimers of EGVYVHPV (1796.3 and 2693.1 Da) can be detected after incubation with laccase (Figure 4B), accompanied by mass peaks showing that a fraction of the oligomerized EG-VYVHPV peptides is dehydrated. Additional mass peaks are observed at 1364.0 and 2260.7 Da. In the presence of ferulic acid no peptide oligomers are observed (Figure 4C and D); instead, a mass peak

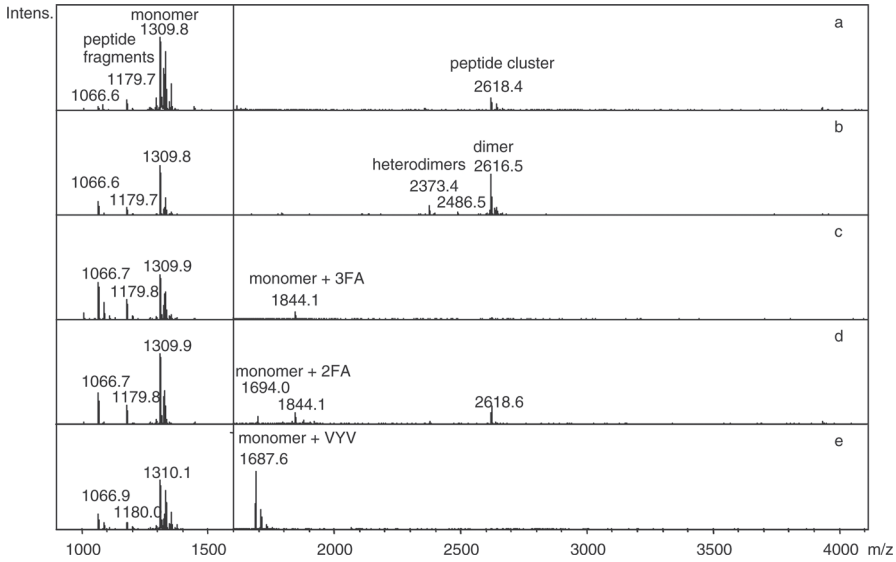

Figure 1. MS spectra of KPSPDRFYGLM (1308.7 Da), incubated for 24 h at room temperature without (A) and with laccase (B), in the presence of ferulic acid (C), ferulic acid and VYV (D) or VYV (E). At masses higher than 1600 Da intensities are enhanced by a factor 50.

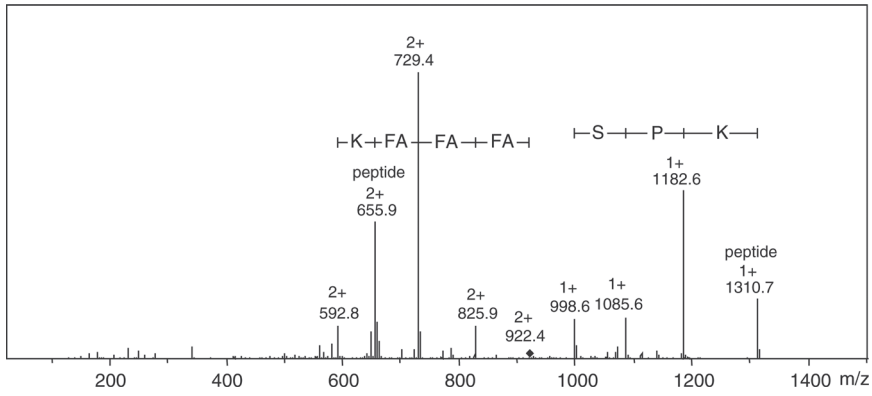

Figure 2. LC-MS/MS spectrum of the 1844.1 Da double-charged ion of KPSPDRFYGLM with three ferulic acid (FA) residues added, of which one is decarboxylated. The parent ion is marked by♦.

(1434.0 Da) corresponding to the addition of an ferulic acid trimer is observed. When VYV and no ferulic acid is present during incubation with laccase (Figure 4E), a small mass peak from the peptide dimer was detected, together with a mass peak at 1276.9 Da, corresponding to the addition of VYV to the peptide.

MS/MS of the additional mass peaks at 1364.0 and 2260.7 Da shows that these adducts consist of a peptide and a peptide dimer, both with addition of an EGVY peptide fragment. An MS/MS spectrum acquired from the dehydrated EGVYVHPV peak reveals that water is lost from either the glutamine (E) or the glycine (G) residue (data not shown). Most probably, a ketene (–CH=C=O) group is formed in glutamic acid during a reaction induced by the MS technique.

