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Identification of Dehydro-Ferulic Acid-Tyrosine in Rye and Wheat: Evidence for a Covalent Cross-Link between Arabinoxylans and Proteins

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To monitor chemical reactions between ferulate and proteins during breadmaking, 8-^{14}C-(*E*)-ferulic acid-(D-galactopyranose-6′-yl)ester was synthesized as a radiotracer and added to wheat and rye flour prior to breadmaking. Breads were lyophilized, extracted by means of a modified Osborne fractionation, and the radioactivity of the fractions was determined by scintillation analysis. The major portion of the radioactivity remained in the water-soluble fraction. However, a significant enrichment of the tracer was also detected in the prolamin and glutelin fractions in comparison to the control experiment. Separation of the prolamin fraction by RP-HPLC and scintillation measurement of the fractions gave evidence for a chemical modification of the tracer. To determine the structure of the reaction product, the prolamin fractions were completely hydrolyzed to free amino acids by means of an enzyme cocktail, and the digests were studied by LC−MS. In one fraction, a newly formed compound was detected. Comparison of its chromatographic and mass spectrometric behavior with a synthetic reference compound gave evidence that the newly identified compound was a dehydroferulic acid−tyrosine cross-link. It is likely that this cross-link represented a covalent linkage between arabinoxylans and cereal proteins. The newly identified cross-link was also identified in wheat and rye flour doughs, which had been prepared without addition of the ferulate tracer. The relative concentration of the dehydroferulic acid−tyrosine cross-link increased during wheat dough preparation.

KEYWORDS: Arabinoxylans; pentosans; ferulic acid; tyrosine; cross-link; bread; wheat; rye

INTRODUCTION

Arabinoxylans (AX) are important components of cereal cell walls. As nondigestible constituents, they are by definition part of the dietary fiber in cereals (*1*), where they make up the main portion of the so-called pentosans. AX consist of linear (1 → 4)-*β*-D-xylopyranosyl-chains, which can be substituted at the O-2 and/or O-3-positions with α-L-arabinofuranose (*2*). A particular minor component of AX is ferulate, which is bound to arabinose as an ester at the O-5-position (*3*). The AX of different cereals can vary substantially in content, substitution pattern, and molecular weight (*4*−*7*).

AX from rye and wheat flour have been the subject of many investigations since they have been shown to have significant influence on the formation and properties of dough and bread. The addition of AX from rye and wheat to flour leads to an increase in water absorption and loaf volume, and to a retardation of bread staling (*8*−*10*). Addition of AX strongly affects rheological parameters of wheat gluten (*11*). The content of xylanase in flour, which can degrade AX, can have a strong effect on the quality of dough and bread, stressing the influence of AX on breadmaking, again (*12*, *13*).

Although there are possible physical explanations for the effects of AX during breadmaking, like the strong gel formation potential and high water holding capacity (*14*), there are several effects that can only be explained by chemical interactions of AX during dough and bread preparation. While the addition of rye and wheat AX results in dough with improved viscoelastic properties, addition of dephenolated AX results in practically unchanged dough (*15*). From flour more AX an be isolated than from dough, and mixing of dough in the presence of air oxygen leads to a decrease of the ferulate content of AX (*16*). Along with overmixing of wheat dough, ferulate is lost, whereas large amounts of exogenous ferulic acid can induce breakdown of the dough (*17*). AX interfere with gluten formation, because they have a negative effect on gluten yield and lead to a less extensible gluten (*18*−*20*). AX can also affect the re-agglomeration of gluten-network. This effect can be weakened by addition of free ferulic acid suggesting a ferulate related chemical interaction (*21*). Covalent cross-linking of proteins and ferulate containing AX induced by peroxidase has been proposed to explain these results (*22*−*24*).

Despite this large number of investigations, no clear structure function relationship of AX during breadmaking has been found. The aim of this study was the investigation of chemical reactions of ferulate present in wheat and rye flours during breadmaking.

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For this reason, a radioactive, [^{14}C]-labeled ferulic acid ester was synthesized as a tracer to monitor chemical modifications.

MATERIALS AND METHODS

Chemicals. All reagents were obtained from commercial suppliers in the highest purity available and used without further purification unless otherwise noted. [^{1}H] and [^{13}C] NMR spectra were obtained on a BRUKER AM-360 spectrometer, using tetramethylsilane as an internal standard.

Radioactivity Analysis. Radioactivity of [^{14}C]-decay was measured by using a 1219 Rack Beta Liquid Scintillation Counter (LKB Wallac, Sweden) with Emulsifier-SAFE Liquid Scintillation Cocktail (Packard BioScience, Boston, MA; 4 mL scintillation cocktail/mL sample solution). Counting time for each measurement was 60 s; standard deviation was ±2.3 dpm.

Synthesis of 8-[^{14}C]-(*E*)-Ferulic Acid. To a suspension of 2-[^{14}C]-disodiummalonate (1.9 mg, 250 μCi, 12.6 μmol; Amersham, Buckinghamshire, UK) in pyridine (4 mL) were added malonic acid (1.25 g, 12.0 mmol), vanilline (1.52 g, 10.0 mmol), and piperidine (98 μL, 1.0 mmol). The reaction mixture was stirred under nitrogen atmosphere at 70 °C for 16 h until TLC-analysis indicated complete reaction. The mixture was poured into hydrochloric acid (c = 6 mol/L, 60 mL), whereby a precipitate formed. The precipitate was centrifuged off, washed with water (2 × 30 mL), and dried in vacuo to give the desired product (1.53 g, 7.87 mmol, 78%) as an offwhite, crystalline solid.

[^{1}H] NMR (360 MHz, methanol-d_4): δ_H 7.59 (d, J = 15.9 Hz, 1H), 7.16 (s, 1H), 7.05 (d, J = 8.2 Hz, 1H), 6.81 (d, J = 8.2 Hz, 1H), 6.31 (d, J = 15.9 Hz, 1H), 4.92 (br. s, 2H), 3.88 (s, 3H). [^{13}C] NMR (90 MHz, methanol-d_4): δ_C 171.3, 150.6, 149.5, 147.2, 128.0, 124.2, 116.7, 116.1, 111.9, 56.7. Activity: 1.2 kBq/mg.

Synthesis of 4-*O*-*tert*-Butyldimethylsilyl-8-[^{14}C]-(*E*)-ferulic Acid. To a solution of 8-[^{14}C]-(*E*)-ferulic acid (408 mg, 2.1 mmol, 492 kBq) in DMF (7 mL) were added *tert*-butyldimethysilyl chloride and imidazole (429 mg, 6.3 mmol). The solution was stirred under nitrogen atmosphere at room temperature for 18 h. The reaction mixture was poured into ice water/phosphoric acid (c = 1 mol/L, 25 mL) and extracted with ethyl acetate (3 × 10 mL). The combined organic phases were washed with water (2 × 10 mL) and saturated sodium chloride solution (10 mL). The organic phase was dried over sodium sulfate and concentrated in vacuo to give the disilylated product. The disilylated product was suspended in acetic acid/water/THF (3+1+1 (v+v+v), 7 mL), and the suspension was stirred at room temperature for 80 min. The reaction was poured into ice water (20 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic phases were washed with water (2 × 10 mL) and saturated aqueous sodium chloride solution (10 mL). The organic phase was dried over sodium sulfate and concentrated in vacuo to give the desired product (626 mg, 2.03 mmol, 97%) as a reddish solid.

