# 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 $\mu$M 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 $\mu$M $H_2O_2$, and final concentrations of peptide ranging from 1 to 5 mM or FA ranging from 20 to 80 $\mu$M 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 $\mu$M $H_2O_2$. 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 $\mu$M in 100 mM KPi buffer, pH 7.4, containing 140 nM HRP and 250 $\mu$M $H_2O_2$ 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-$\mu$L 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 $\mu$M $H_2O_2$ in a stirred solution. Five times a 10 $\mu$L aliquot of hydrogen peroxide was added. Twice a 10 $\mu$L aliquot of HRP was added. The sequence of addition of $H_2O_2$ and HRP was in analogy to the heteroincubation 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 $\mu$M $H_2O_2$, and 400 $\mu$M 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 $\mu$M $H_2O_2$ 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-$\mu$L aliquots of FA were added while the reaction process was monitored. In total, seven 10-$\mu$L aliquots of $H_2O_2$ and two 10-$\mu$L 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 $\mu$L of the crude incubation mixture onto an Inertsil ODS-2 5 $\mu$m 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 $\mu$L 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

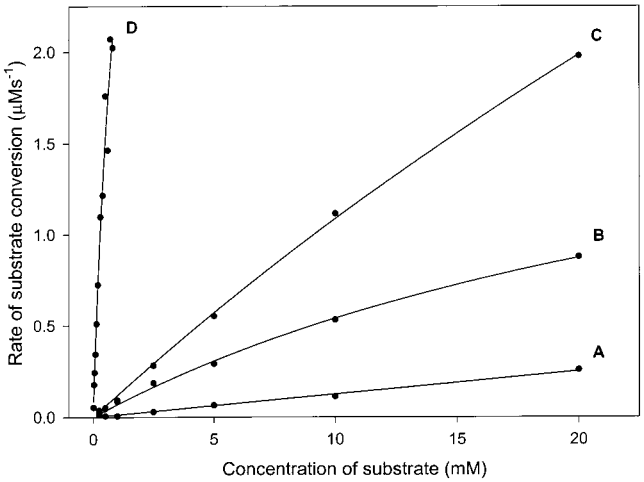


**Figure 1.** Rate of tyrosine conversion for GYG (A), YGG (B), GGY (C), and FA (D) as a function of the substrate concentration upon incubation with HRP and $H_2O_2$.