The unmodified peptide VEPIPY is observed as a mass peak at 717.6 Da. Di-, tri-, quadro- and pentamers can be seen to be formed from VEPIPY after incubation with laccase (Figure 5B). In the presence of ferulic acid and/or VYV, only dimers are detected (Figure 5C-E). When VYV is present during incubation with laccase (Figure 5D and E), a mass peak (1094.7 Da) corresponding to the addition of VYV to VEPIPY is seen. What appears to be a dimer is also observed after incubation without laccase and with laccase in the presence of VYV (Figure 5A and E), but the mass and the pronounced exchange of hydrogen with sodium identify the peak as arising from a cluster of two peptides.

An MS/MS spectrum of the 2146.3 Da mass peak assigned as a cross-linked peptide trimer reveals the cross-link to be located between tyrosine residues (Figure 6). Additions of ferulic acid to the peptide, which could be observed to both KPSPDRFYGLM and EGVYVHPV, were not observed in the MALDI-MS spectra of VEPIPY. However, by a specific search for masses corresponding to ferulic acid-added peptides in the LC-MS run, a mass peak is found at 1101.8 Da corresponding to the peptide with a ferulic acid dimer added. MS/MS of this peak reveals the diferulic acid to be located on the tyrosine residue (data not shown).

The unmodified peptide YPYDVPDYA is observed at a mass peak at 1101.5 Da (Figure 7). Dimers and

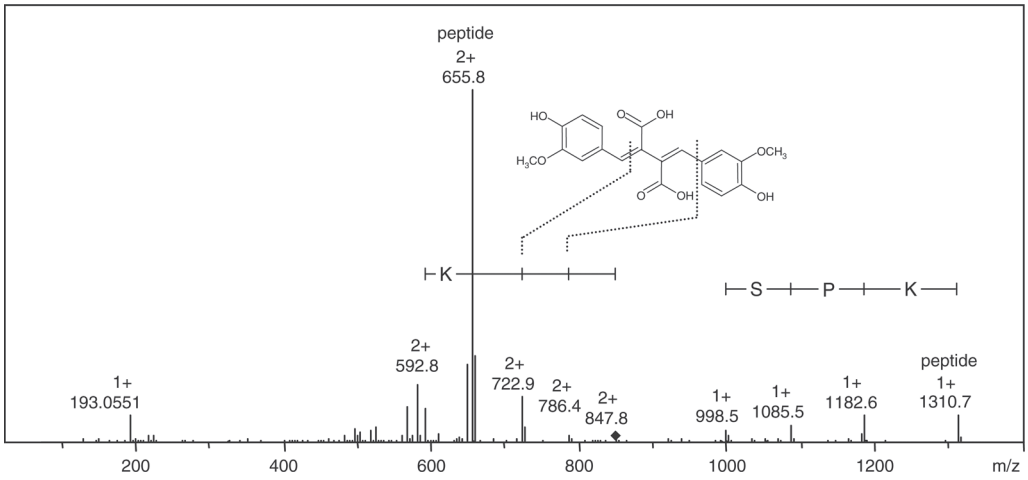

Figure 3. LC-MS/MS spectrum of the 1694.0 Da double-charged ion of KPSPDRFYGLM with two ferulic acid residues added. The parent ion is marked by ♦.

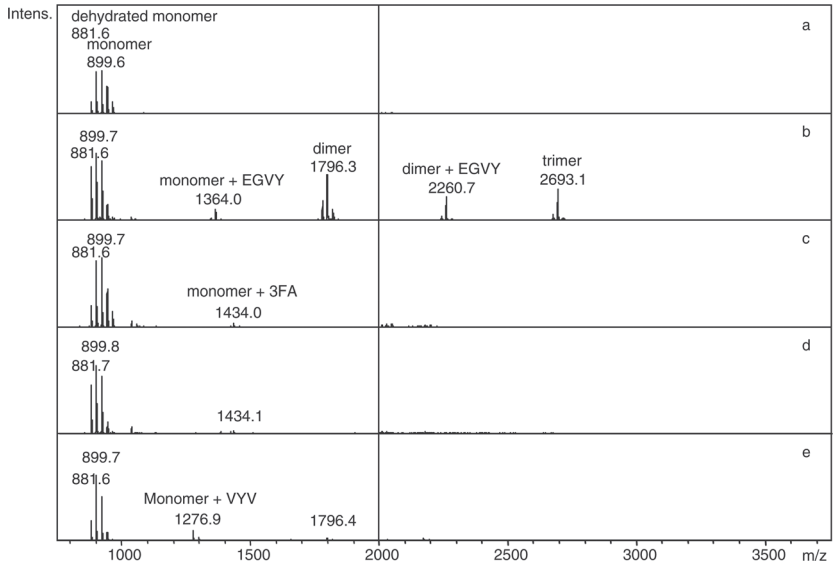

Figure 4. MS spectra of EGVYVHPV (898.5 Da), incubated for 24 h at room temperature without (A) and with laccase (B), in the presence of ferulic acid (C), ferulic acid (FA) and VYV (D) or VYV (E). At masses higher than 2000 Da intensities are enhanced by a factor 10.