[^{1}H] NMR (360 MHz, $CDCl_3$): δ_H 7.73 (d, J = 15.7 Hz, 1H), 7.07−7.03 (m, 2H), 6.85 (d, J = 8.6 Hz, 1H), 6.31 (d, J = 15.8 Hz, 1H), 3.84 (s, 3H), 0.99 (s, 9H), 0.18 (s, 6H). [^{13}C] NMR (90 MHz, CDCl3): δ_C 172.9, 151.3, 148.0, 147.1, 127.9, 122.7, 121.1, 115.0, 111.1, 55.4, 25.6, 18.5, −4.6.

Synthesis of 8-[^{14}C]-(*E*)-Ferulic Acid-(1,2:3,4-di-isopropylidene-D-galactopyranose-6-yl)ester. To a solution of 4-*O*-*tert*-butyldimethylsilyl-8-[^{14}C]-(*E*)-ferulic acid (616 mg, 1.99 mmol) in dichloromethane (20 mL) were added 1,2:3,4-di-isopropylidene-D-galactopyranose (551 mg, 2.11 mmol), dicyclohexylcarbodiimide (433 mg, 2.10 mmol), and 4-(dimethylamino)pyridine (24 mg, 0.20 mmol). The solution was stirred at room temperature under nitrogen atmosphere for 20 h until TLC analysis showed complete reaction; meanwhile a white precipitate formed. The mixture was filtered, and the filtrate was washed with aqueous acetic acid (w = 5% (m/m), 2 × 15 mL), and half saturated aqueous sodium chloride solution (15 mL). The organic phase was dried over sodium sulfate and concentrated in vacuo to obtain the silylated ester. To a solution of the silylated ester in DMF (15 mL) were added potassium fluoride (233 mg, 4.0 mmol) and hydrobromic acid (w = 47% (m/m), 46 μL, 0.4 mmol). The mixture was stirred at room temperature for 1 h. The reaction was poured into aqueous acetic acid (w = 5% (m/m), 60 mL) and extracted with ethyl acetate (3 × 30 mL). The combined organic phases were washed with half concentrated aqueous sodium chloride solution (2 × 20 mL). The organic phase was dried over sodium sulfate and concentrated in vacuo. The crude material was purified by flash chromatography (silica gel 60, hexane/ethyl acetate 60/40 (v/v)) to give the desired product (667 mg, 1.53 mmol, 76%) as a white solid.

[^{1}H] NMR (360 MHz, $CDCl_3$): δ_H 7.63 (d, J = 16.0 Hz, 1H), 7.05−7.03 (m, 2H), 6.91 (d, J = 8.0 Hz, 1H), 6.34 (d, J = 16.0 Hz, 1H), 5.56 (d, J = 5.0 Hz, 1H), 4.64 (dd, J = 7.9 und 2.5 Hz, 1H), 4.44 (dd, J = 11.6 and 4.6 Hz, 1H), 4.34 (dd, J = 5.0 and 2.6 Hz, 1H), 4.31 (t, J = 1.7 Hz, 1H), 4.29 (t, J = 1.9 Hz, 1H), 4.13−4.09 (m, 1H), 3.93 (s, 3H), 1.53 (s, 3H), 1.47 (s, 3H), 1.36 (s, 3H), 1.34 (s, 3H). [^{13}C] NMR (63 MHz, $CDCl_3$): δ_C 167.1, 148.0, 146.8, 145.2, 126.9, 123.3, 115.1, 114.7, 109.6,109.2, 108.8, 96.3, 71.1, 70.7, 70.4, 66.1, 63.3, 55.9, 25.5, 24.9, 24.8, 24.4.

Synthesis of 8-[^{14}C]-(*E*)-Ferulic Acid-(D-galactopyranose-6-yl)-ester. To a solution of 8-[^{14}C]-(*E*)-ferulic acid-(1,2:3,4-di-isopropylidene-D-galactopyranose-6-yl)ester in 1,4-dioxane (11.4 mL) was added hydrochloric acid (c = 1 mol/L, 2.8 mL). The solution was stirred under nitrogen atmosphere in a sealed tube for 1 h at 100 °C until TLC analysis showed complete reaction. The solvent and hydrochloric acid were removed under a nitrogen gas flow and finally dried in vacuo to give the unprotected sugar ester (1.5 mmol, quantitative) as an orange oil. For more convenient handling, the product was dissolved in DMSO (10.0 mL).

MS (ESI pos) (m/z, rel intensity): 357.1 ([M + H]$^+$, 100%), 177.0 ([M − $C_6H_{11}O_6$]$^+$, 88%). MS (ESI neg) (m/z): 355.0 ([M − H]$^-$). Activity: 24.6 kBq/mL.

Wheat Breadmaking Procedure. Baking experiments were performed following the 10 g micro-version of the rapid-mix-test developed by Kieffer et al. (*25*). To wheat flour (10 g, cv. Soissons, ash content 0.55% in dry mass), sodium chloride (0.2 g), sucrose (0.1 g), baker's yeast (0.7 g, Wieninger, Passau, Germany), and water (6.15 mL) was added 8-[^{14}C]-(*E*)-ferulic acid-(D-galactopyranose-6-yl)ester-solution (100 μL, 2.5 kBq). The compounds were mixed in a micro-rapid-mixer for 45 s at 1250 rpm. The dough was fermented for 20 min at 30 °C and 90% rel. humidity. Afterward, a spherical dough piece was formed, and proofed for 45 min at 30 °C and 90% rel. humidity. Baking was performed at 230 °C for 10 min. The bread was lyophilized, ground, and defatted by extraction with hexane (2 × 30 mL).

Rye Breadmaking Procedure. For the preparation of sour dough, rye flour (100 g, cv. Nikita, ash content 1.15% in dry mass), sour dough deep freeze starter (10 g, Böcker, Minden, Germany), and water (90 mL) were mixed in a 500 mL beaker for 5 min and proofed at 28 °C and 90% rel. humidity for 18 h to give sour dough. For the preparation of the bread, to rye flour (5.72 g, cv. Nikita, ash content 1.15% in dry mass), sour dough (4.29 g), sodium chloride (0.7 g), and water (4.8 mL) was added 8-[^{14}C]-(*E*)-ferulic acid-(D-galactopyranose-6-yl)ester-solution (300 μL, 7.4 kBq). The compounds were mixed in a micro-rapid-mixer for 45 s at 1250 rpm. The dough was fermented for 15 min at 29 °C and 90% rel. humidity. The dough was given into a micro baking tin (2.5 × 5 cm), and proofed for 50 min at 29 °C and 90% rel. humidity. Baking was performed at 220 °C for 10 min. The bread was lyophilized, ground, and defatted by extraction with hexane (2 × 30 mL).

Modified Osborne Fractionation. Osborne fractionations were performed following the method developed by Wieser et al. (*26*) with some modifications. Lyophilized and defatted bread samples (1000 mg) were stepwise extracted with 0.4 mol/L NaCl/0.067 mol/L $HKNaPO_4$ (pH 7.6) (3 × 10 mL), with 60% (v/v) aqueous ethanol (3 × 10 mL), with 0.05 mol/L NaH_2PO_4/1% (w/v) SDS (pH 6.9) (3 × 10 mL), and finally with 50% (v/v) aqueous 1-propanol/2 mol/L urea/0.05 mol/L Tris-HCl (pH 7.5)/1% (w/v) dithioerythritol under nitrogen and increased temperature (60 °C) (3 × 10 mL).