**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 $\mu$L 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

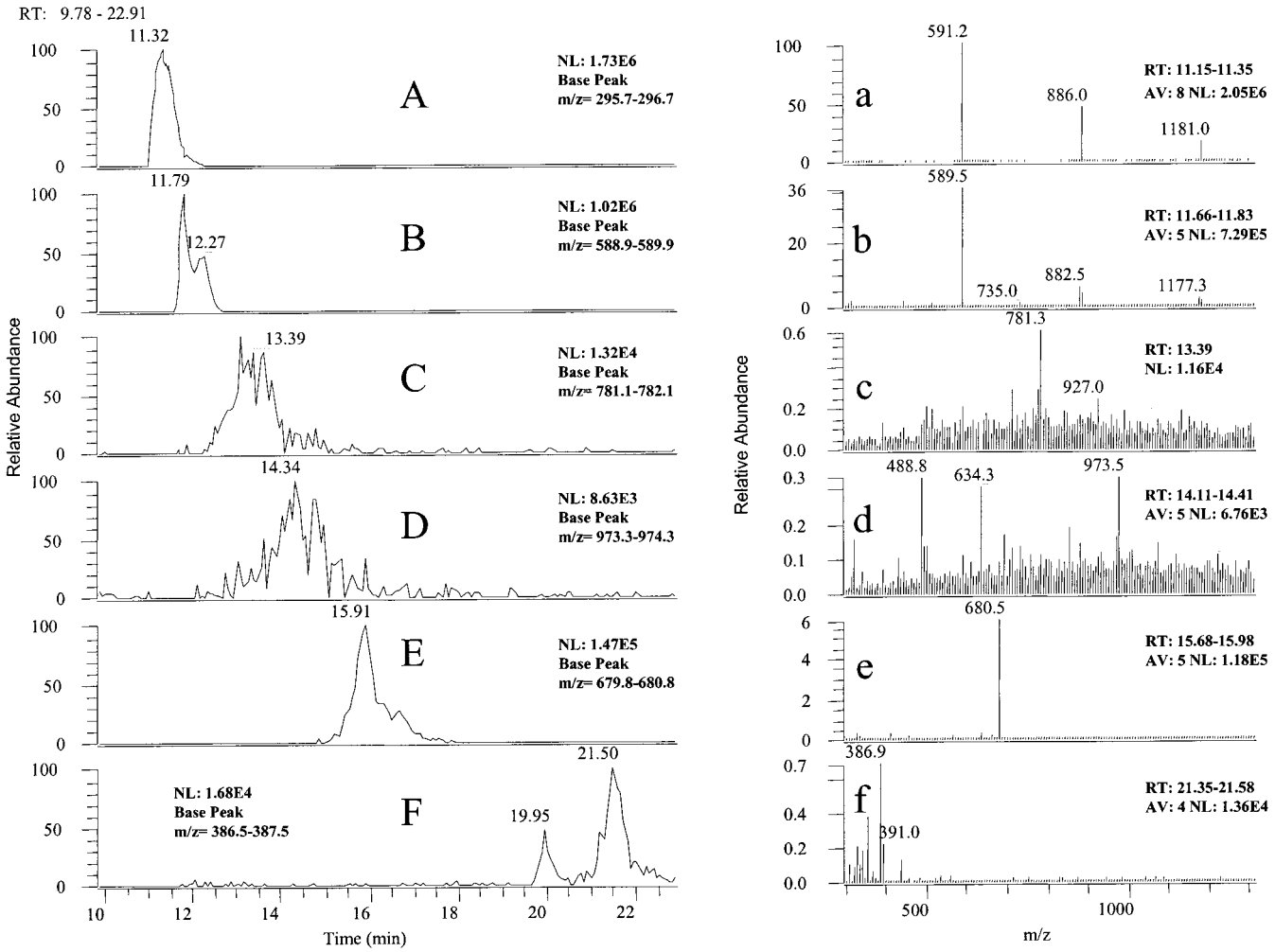


**Figure 2.** Base-peak RP-HPLC chromatograms of the kinetically controlled heteroincubation of GYG and FA (A−F), with RP-HPLC elution of the mass indicated in each chromatogram and corresponding selected mass spectra of these eluates (a−f): (A/a) GYG; (B/b) $(GYG)_2$; (C/c) FA-$(GYG)_2$; (D/d) FA-$(GYG)_2$-FA;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