trimers of the peptide can be detected after incubation with laccase (Figure 7B). In the presence of VYV (Figure 7E), the peptide dimers and trimers are also observed together with mass peaks (1480.2, 2580.1 Da) corresponding to addition of VYV to YPYDVPDYA monomers and dimers, respectively. What appears to be a dimer is observed without laccase and after incubation with laccase in the presence of ferulic acid (Figure 7A, C and D), but the mass and the pronounced exchange of hydrogen with sodium identify the peak as arising from a cluster of two peptides. Thus, in the presence of ferulic acid neither oligomerization nor any additions to the peptide were observed.

In the MS/MS spectrum acquired from the dimer peak (2202.3 Da) (spectrum not shown), a complex mixture of y and b ions is seen, which does not allow pointing out the exact position of the cross-link between the two peptides. Additions of ferulic acid to the peptide observed in the other tyrosine-containing peptides were not observedin the MALDI-MS spectra of YPYDVPDYA. However, by a specific search for masses corresponding to ferulic acid-added peptides in the LC-MS run, double charged mass peaks are found at 819.2 and 745.1 Da corresponding to the peptide with a decarboxylated ferulic acid trimer and a ferulic acid dimer added, respectively. MS/MS of the peaks does not reveal the location of the additions,

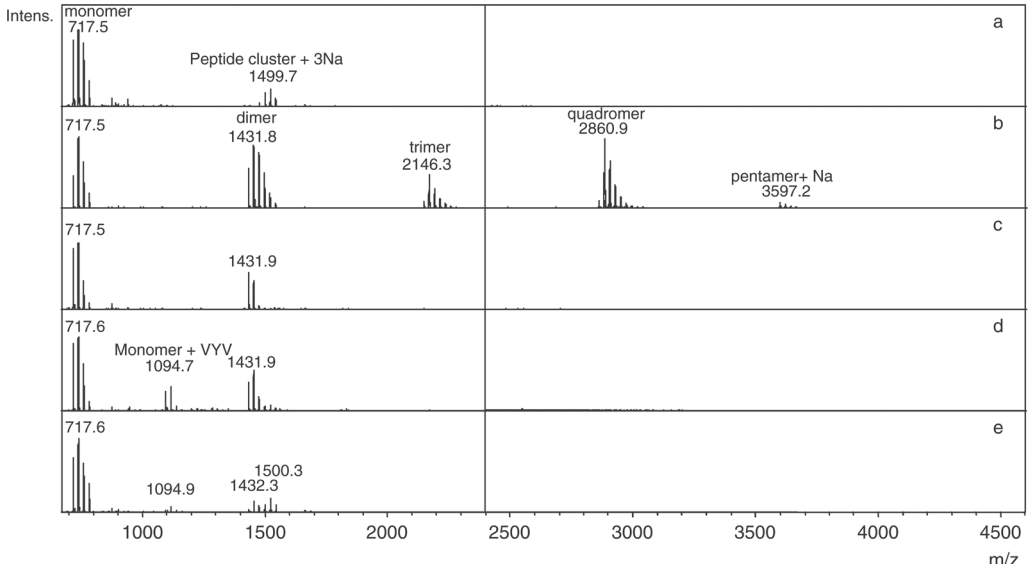

Figure 5. MS spectra of VEPIPY (716.4 Da), incubated for 24 h at room temperature without (A) and with laccase (B), in the presence of ferulic acid (C), ferulic acid and VYV (D) or VYV (E). At masses higher than 2400 Da intensities are enhanced by a factor 5.