HPLC Analysis of Bread Prolamins. HPLC analyses of the bread prolamins were performed on a Kontron Instruments System, which was equipped with a data system D450, two HPLC 420 pumps, and an HPLC 432 detector (Kontron Biotek, Neufahrn, Germany). The chromatographic separation of the hydrolyzates was carried out on a Nucleosil 300-5-C_8 (250 × 4.6 mm, 5 μm) column (Macherey-Nagel, Düren, Germany) at 50 °C. The mobile phase was as follows: eluent

A, TFA in water (0.1% (v/v)); eluent B, TFA in acetonitrile (0.1% (v/v)). A linear gradient (0−50 min, 24−56% B) was applied, flow rate was 1.0 mL/min, UV-detection was at 210 nm. Injection volume of the sample was 50 μL.

Enzymatic Hydrolysis of Bread Samples. The enzymatic hydrolysis of the bread samples was performed with some modifications following a procedure developed by Schmitz et al. for the hydrolysis of milk proteins (*25*). Sample material (150−200 mg) was weighed into test tubes (20 × 100 mm, Pyrex, UK) and suspended in hydrochloric acid (5 mL, c = 20 mmol/L). A solution of pepsin (200 μL, 6.5 mg/mL; Roche, Basel, Switzerland) and a small crystal of thymol were added. Incubation was carried out in a water bath at 37 °C for 24 h. After addition of sodium hydroxide (200 μL, c = 5 mol/L), the mixture was incubated at room temperature for 2 h to hydrolyze ester bonds. Hydrochloric acid (200 μL, c = 5 mol/L) and Tris/HCl-buffer (600 μL, c = 2 mol/L, pH 8.2) were added, followed by an α-amylase solution (200 μL; 2.8 mg/mL, from bacillus licheniformis, 631 units/mg). The mixture was heated at 90 °C for 5 min in a water bath and incubated at room temperature for another 1.5 h. After addition of an amyloglucosidase-solution (200 μL, 2.8 mg/mL, from aspergillus niger, 7.9 units/mg) and a β-xylanase-suspension (10 μL, in 3.2 mol/L ammoniumsulfate, from T. longibrachiatum, 2740 units/mL, Megazymes, Bray, Ireland), the mixture was incubated at 37 °C for 24 h, after which a solution of Pronase E (200 μL, protease from streptomyceus griseus, 5.9 mg/mL, 5.6 units/mg) was added. Incubation was carried out for another 24 h at 37 °C. A prolidase solution (125 μL, from porcine kidney, 2.0 mg/mL, 88 units/mg) was added and the incubation carried out another 24 h at 37 °C. Finally, a leucine aminopeptidase suspension (70 μL, microsomal, from porcine kidney, 25 units/180 μL suspension (in 3.5 mol/L ammoniumsulfate, 10 mol/L $MgCl_2$, pH 7.7)) was added to the hydrolyzate mixture and incubated for 24 h at 37 °C. After hydrolysis, samples were lyophilized, dissolved in acetonitrile/water (15/85 (v/v)), and filtered (0.45 μm membrane filter) prior to HPLC analysis.

HPLC Analysis of Bread Hydrolyzates. HPLC analyses of the bread hydrolyzates were performed on a Kontron Instruments System, which was equipped with a System 522 pump and an HPLC 535 detector (Kontron Biotek, Neufahrn, Germany). The chromatographic separation of the hydrolyzates was carried out on a HyperClone 5 μ ODS (C_{18}) (250 × 4.6 mm, 5μm) column (Phenomenex, Aschaffenburg, Germany) at room temperature. The mobile phase was as follows: eluent A, TFA in water (0.1% (v/v)); eluent B, TFA in acetonitrile (0.1% (v/v)). A linear gradient (1−40 min, 5−95% B) was applied; flow rate was 0.8 mL/min, UV-detection was at 310 nm. Injection volume of the sample was 100 μL.

HPLC/MS Analysis. Mass spectra were recorded by means of an ion trap mass spectrometer (LCQ classic, Finnigan MAT, Dreieich, Germany) coupled to a liquid chromatography system (Spectra System P4000 HPLC pump and Spetra System UV1000 Detector, Finnigan MAT, Dreieich, Germany) equipped with an autosampler and a HyperClone 5 μ ODS (C_{18}) (250 × 4.6 mm, 5 μm) column (Phenomenex, Aschaffenburg, Germany). The mobile phase was composed as follows: eluent A, formic acid in water (0.1% (v/v)); eluent B, formic acid in acetonitrile (0.1% (v/v)). A linear gradient (1−21 min, 15−42% B) was applied; flow rate was 0.8 mL/min, UV-detection was at 310 nm. The effluent between 4.0 and 19.2 mL was directed into the electrospray interface. The mass spectrometer was operated in the positive electrospray ionization mode (ESI^+) with a spray needle voltage of 5.0 kV and a spray current of 80 μA. The temperature of the capillary was 200 °C, and the capillary voltage was 10 V. The sheath and auxiliary gas (nitrogen) was adjusted to 80 and 20 arbitrary units, respectively. The compounds in the hydrolyzed samples were characterized by means of their molecular masses obtained in the full scan mode (range m/z 105−1000).

HPLC/MS−MS Analysis. Mass spectra were recorded by means of a triple quadrupole tandem mass spectrometer (TSQ Quantum Discovery, Thermo Electron, Dreieich, Germany) coupled to a Surveyor HPLC system (Thermo Finnigan, Dreieich, Germany) equipped with a thermostated (20 °C) autosampler and a Synergi Polar-RP 80 Å HPLC column (150 × 2.0 mm, 4 μm, Phenomenex, Aschaffenburg, Germany; kept at 30 °C) and a Polar-RP precolumn (4 × 2.0 mm, Phenomenex). The sample (10 μL) was separated at a flow rate of 0.2 mL/min. The solvent system was composed of (A) formic acid in water (0.1% (v/v)) and (B) formic acid in acetonitrile (0.1% (v/v)). A linear gradient (3−20 min, 10−42% B) was applied. The effluent between 1.5 and 10.0 mL was directed into the electrospray interface. The mass spectrometer was operated in the positive electrospray ionization mode (ESI^+) with a spray needle voltage of 3.0 kV and a spray current of 5 μA. The temperature of the capillary was 300 °C, and the capillary voltage was 35 V. The sheath and auxiliary gas (nitrogen) was adjusted to 35 and 7 arbitrary units, respectively. The collision cell was operated at a collision gas (argon) pressure of 6.7×10^{-2} Pa. The scan time for each transition and single reaction monitoring was 0.60 s.