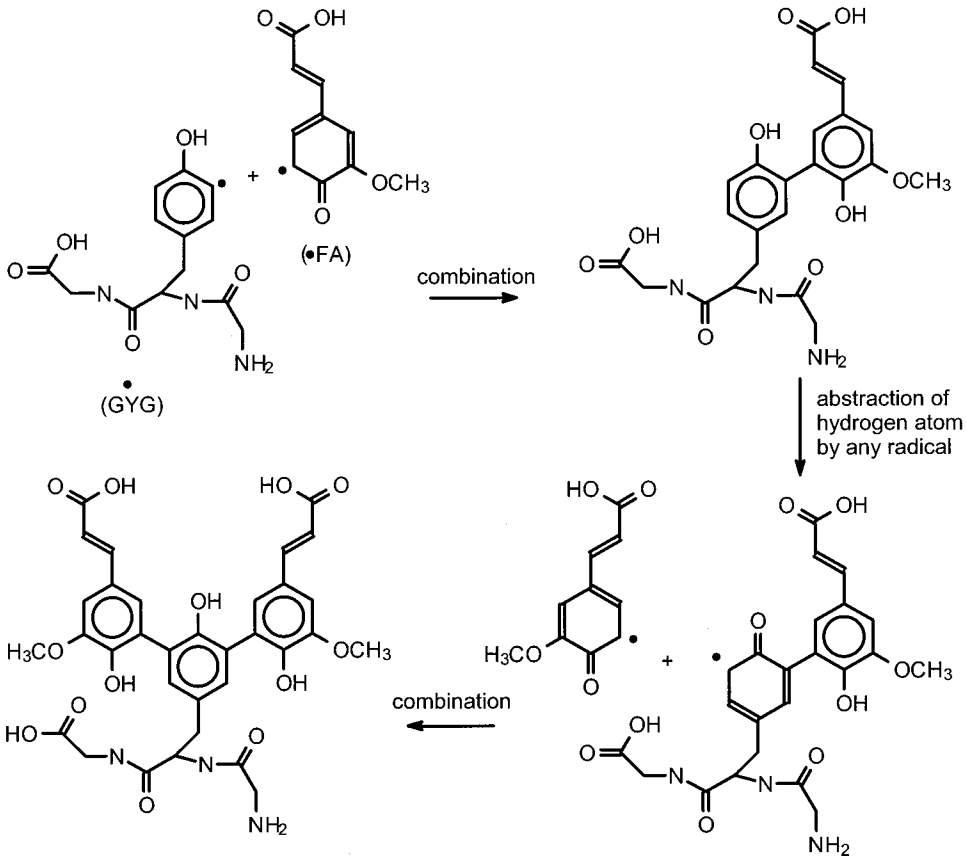


**Figure 3.** Proposed mechanism for the formation of the most predominant heteroadduct, FA-GYG-FA.

determined. This $\epsilon$ has limitations for detailed understanding of the kinetics of the process but is sufficient as a tool to merely compare substrates. As was stated by Michon et al. (*8*) in studies dealing with oxidation of tyrosines, it is generally not taken into account that an increase in absorbance or fluorescence results from contributions of different polymers. Monitoring a change in absorbance takes neither the differences in populations of oligomers for the different peptides into account nor the differences in wavelength shift of each oligomer.

As can be seen in Figure 1 the GYG peptide is at all concentrations the slowest oligomerizing tyrosine. Because $V_{max}/K_M$ for FA was 1.66, this compound is a much better substrate for the enzyme than GYG. Therefore, to achieve heterocoupling, GYG must be present in a high concentration in the incubation mixture and FA at a much lower concentration, resulting in the formation of approximately equal concentrations of radicals. However, to achieve a complete conversion, the final concentration of FA must be at least equal to the tyrosine concentration. This necessitates a kinetic control of the reaction.

The rate of oligomerization of YGG, GYG, and GGY is greatly influenced by the tyrosine position in the peptide, in agreement with what was found by Michon et al. (*8*). The monomeric, dimeric, and trimeric radicals of the peptide can, upon condensation with any oligomeric radical, lead to the higher oligomers that have indeed been found in the present study. The predominant mechanism of GYG oligomerization is the continuous generation of GYG free radicals by the enzyme, followed by hydrogen abstraction from any of the other oligomers present in the incubation mixture or combination with any of the radicals present. Contrary to Michon et al. (*8*), we assume that, for example, combination of monomeric and trimeric radicals contributes substantially to the population of tetramers and that not exclusively the combination of dimeric radicals (stemming from direct oxidation of the dimers by the peroxidase) is responsible for tetramer formation. The huge excess of GYG at each moment during incubation renders the oxidation of GYG the predominant one, even though the enzyme might possess a higher affinity for the small oligomers. Therefore, the elongation of the tyrosine-containing oligomers might be due to the continuous oxidation of monomers rather than the oxidation of oligomers. Kinetic studies are seriously complicated by these phenomena.

**Product Identification Homoincubations GYG and FA.** The one-electron oxidation by HRP of GYG generates radicals continuously. Therefore, a wide variety of products results from incubations of GYG because each newly generated radical can abstract a proton from a previously formed oligomer. The oligomers found here (Table 2) are in accordance with what has been found using MALDI-TOF in similar studies for tyrosine (*20*) and our own results for tyrosine and tyrosine derivatives using LC-MS. Isomers of the oligomers cannot be identified with absolute certainty using this technique, although the difference in elution as seen in the base peak chromatogram of, for example, the dimer (Figure 2B) does indicate the presence of isomers. The masses of the oligomers and their coelution are consistent with theoretical values (eq 1) for dehydrogenative cross-linking.

For FA only dimers are found (data similar to those in Figure 2F). Five different isomers of these dimers are known to exist (*10*). Higher polymers of FA are not observed in the present study. This can be explained by the fact that the phenolic radicals that are generated