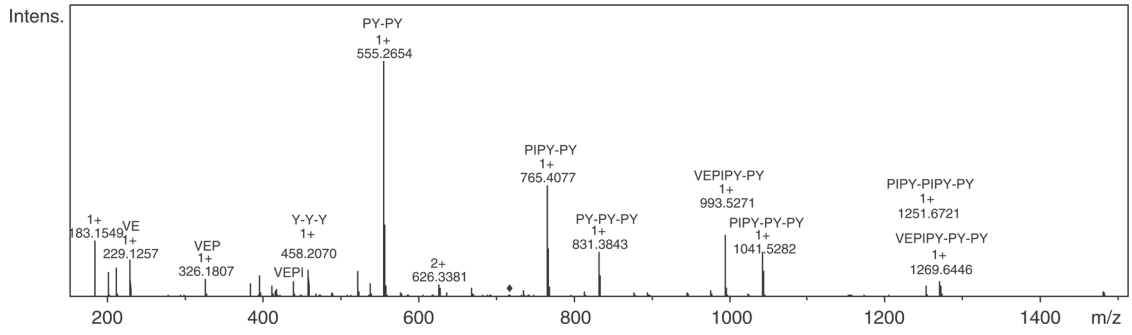

Figure 6. LC-MS/MS spectrum of the 716.5 Da triple-charged trimer ion of VEPIPY. The parent ion is marked by ♦.

as ferulic acid is cleaved off prior to fragmentation of the peptide, as exemplified in Figure 8.

A major part of the unmodified peptide NRCSQGSCWN can be observed as a mass peak at 1152.7 Da (Figure 9), corresponding to the pep-tide having lost two hydrogen atoms, which indicates that an internal disulphide bridge has been formed during incubation without laccase in the initially reduced peptide sample. Dimers and trimers of NRCSQGSCWN are observed after incubation with laccase (Figure 9B) and they are most likely arising from intermolecular sulphide bridges. This is confirmed by the disappeance of these oligomers after introduction of reducing conditions (data not shown). Identical oligomerization is observed in the presence of ferulic acid together with VYV (Figure 9D). In the presence of ferulic acid, quadro-, penta-and hexamers also appeared (Figure 9C). A closer examination of oligomer-masses shows that where monomers and dimers form rings involving all cysteines, the higher oligomers exist as a strand in which two cysteines are not involved in any sulphide bridges. No polymerization of or additions to the peptide were observed in the presence of the tripeptide VYV (Figure 9D and E).

In the MS/MS spectrum acquired from the peptide oligomes (1152.7 Da and 2305.3 Da) (spectra not shown), the formation of disulphide bridges is confirmed, as only fragmentation of the two outermost amino acids could be observed.

The unmodified peptide PTHIKWGD is observed as a mass peak at 953.6 Da (Figure 10). No oligomerization of the peptide PTHIKWGD was observed after incubation with laccase. As this peptide does not contain tyrosine or cysteine, it is naturally unable to form tyrosine or cysteine cross-links. Some auto-oxidation of the peptide is observed in the Maldi MS spectra of PTHIKWGD as mass peaks corresponding to the peptides with additions of one or two oxygen atoms (969.7 Da, 985.7 Da). When unravelling the peptide sequence by MS/MS, the auto-oxidation with

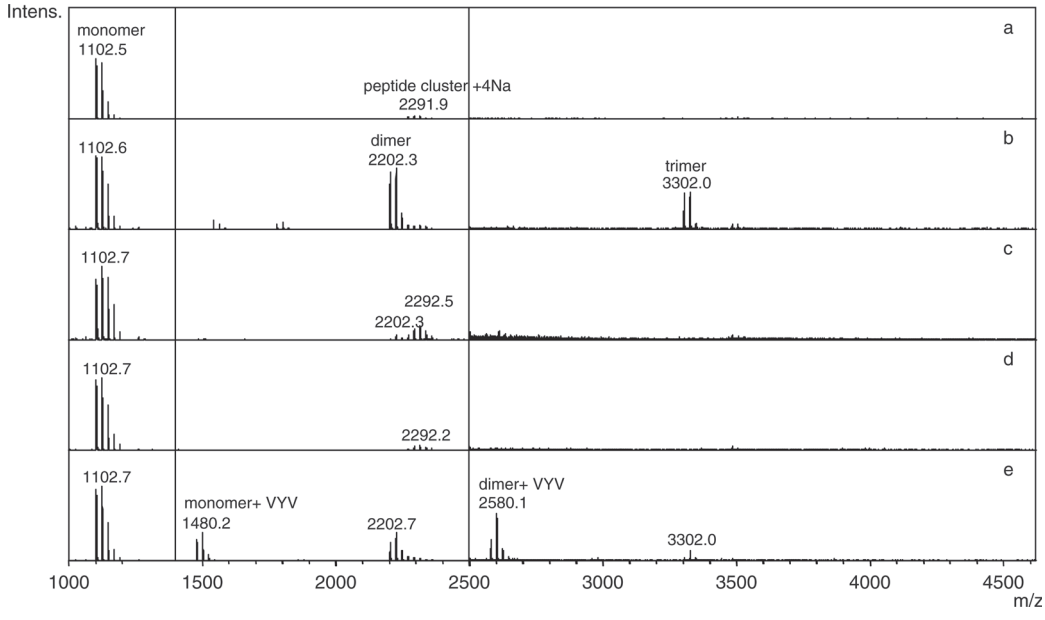

Figure 7. MS spectra of YPYDVPDYA (1101.5 Da), incubated for 24 h at room temperature without (A) and with laccase (B), in the presence of ferulic acid (C), ferulic acid and VYV (D) or VYV (E). At masses higher than 1400 Da intensities are enhanced by a factor 5 and at masses higher than 2500 intensities are enhanced by a factor 100.