Solid-Phase Synthesis of Dehydro-Ferulic Acid-Tyrosine. To a suspension of Tentagel S-OH (500 mg, 90 μm, Rapp Polymere, Tuebingen, Germany) in dichloromethane (5 mL) were added 4-*O*-*tert*-butyldimethylsilyl-(*E*)-ferulic acid (10.2 mg, 33 μmol), diisopropylcarbodiimide (13 μL, 83 μmol), and 4-(dimethylamino)pyridine (4.0 mg, 33 μmol). The mixture was shaken under nitrogen atmosphere at room temperature for 20 h. The suspension was centrifuged off, and the solvent was removed. The solid phase was washed with dichloromethane (2 × 5 mL) and THF (2 × 5 mL). The solid phase was suspended in THF (3 mL). Glacial acetic acid (50 μL) and a solution of tetrabutylammoniumfluoride (330 μL, c = 1 mol/L in THF) were added and stirred for 1 h at 0 °C to deprotect the ferulate. The suspension was centrifuged off, and the solvent was removed. The solid phase was washed with THF (3 × 5 mL) and dried under a nitrogen gas flow. The solid phase was washed with ammonium formiate-buffer (5 mL, c = 135 mmol/L, pH 9.2) and afterward suspended again in ammonium formiate-buffer (5 mL, c = 135 mmol/L, pH 9.2). L-Tyrosine (50 mg) and peroxidase (from horseradish) (1.1 mg) were added. The suspension was shaken for 2 h at room temperature. Hydrogen peroxide solution (200 μL, c = 0.3 mol/L) was added, and the mixture was shaken for 3 h at room temperature. The mixture was centrifuged off, and the solvent was removed. The solid phase was washed with 0.1% aqueous formic acid (3 × 5 mL) and water (5 mL). To the solid phase was added acetonitrile/water (3 mL, 1/1 (v/v))/1% sodium hydroxide (m/m) to hydrolyze the ester. The suspension was shaken for 2 h at room temperature. The suspension was centrifuged off, and the supernatant was collected. The solid phase was washed with acetonitrile/water (1/1 (v/v))/1% (m/v) sodium hydroxide (2 × 3 mL) and centrifuged off. To the combined supernatants was added concentrated formic acid (100 μL). The solution was dried under a nitrogen gas flow to give a white solid, which was dissolved in 0.1% aqueous formic acid (1.0 mL). For further purification, a solid-phase extraction workup was carried out.

A Strata C_{18}-T SPE-tube (500 mg, 3 mL) (Phenomenex, Aschaffenburg, Germany) was conditioned by elution with formic acid in acetonitrile (0.1% (v/v)) (2 mL), Na_2HPO_4-buffer (c = 0.05 moL/L, pH 7.4) (3 × 2 mL), and formic acid in water (0.1% (v/v)) (3 × 2 mL). Afterward, the sample solution was applied onto the SPE tube and eluted with formic acid in water/acetonitrile (99/1 (v/v))/0.1% (v/v) formic acid to remove polar compounds. The desired mixture containing several tyrosine-ferulic acid products was eluted with water/acetonitrile (55/45 (v/v))/0.1% (v/v) formic acid. The eluent was separated by RP-HPLC, and the desired product (**Figure 6**, peak #6) was detected by MS and MS−MS.

MS (ESI pos.)(m/z, rel intensity): 769.1 ($[2M + Na]^+$, 85%), 747.0 ($[2M + H]^+$, 100%), 396.1 ($[M + Na]^+$, 22%), 374.0 ($[M + H]^+$, 87%). MS−MS (374.0; ESI pos., collision energy 27%) (m/z, rel intensity) (315.3, 5%; 310.0, 20%; 292.9, 50%; 283.8, 80%; 271.0, 100%).

Synthesis of 3-OMe-[^{2}H]$_3$-(*E*)-Ferulic Acid. To a solution of 3-[^{2}H]$_3$-methoxy-4-hydroxybenzaldehyd ($M_R(155)/M_R(152)$ = 99.67%) (donation of Ms. C. Scheidig) (200 mg, 1.29 mmol) and malonic acid (105 mg, 1.55 mmol) in pyridine (2 mL) was added piperidine (13 μL). The reaction mixture was stirred under nitrogen atmosphere at 70 °C for 18 h until TLC analysis indicated complete reaction. The mixture was poured into hydrochloric acid (c = 6 mol/L, 30 mL) and extracted with ethyl acetate (3 × 10 mL). The combined organic phases were washed with 1 mol/L hydrochloric acid (2 × 15 mL) and saturated aqueous sodium chloride solution (15 mL). The organic phase was dried over sodium sulfate and concentrated in vacuo. The crude material was

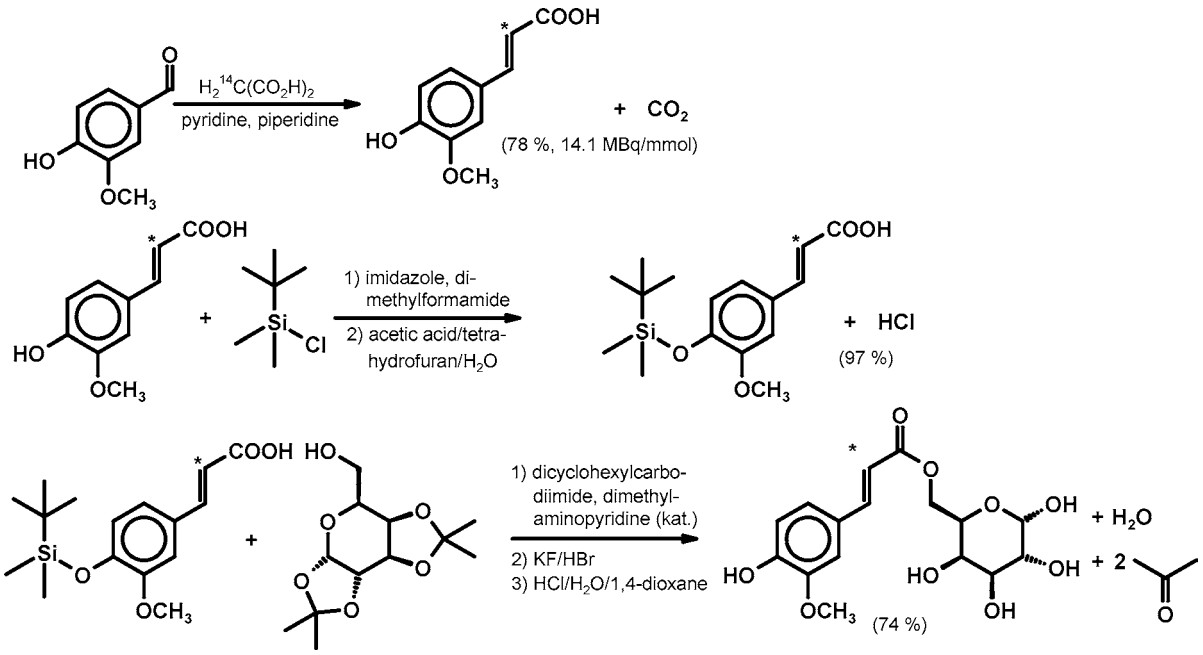

Figure 1. Synthesis of 8-[^{14}C]-(*E*)-ferulic acid-(D-galactopyranose-6-yl)ester. ∗: ^{14}C label.