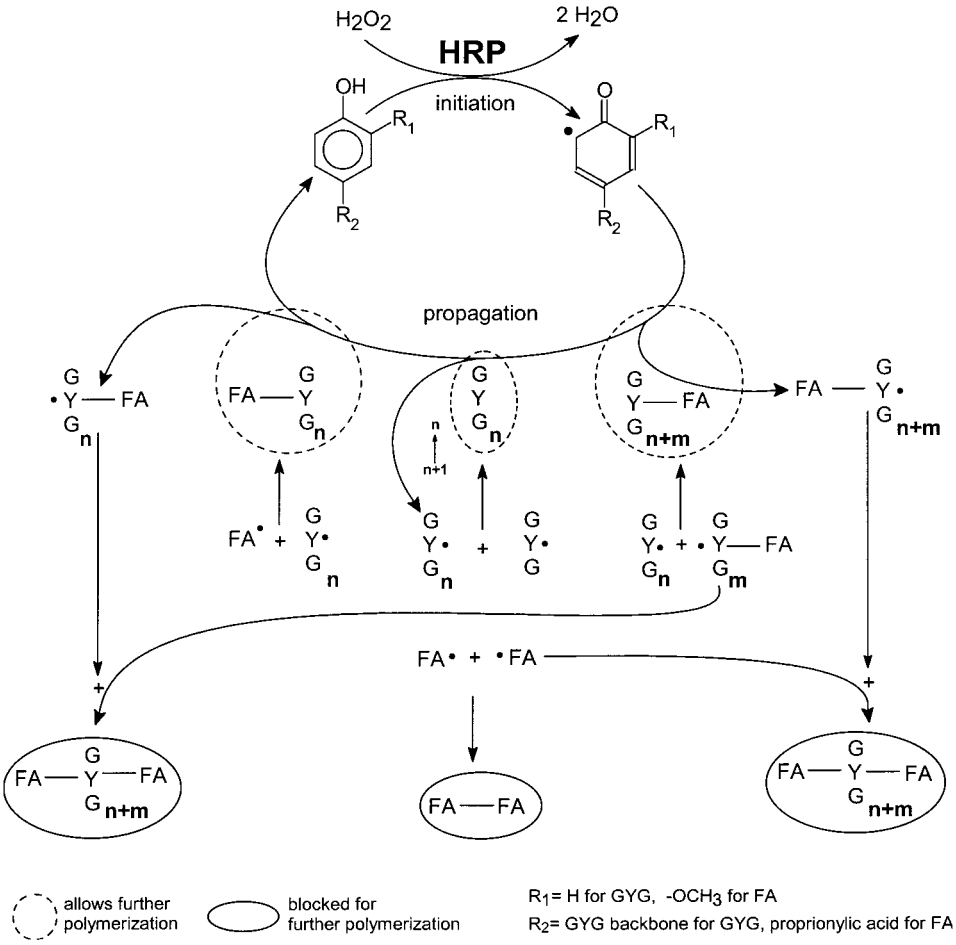


**Figure 4.** Cycles of radical reactions in the heteroincubation mixture of GYG and FA explaining the GYG oligomers and heteroadducts.

by the enzyme are not able to abstract a hydrogen from the vinylic hydrogen at the 8-position of the FA dimer. The ortho position of FA is blocked by the methoxy group.

**Heteroincubation of GYG and FA.** The increase in absorbance at 318 nm of an incubation mixture of GYG stops after addition of an aliquot of FA and is resumed only later. This indicates that tyrosine oligomerization, resulting in absorbance at this wavelength, is stopped temporarily and is resumed when all FA has been converted. This can be explained by the fact that FA is a much better substrate for the enzyme than GYG and by the chemical reactions in the incubation mixture after the addition of FA. Because of the higher $(V/K)_{app}$, FA will compete with GYG for oxidation by the enzyme, decreasing the combination of GYG radicals directly as well as indirectly—directly because a reduced number of GYG radicals is produced and is not available for combination with other GYG radicals, and indirectly because of diminished propagation of GYG oligomers after hydrogen abstraction by monomeric GYG radicals. GYG radicals already present in the incubation mixture will readily abstract a hydrogen from FA (*14*) and, therefore, no longer contribute to GYG combination of radicals. Furthermore, oligomer-terminating heteroreactions, as outlined below and in Figures 3 and 4, lead to a decrease in GYG oligomerization until all FA is consumed.

Because the spectral changes in the heteroincubation differ from those in the homoincubation, the fates of the FA added must be different. The mass spectra of the eluate of the heteroincubation revealed compounds of masses that were not present in the homoincubations. Also, theoretically no polymerization of either GYG or FA could lead to compounds of these masses. A combination of two FA molecules and one GYG covalently linked with loss of four hydrogens leads theoretically to a mass of *m*/*z* 680.6, which can be explained by the mechanism proposed in Figure 3. At the moment that FA is added, GYG polymerization is taking place and GYG radicals as well as GYG oligomer radicals are present in the reaction mixture. FA, because it is the better substrate, is then rapidly converted into semiquinone radicals. Because of the large excess of GYG initially present, also more GYG and GYG oligomer radicals are present than semiquinone radicals or mesomeric structures thereof. Besides side reactions, a combination of the semiquinone radical and a GYG radical (or GYG oligomer radical) at this moment is likely to take place. From such a heteroadduct another hydrogen can be abstracted (Figure 3). This can combine now with either another GYG (oligomer) radical or a semiquinone radical or another heteroadduct radical. As shown in Figure 4, combination with an FA radical would now lead to the compound consisting of two FA molecules covalently linked to the ortho positions of one GYG tyrosine or GYG oligomer. Isomers of this product from linkage at the oxygen are also possible and are indeed found in the base peak chromatogram (Figure 2E). This product is blocked for further polymerization and will therefore be present at the end of the incubation in a significant amount due to accumulation and

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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