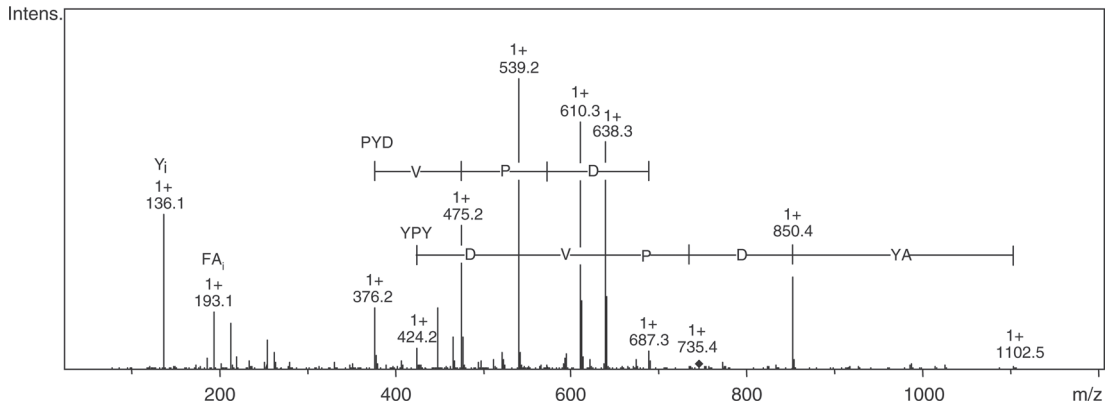

Figure 8. LC-MS/MS spectrum of the 745.1 Da double-charged ion of YPYDVPDYA with two ferulic acid residues added. The parent ion is marked by ♦.

one or two oxygen atoms can both be located to the tryptophan (W) residue.

Detection of ferulic acid monomers and dimers shows that less than 10% of the added ferulic acid can be detected after incubation with laccase (Figure 11). Significantly less ferulic acid was detected in the absence of any peptides, regardless of the concentration of added ferulic acid. This is probably due to the ability of ferulic acid radicals to polymerize. When ferulic acid and peptide were used in equal concentrations, some significant differences between the peptides appear. The least amount of ferulic acid is found with the peptides NRCSQGSCWN and VE-PIPY, more with PTHIKWGD followed by KPSPDRFYGLM, and most ferulic acid was found in the presence of YPYDVPDYA and EGVYVHPV. After incubation with a lower concentration of ferulic acid than of peptide, significantly more ferulic acid was detected in the presence of the peptide YPYDVPDYA than with any of the other peptides. When ferulic acid is added in a higher concentration than the peptide, no significant differences appear between the peptides.

Measurements of dityrosine was performed. It was evident that only a small amount of the available tyrosine (<0.2%) forms dityrosine upon laccase-catalysed oxidation in the absence of ferulic acid, whereas no detectable dityrosine is formed if ferulic acid is present during the incubation with laccase (data not shown).