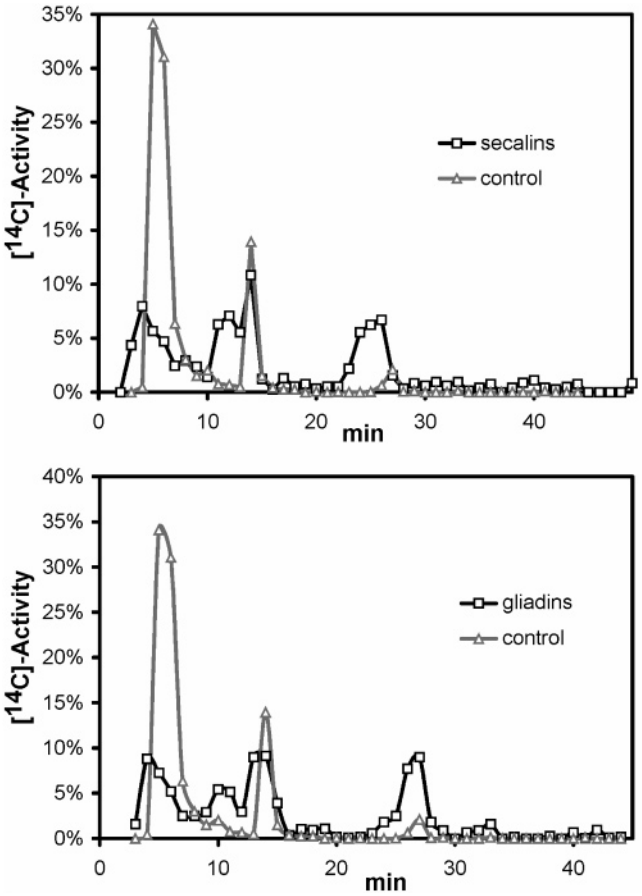

Figure 2. RP-HPLC/scintillation chromatogram of secalins and gliadins isolated by modified Osborne fractionation of rye and wheat bread. Each data point corresponds to the radioactivity present in the eluate collected during 1 min of elution time. Mean coefficient of variation <3%.

purified by flash chromatography (silica gel 60, ethyl acetate/hexane/acetic acid, 80+20+1 (v+v+v)) to give the desired product (235 mg, 1.19 mmol, 93%) as an off-white solid.

[^{1}H] NMR (360 MHz, methanol-d_4): δ_H 7.59 (d, J = 15.9 Hz, 1H), 7.16 (d, J = 1.8 Hz, 1H), 7.05 (dd, J = 8.2 and 1.9 Hz, 1H), 6.81 (d, J = 8.2 Hz, 1H), 6.30 (d, J = 15.9 Hz, 1H), 4.86 (br. s, 2H). [^{13}C] NMR (90 MHz, methanol-d4): δ_C 171.3, 150.8, 149.6, 147.2, 128.1, 124.3, 116.7, 116.2, 112.0.

MS (ESI pos) (*m*/*z*, rel intensity): 198.0 ([M + H]$^+$, 100%), 180.2 ([M − H_2O + H$^+$], 44%).

Solid-Phase Synthesis of [^{2}H]$_3$-Dehydro-(*E*)-Ferulic Acid-Tyrosine. *Synthesis of [^{2}H]$_3$-4-O-tert-Butyldimethylsilyl-8-(E)-Ferulic Acid.* The synthesis was performed as described for the undeuterated compound. 3-OMe-[^{2}H]$_3$-(*E*)-ferulic acid (191 mg, 0.97 mmol) was transformed after reaction with *tert*-butyldimethylsilyl chloride (377 mg) and imidazole (68 mg) to the desired product (277 mg, 0.89 mmol, 92%) as an off-white solid.

[^{1}H] NMR (360 MHz, $CDCl_3$): δ_H 7.75 (d, J = 15.9 Hz, 1H), 7.07−7.04 (m, 2H), 6.86 (d, J = 8.6 Hz, 1H), 6.32 (d, J = 15.9 Hz, 1H), 1.00 (s, 9H), 0.18 (s, 6H). [^{13}C] NMR (90 MHz, $CDCl_3$): δ_C 172.6, 151.3, 148.0, 147.1, 128.0, 122.7, 121.1, 115.0, 111.1, 25.6, 18.5, −4.6.

Synthesis of [^{2}H]$_3$-Dehydro-(E)-Ferulic Acid-Tyrosine. The synthesis was performed as described for the undeuterated compound. [^{2}H]$_3$-4-*O*-*tert*-Butyldimethylsilyl-8-(*E*)-ferulic acid (40.7 mg, 0.13 mmol) was coupled to Tentagel S-OH (2.0 g) with diisopropylcarbodiimide (51 μL) and (dimethylamino)pyridine (4.0 mg). The ester was desilylated with tetrabutylammoniumfluoride (1.32 mmol) and afterward brought to reaction with L-tyrosine (200 mg), horseradish peroxidase (3.0 mg), and hydrogen peroxide (0.13 mmol). After alkaline release and solid-phase extraction workup, a solution of [^{2}H]$_3$-dehydro-(*E*)-ferulic acid-tyrosine and other deuterated tyrosine-ferulic acid products (12 mL) was obtained. The concentration of the desired isomer (**Figure 6**, peak # 6) was estimated by HPLC/UV-detection at 310 nm. Because no identical standard compound was available, ferulic acid was used for the generation of a calibration curve. Under the assumption of an identical molar absorption coefficient of ferulic acid and DFT at 310 nm (as tyrosine does not absorb light at 310 nm), the concentration of the desired isomer (**Figure 6**, peak # 6) was 978 ng/mL.

MS (ESI pos.) (*m*/*z*, rel intensity): 377.0 ([M + H]$^+$, 100%). MS/MS (377.0; ESI pos., collision energy 21%) (*m*/*z*, rel intensity) (377.0, 12%; 359.1, 45%; 313.1, 56%; 296.2, 100%).

Quantitation of Dehydro-Ferulic acid-Tyrosine in Wheat and Rye Flour, Dough, and Bread. Wheat and rye dough and bread were made as described in the baking procedures, except no ferulic acid was added. Flour, dough, and bread samples were lyophilized and ground (<0.2 mm). 10−20 mg of the sample was weighed into a sealed tube. Hydrochloric acid/phenol (c(HCl) = 6 mol/L, w(phenol) = 0.1% (m/v)) was added, and the material was hydrolyzed under nitrogen atmosphere at 110 °C for 24 h. Hydrochloric acid was removed at room

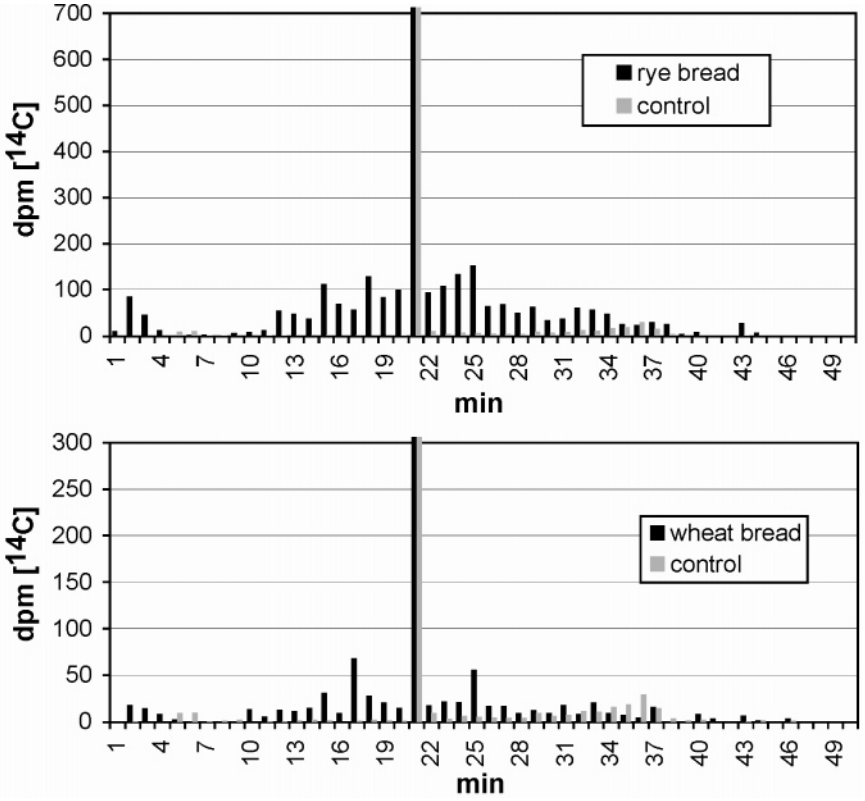

Figure 3. RP-HPLC/scintillation chromatogram of rye and wheat bread enzymic hydrolyzates. Each data bar corresponds to the radioactivity present in the eluate collected during 1 min of elution time. Mean coefficient of variation <3%.

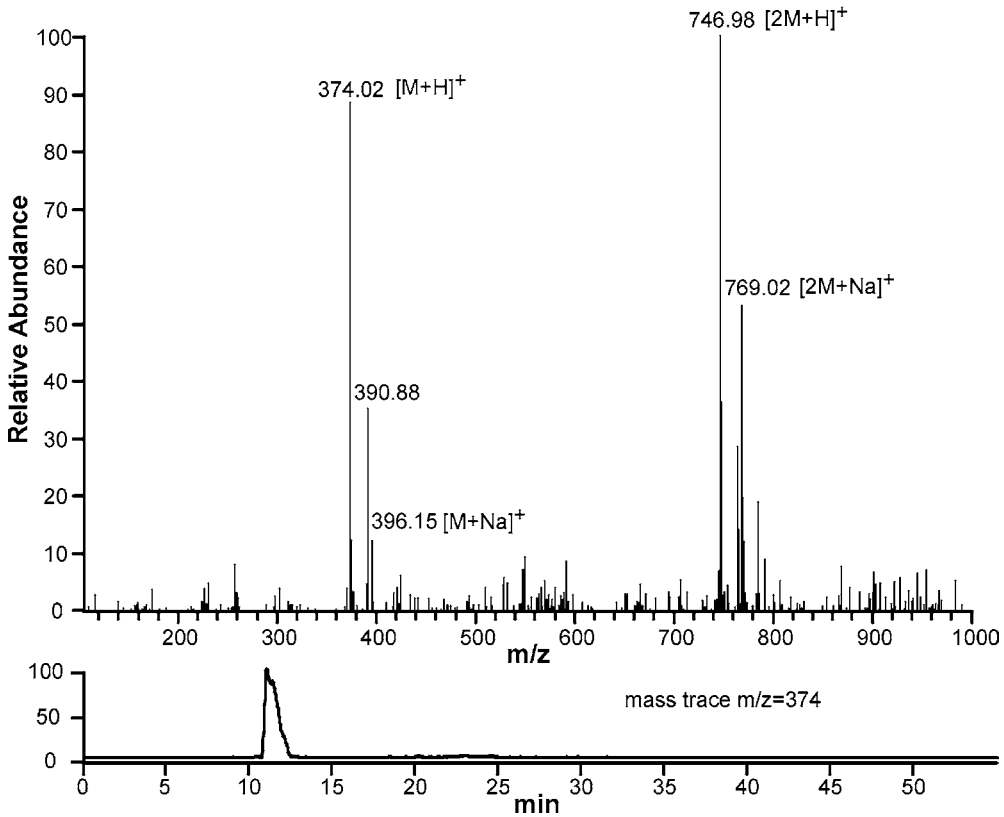

Figure 4. RP-HPLC/ESI-MS of dehydro-ferulic acid-tyrosine present in a wheat bread enzymic hydrolyzate. Signals are assigned to the structure of the ions. M: molecular mass of dehydro-ferulic acid-tyrosine (373 g/mol).

temperature under nitrogen gas flow. To the dry hydrolyzate were added the identified isomer of $[^2H]_3$-dehydro-(*E*)-ferulic acid-tyrosine (9.8 ng) and aqueous formic acid (1.0 mL, 0.1% (v/v)). The mixture was homogenized using an ultrasonic bath for 15 min, followed by a solid-phase extraction workup as described for the synthesis of DFT. The eluent of the workup was lyophilized and dissolved in acetonitrile/water/formic acid (250 μL, 10/90/0.1 (v/v/v)). The concentration of DFT was determined by means of HPLC/MS−MS analysis.

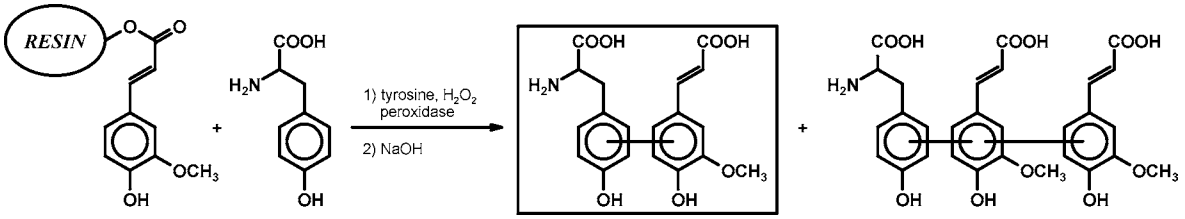

Figure 5. Solid-phase synthesis of dehyro-ferulic acid tyrosine.

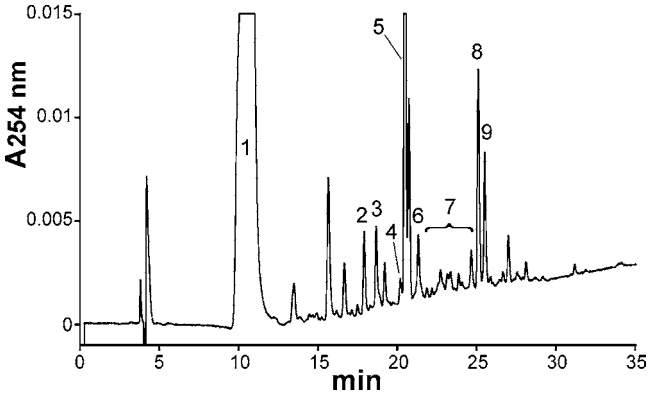

Figure 6. RP-HPLC chromatogram of the solid-phase product mixture after alkaline release. 1, tyrosine; 2, 3, 4, and 6, isomers of dehydro-ferulic acid-tyrosine; 5, ferulic acid; 7, isomers of dehydro-diferulic acid-tyrosine and -triferulic acid-tyrosine; 8 and 9, isomers of decarboxylated dehydro-triferulic acid-tyrosine.

Table 1. Retention of the Ferulate Radio Tracer within Protein Fractions

	protein fraction			
	albumins/ globulins	prolamins	SDS-soluble glutelins	SDS-insoluble glutelins
control[a]	95.8%	3.4%	0.5%	0.3%
wheat bread[a]	70.5%	24.5%	1.2%	3.8%
rye bread[a]	78.6%	19.8%	0.7%	0.9%

[a] Number of experiments $n = 3$, mean coefficient of variation <3%.

RESULTS

Synthesis of the [^{14}C]-Labeled Tracer. Starting from vanilline and 2-[^{14}C]-labeled malonic acid, 8-[^{14}C]-labeled (*E*)-ferulic acid was obtained in a Knoevenagel condensation. Because free ferulic acid is poorly water soluble and has different electronic properties than an ester, the acid was converted into 8-[^{14}C]-(*E*)-ferulic acid-(D-galactopyranose-6-yl)ester as outlined in **Figure 1**. After acidic hydrolysis of the ketal, the product was obtained as a mixture of diastereomers of the carbohydrate unit. Therefore, it was not possible to characterize the product by NMR. The product was used as a radio tracer in the subsequent studies.