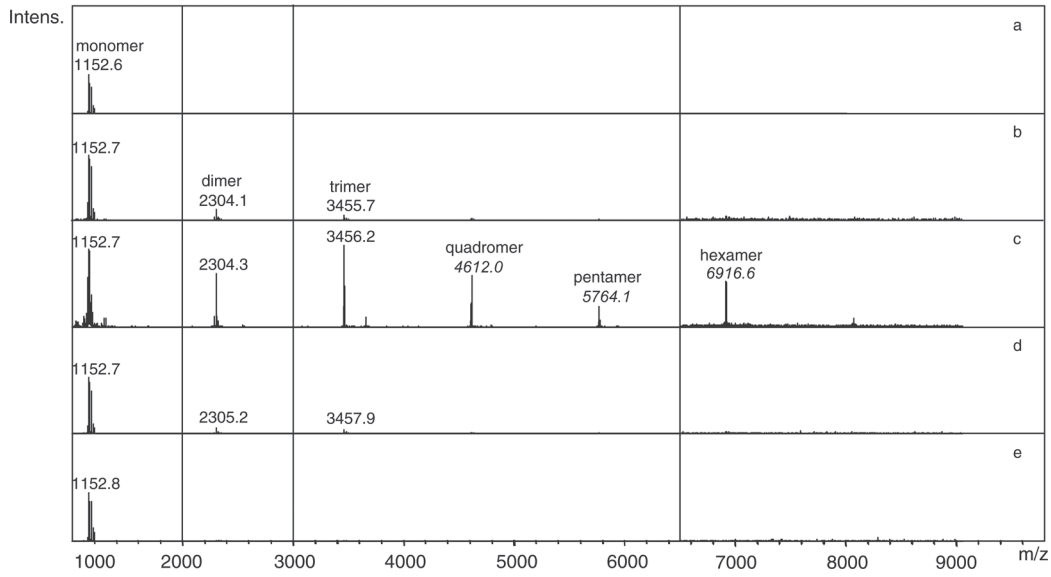

Figure 9. MS spectra of NRCSQGSCWN (1153.4 Da), incubated for 24 h at room temperature without (A) and with laccase (B), in the presence of ferulic acid (C), ferulic acid and VYV (D) or VYV (E). At masses higher than 2000 Da intensities are enhanced by a factor 5, at masses higher than 300 by a factor 20 and at masses higher than 6500 intensities are enhanced by a factor 200. Masses given in italics are average instead of mono-isotopic masses.

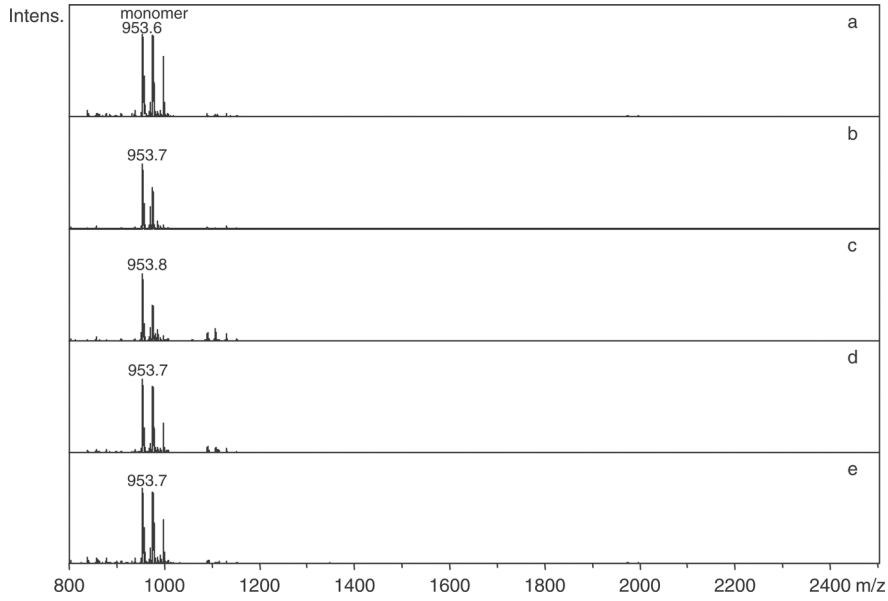

Figure 10. MS spectra of PTHIKWGD (952.5 Da), incubated for 24 h at room temperature without (A) and with laccase (B), in the presence of ferulic acid (C), ferulic acid and VYV (D) or VYV (E).

Discussion

From the Maldi-MS results, it can be seen that laccase catalyses cross-linking of the cysteine-containing pep-tide NRCSQGSCWN and of the tyrosine-containing peptides KPSPDRFYGLM, EGVYVHPV, VEPIPY and YPYDVPDYA, of which the peptide with the c-terminal tyrosine showed the most elaborate cross-linking. By MS/MS the cross-links in the tyrosine-containing peptides are, with one exception (YPYDVPDYA), located in the tyrosine residues and could at least partly be dityrosine bonds as a small amount of dityrosine is detected in tyrosine-containing peptides incubated with laccase. Laccase-catalysed cross-linking has previously been shown in tyrosine-containing tripeptides [4], where the reaction intermediate is shown to be tyrosyl radicals. In a different case of peptide cross-linking, the formed tyrosyl radicals are suggested to form dityrosine bonds as well as isodityrosine bonds [21].