Modified Osborne Fractionation of Tracer Breads. After adding the radio tracer to the flour, wheat and rye breads were prepared in a 10 g scale. The breads were extracted by a modified Osborne fractionation. The albumin/globulin-, prolamin-, SDS-soluble, and SDS-insoluble glutelin fractions were examined by means of liquid scintillation measurements to monitor the retention of the radio tracer. The results are shown in **Table 1**. As a control experiment, the radio tracer was added to wheat starch, which was chosen as a control instead of flour, to exclude the possible formation of artifacts especially during the extraction of the albumins/globulins. As the unmodified tracer was rather water soluble, more than 95% of the tracer in the control experiment was found in the aqueous extract, and less than 4% in the ethanol extract. In comparison to the control experiment, the prolamin fraction (ethanol extract) of the rye and wheat bread was strongly enriched with the tracer. The glutelin fractions (SDS extract without and with reducing agent added) of the wheat bread also contained significantly higher amounts of the tracer than the corresponding extracts of the control experiment. These findings indicated that ferulate underwent chemical modifications during breadmaking, which led to an enrichment of ferulate in the protein fractions of the bread.

HPLC/Scintillation of Prolamins. As higher radioactivity was found in the prolamin fractions than in the glutelin fractions, the prolamins of rye and wheat bread were further investigated by HPLC/scintillation measurements. First, the isolated secalins (rye prolamins) and gliadins (wheat prolamins) of the tracer-containing breads were separated by means of reversed phase HPLC. As a control experiment, the unmodified tracer was examined in the same manner. Fractions of the HPLC separation were further analyzed by scintillation measurements to obtain HPLC/scintillation chromatograms. Both the chromatograms of secalins and gliadins showed signals that were not present in the control experiment (**Figure 2**). These results confirmed the assumption that ferulate was chemically modified during rye and wheat bread preparation.

HPLC/Scintillation of Bread Hydrolyzates. To isolate the chemically modified ferulate compound, the bread samples had to be hydrolyzed. Whole bread samples were used rather than Osborne fractions because the former had a higher radioactivity than the prolamin and glutelin fractions. As described above, a modified enzymatic procedure by Schmitz et al. (*27*) was used to hydrolyze the bread samples and, therefore, to release the chemically modified tracer. Again, unmodified ferulate tracer was used in the control experiment. The hydrolyzed samples were separated by means of reversed phase HPLC, and fractions of the separation were examined by scintillation measurement to monitor the ferulate tracer. The results are shown in **Figure 3**. The largest signals in all measurements at $t = 21$ min were due to unchanged ferulate tracer. Additionally to the unchanged tracer, both rye and wheat bread samples showed numerous signals that were not present in the control experiment. All of these signals originated from ferulate modifications formed during breadmaking.

HPLC/MS. To determine the structure of the modified ferulate compounds, all radioactive fractions of the bread hydrolyzates were examined by means of HPLC/MS measurements. In addition, MS−MS spectra were recorded from the eluted peaks. To ensure sensitive measurement of possible ferulic acid-amino acid cross links, 7-*S*-cysteinohydroferulic acid was synthesized (*28*) and used as a tuning standard for the ESI mass spectrometer. LC/MS analysis of the fractions $t = 18-20$ min of the HPLC separation of both the rye and the wheat bread hydrolyzates (**Figure 4**) gave a signal with m/z $[M + H]^+ = 374$, which corresponded to the structure of a dehydro-ferulic acid-tyrosine cross-link. To verify the structure

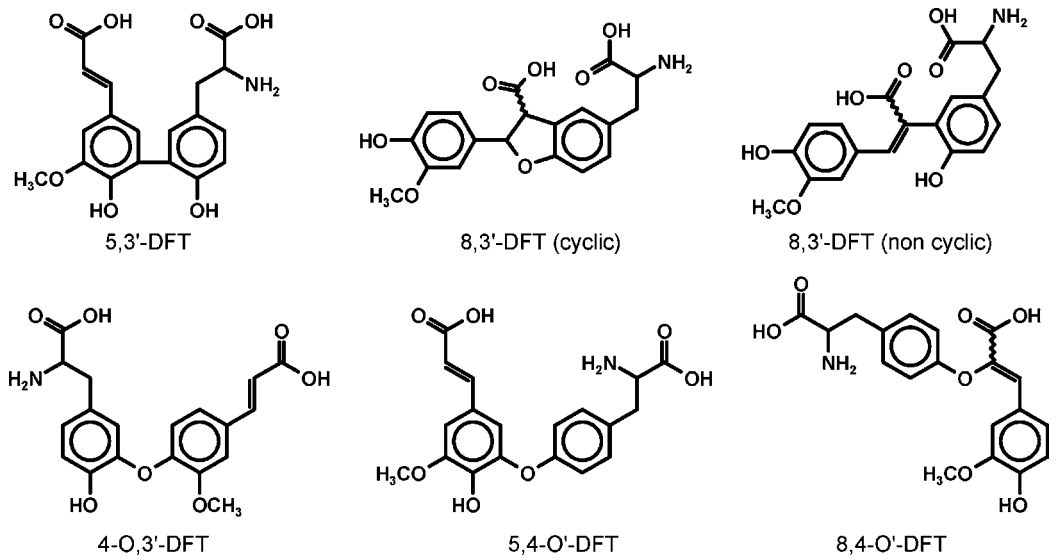

Figure 7. Speculative structures of dehydro-ferulic acid-tyrosine heterodimers related to those known from ferulate homocoupling.

of this compound, dehydro-ferulic acid-tyrosine was synthesized by treating polymer-bound ferulate with tyrosine and hydrogen peroxide in the presence of horseradish peroxidase and subsequent alkaline hydrolysis (**Figure 5**). After SPE purification, the products were analyzed by HPLC/MS. As depicted in **Figure 6**, the synthesis led to a product mixture. The major components were several peaks giving signals at $m/z = 566.1$ ($[M + H]^+$). From the molecular masses and the fragmentation pattern of MS−MS experiments, it could be assumed that this class of compounds was different isomers containing two ferulic acid and one tyrosine unit. Another class of compounds was represented by four peaks, all having signals at $m/z = 374$ of the $[M + H]^+$ ions and all showing similar fragmentation patterns (m/z 374 → m/z 310 and m/z 293); one isomer (peak #6) additionally showed the fragmentation m/z 374 → m/z 315. From the mass spectrometry data, it could be concluded that these four compounds were different isomers of dehydro-ferulic acid-tyrosine (DFT) representing different types of linkage. As outlined in **Figure 7**, structures of the heterodimers related to those known from ferulate homocoupling are possible. However, as the spectra of all four compounds were very similar, an assignment of the type of linkage between ferulic acid and tyrosine was not possible. Furthermore, it was not possible to obtain NMR spectra of these compounds, because a certain degree of isomerization could always be detected after HPLC purification and re-injection. Current investigations are underway to determine which isomer of dehydro-ferulic acid-tyrosine was formed.