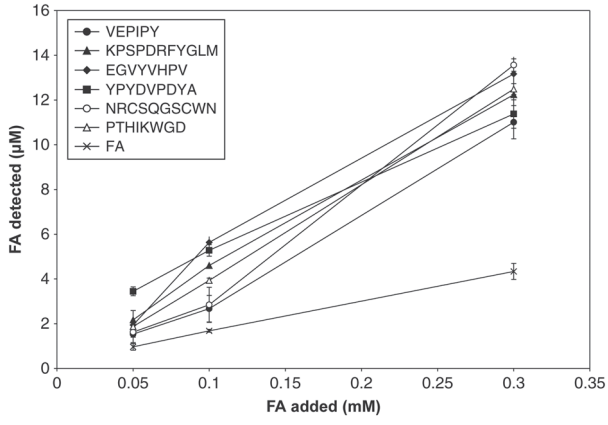

Figure 11. The total concentration of ferulic acid monomers and dimers detected after incubation of peptides with laccase as a function of added ferulic acid (FA) concentrations.

$$Y \rightarrow Y^{\bullet} \text{ (catalysed by laccase)}$$
$$2Y^{\bullet} \rightarrow Y\text{-}Y$$

The peptide YPYDVPDYA has three tyrosine residues, thus several differently located tyrosine-cross-links are theoretically possible. The mass of the formed multimers shows that cross-linking of the peptides is caused by only one cross-link between each peptide, but it was not possible to locate the position of the cross-links. This indicates that the detected mass peaks are most likely to be a mixture of differently cross-linked YPYDVPDYA oligomers. The formation of only one cross-link between peptides, where more linkages ought to be possible, is puzzling and could be related to the configuration of the peptides. It also indicates that closely-spaced tyrosines in proteins could result in only one tyrosine cross-link.

Fragmentation was only observed in one peptide. The observed fragmentation of a part of the cross-linked EGVYVHPV peptides suggests that this is correlated to the amino acids present in the peptide. In this peptide the tyrosine residue is neighboured by two bulky valine residues, thus it is likely that fragmentation would be favoured due to steric stress during formation of tyrosine cross-links.

Incubation of the peptides with laccase in the presence of the tri-peptide VYV results in hetero-dimers with the tyrosine-containing peptides. Here the cross-links are also likely to be between tyrosine residues, whereby the tri-peptide acts as a substitute for the peptides.

Laccase-catalysed oxidation of ferulic acid (FA) produces a reactive ferulic acid radical ($FA^{\bullet}$) [4].

$$FA \rightarrow FA^{\bullet} \text{ (catalysed by laccase)}$$

The action of ferulic acid as a mediator of laccase-catalysed oxidation reactions being able to transfer radicals to the substrate is used to explain why the effect of laccase as a cross-linking agent in bread is enhanced in the presence of free ferulic acid [2,3]. A similar mediator effect of ferulic acid is observed during the oxidation of the cysteine-containing peptide NRCSQGSCWN (Figure 7). As the presence of ferulic acid caused a formation of higher peptide oligomers, but not cross-linking of significantly more peptide residues, it is likely that ferulic acid is also able to reduce already formed cystine (R-S-S-R) into cysteine (R-SH), allowing other peptides to be linked into the oligomer. Thus, ferulic acid should participate in the following equilibrium reaction, where the left-hand direction represents ferulic acid-mediated laccase cross-linking, while the right-hand direction suggests how the presence of ferulic acid could cause breaking of already formed cross-links and thus subsequent formation of higher peptide oligomers.

$$2FA + R - S - S - R \leftrightarrows 2R - SH + 2FA^{\bullet}$$

Opposed to the mediator effect of ferulic acid toward cross-linking of cysteine-containing peptides, ferulic acid shows an antioxidative effect towards laccase-catalysed oxidation of tyrosine-containing peptides. The small amount of dityrosine formation otherwise found after laccase-catalysed oxidation disappeared in the presence of ferulic acid and no cross-linking of tyrosine-containing peptides was detected in the presence of ferulic acid. As ferulic acid has previously been shown to be a much better substrate for laccase than tyrosine [2], ferulic acid is likely to be oxidized prior to tyrosine, resulting in less dityrosine formation. Alternatively the antioxidative effect of ferulic acid can be explained if a radical transfer reaction occurs from tyrosine radicals to ferulic acid, but not from ferulic acid to tyrosine, whereby ferulic acid prevents tyrosyl radicals from forming linkages. This would be the case if the redox potential of the peptide-bound $Y^{\bullet}/Y$ couple is higher than the redox potential of the FA /FA couple.

$$Y^{\cdot} + FA \rightarrow FA^{\bullet} + Y$$

The peptide VEPIPY, which showed the most elaborate cross-linking without ferulic acid, is also less affected by the antioxidative effect of ferulic acid. VEPIPY is still able to form dimers or heterodimers with VYV in the presence of ferulic acid. The reason for this exceptional reactive behaviour of the c-terminal tyrosine in the VEPIPY peptide could be sought in the redox potential, but no significant differences in redox potential have been found between differently located peptide-bound tyrosine radicals [21]. However, previous studies have shown that the laccase-catalysed oxidation rates of ferulic acid and of c-terminal tyrosine in a tri-peptide are comparable in magnitude [4]. This could indicate that laccase has an equal affinity towards ferulic acid and c-terminal tyrosine, as opposed to the reasonable assumption that tyrosine residues located within a peptide strand are probably less easily accessible for the enzyme. The antioxidative effect of ferulic acid in relation to the cross-linking of tyrosine-containing peptides could be a consequence of a relatively high concentration of ferulic acid (1:1) or the fact that the used peptides are too small to have amino acids that are really inaccessible to laccase, whereby ferulic acid could adapt the role of a competitor rather than a mediator of oxidation.

The mediator effect of ferulic acid towards cross-linking of cysteine-containing peptides is not observed when VYV is present. The presence of VYV counteracts the mediator effect of ferulic acid. However, although VYV is present in the same concentration as the peptide and is capable of forming cross-links upon enzymatically catalysed oxidation [22], no oligomerization of the tripeptide was observed. Thus, the tyrosyl radicals that are supposedly generated must interfere with the reactions where ferulic acid assists in reducing and oxidizing cysteine/cystine. If ferulic acid will sooner reduce tyrosyl radicals to tyrosine than reduce cystine to cysteine, no tyrosine cross-links will be generated and no cystines will be reduced, allowing no additional peptides to be added to the peptide oligomers.

In the absence of ferulic acid, VYV prevents oligomerization by cysteine cross-links. This inability to cross-link both cysteine and tyrosine when both are present is most intriguing, as on account of similarities between ferulic acid and tyrosine, interactions between the two could be expected to confer to the following equilibrium reaction, whereby some cross-linking might be expected.

$$2Y + R - S - S - R \equiv 2R - SH + 2Y^{\bullet}$$

Some dipeptides have been shown to inhibit laccase by their ability to form complexes with Cu(II) in laccase. Especially dipeptides with c-terminal tyrosine groups were found to effectively inhibit laccase-catalysed oxidation of ferulic acid by laccase [23]. If VYV fragments upon laccase-catalysed oxidation in the presence of cysteine-containing peptides, it is possible that inhibitory dipeptides could be formed, thus explaining the lack of cross-linking. Laccase has previously been reported to fragment proteins as well as cross-linking them [24] and laccase-induced fragmentation was observed in one peptide; EGVYVHPV, where cross-linking between the

peptide and the fragment EGVY was observed. Here, the fragmentation occurs next to the site of cross-linking, rendering the formation of an inhibitory dipeptide by a similar fragmentation of VYV likely. In proteins both cysteine and tyrosine residues are expected to be present, but even if fragmentation will occur, it is unlikely that inhibitory dipeptides will be formed hereby.

Apart from affecting the cross-linking extent, the presence of ferulic acid when treating peptides with laccase also caused incorporation of ferulic acid into the tyrosine-containing peptides. The location of the incorporation could only be determined for the pep-tide VEPIPY, where it is found on the tyrosine residue. As this tyrosine residue is c-terminal, the addition of ferulic acid could involve either the phenolic group of the tyrosine residue or the c-terminal acid group. Both types of groups are known to be involved in dimeriza-tion of ferulic acid [23].

The amount of ferulic acid detectable as monomers and dimers is lowered remarkably after incubation with laccase. Due to the different possible sites of dimerization within the ferulic acid molecule and the known ability of ferulic acid to form not only dimers but also trimers, it is an obvious assumption that a further polymerization takes place, which can explain the lowering of detectable ferulic acid. The lowering is less pronounced when peptides are also present in the reaction volume. No correlation exists between incorporation of ferulic acid and the amount of detectable ferulic acid after incubation though.

In a previous study of laccase-catalysed oxidation of proteins [10], carbonyl formation was shown on histidine, tryptophan and methionine. Neither these modifications nor any other laccase-catalysed modifications that would change the mass of an amino acid were observed as a result of laccase-catalysed oxidation in the examined peptides. This could indicate that although such modifications to single amino acid residues occur and can be detected by sensitive analytical methods, they are not major reaction products of laccase-catalysed oxidation. This must infer that the possible quality impairment imposed by unspecific oxidation during laccase-catalysed cross-linking of food proteins should be minor. Thus, cross-linking by tyrosine or cysteine residues along with occasional fragmentation next to the site of cross-linking and possible incorporation of ferulic acid when this is used as a mediator should be the major results of laccase-catalysed oxidation of proteins.

Declaration of interest: The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 21 September 2009.