Comparing the LC/MS and LC/MS−MS data of the synthesized and the isolated compounds, only one of the substances from the synthesis showed a MS pattern identical to that of the isolated product. However, these two substances did not have the same retention time during HPLC. Therefore, additional experiments for the identification of dehydro-ferulic acid-tyrosine had to be performed. For this reason, $[^2H]_3$-ferulic acid was synthesized by a Knoevenagel reaction of $[^2H]_3$-vanilline and malonic acid. 10.8 mg of $[^2H]_3$-ferulic acid was added to 10.0 g of wheat flour, and bread was baked from this mixture. After hydrolysis, the HPLC/MS signal for $m/z = 374$ decreased in favor for a new signal at $m/z = 377$, which is a strong indication for the formation of dehydro-ferulic acid-tyrosine from the added $[^2H]_3$-ferulic acid. For this experiment, the free acid ($[^2H]_3$-ferulic acid) was added because it led to the formation

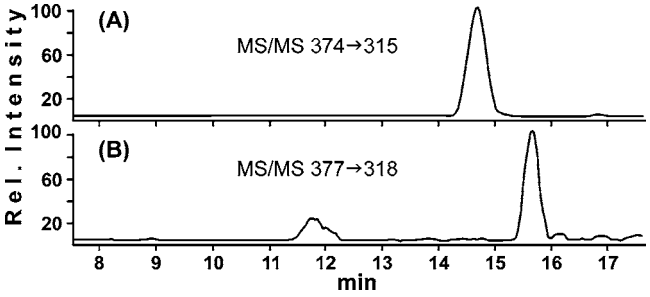

Figure 8. Differences in retention time of unlabeled and $[^2H]_3$ labeled dehydro-ferulic acid-tyrosine. RP-HPLC/MS–MS chromatogram of (A) an enzymic wheat bread hydrolyzate containing dehydro-ferulic acid-tyrosine and (B) synthetic $[^2H]_3$-dehydro-ferulic acid-tyrosine.

of a product with the mass of $[^2H_3]$-labeled DFT. Therefore, there was no need to synthesize the labeled galactose ester.

Relative Quantitation of Dehydro-Ferulic Acid-Tyrosine. DFT was detected in all hydrolyzed samples of wheat and rye (flour, dough, bread). This was also the case for samples that had been prepared without any addition of the exogenous ferulate tracer. To get information on the formation of DFT within the course of bread preparation, the relative changes of the concentrations of DFT were determined in the hydrolyzates of flour, dough, and bread using a deuterium labeled isomer of DFT (**Figure 6**, peak # 6) as an internal standard, which also allowed the estimation of the magnitude of the amount of DFT by assuming a similar response factor of the exogenous and natural DFT-isomer. To do so, $[^2H]_3$-dehydro-ferulic acid-tyrosine was synthesized from $[^2H]_3$-ferulic acid and L-tyrosine using hydrogen peroxide and horseradish peroxidize as already described for the nondeuterated compound. Addition of $[^2H]_3$-DFT to the hydrolyzed samples allowed the estimation of the content of DFT in the hydrolyzates by HPLC/MS−MS analysis (**Figure 8**). Dough and bread were prepared from wheat and rye flour without the addition of exogenous ferulate compounds. The relative changes of the content of DFT from flour to dough and bread from wheat and rye, respectively, are shown in **Table 2**. The influence of L-ascorbic acid, which is widely used as a dough improver, on the formation of DFT was also examined in these experiments.

It is evident that the concentration of DFT increased by more than 300% during the formation of wheat dough from roughly

Table 2. Relative Quantitation of Dehydro-Ferulic Acid-Tyrosine (DFT) in Wheat (cv. Soissons) and Rye (cv. Nikita) Flour, Dough, and Bread (Content of DFT in Wheat Flour = 100%)

	DFT (%, in relation to wheat flour)			
	wheat (cv. Soissons)		rye (cv. Nikita)	
	no additive	L-ascorbic acid[a]	no additive	L-ascorbic acid[a]
flour[b]	100.0 ± 18.8		16.2 ± 1.5	
dough[b]	376.1 ± 56.3	544.7 ± 91.3	16.8 ± 4.6	17.3 ± 3.6
bread[b]	370.6 ± 106.5	578.7 ± 32.0	20.3 ± 2.0	23.4 ± 5.6

[a] 100 mg/kg of flour. [b] Number of experiments $n = 3$.

0.2 μg/g in flour to 0.7 μg/g in dough, and then remained almost constant during baking. Addition of L-ascorbic acid (100 mg/kg flour) led to an increase of over 40% in the formation of DFT during wheat dough preparation. With rye, a much lower amount of DFT was detected in flour compared to wheat. The content of DFT in rye flour was only about a sixth of the content of DFT in wheat flour with roughly 0.03 μg/g. More important, no significant increase of DFT in rye was observed during dough preparation and baking. A possible explanation for this observation might be the lower tyrosine content of rye flour as compared to wheat flour (0.29% vs 0.33% (m/m)). Furthermore, the concentration of ferulate in rye arabinoxylans is lower than that in wheat arabinoxylans (450 μg/g vs 700 μg/g water soluble arabinoxylan).

DISCUSSION

Previous studies (*17, 21*) revealed that ferulate undergoes chemical modification within the course of the breadmaking process. In this study, one possible product of this modification, the dehydro-ferulic acid-tyrosine cross-link, has been identified for the first time in wheat and rye bread. However, it was necessary to use a radioactive ferulic acid-galactose ester as a tracer to enable selective and sensitive detection of ferulate modifications during breadmaking. A galactose ester was used instead of free ferulic acid, because the latter is poorly water soluble and has different chemical properties than an ester. Furthermore, the acetone ketal of galactose was commercially available as one of the starting materials for the synthesis. The product of the synthesis was water soluble and contained ferulic acid as an ester, similar to ferulate present in AX.

In the first experiments, Osborne fractions were investigated for the presence of ferulate modifications, as, especially in the prolamin and glutelin fractions, increased radioactivity would indicate protein-bound ferulate. The experiments clearly showed incorporation of ferulate into the protein fractions. The fact that prolamin fractions were stronger affected than the glutelin fractions cannot be explained up to now. Possibly, in a dough, the monomeric prolamins are more mobile than the polymeric glutelins. Whole bread was then used for the identification studies to identify all modifications present in bread.

The identification of the cross-link was based on mass spectrometric data. It was not possible to record NMR spectra of the isolated compound because it was present in very low amounts. To verify the identification of the cross-link, its structure was confirmed by chemical synthesis. However, this was difficult to achieve as complex mixtures of products were formed. As initial experiments in the liquid phase gave even more complex mixtures, a polymer-based solid-phase synthesis was carried out which generated a more limited mixture, which, nevertheless, contained more than 20 individual compounds. These compounds represented different isomers of substances based on two, three, and four tyrosine and ferulic acid building blocks. Isolation of the relevant compound by HPLC showed that its structure was not stable, as isomerization was detected by rechromatography of the compounds. This phenomenon still has to be clarified.

The newly identified cross-link contains ferulic acid and tyrosine. As low amounts of free tyrosine are present in flour and as ferulate is present in endosperm-AX as well as in lignin originating from the outer layers of the kernel, it might be possible that DFT comes from these sources. However, as the major portions of ferulate and tyrosine are present in AX (*29*−*31*) and (gluten) proteins, respectively, it is very likely that DFT represents a new covalent cross-link between AX and proteins in cereal flour. The increase of the DFT concentration during wheat dough mixing shows that ferulate may have an impact on the dough properties by forming cross-links during bread preparation. As the radioactive tracer experiments showed that ferulate was also incorporated in the rye flour proteins, further investigations need to be performed to identify chemical modifications of ferulate in rye dough and bread. Obviously, mixing and/or air are required for the cross-link reaction, because baking of the dough did not affect the DFT concentration. The fact that ascorbic acid led to an increase in the formation of DFT may be explained with the possible role of dehydroascorbic acid as a hydrogen acceptor during the oxidative coupling of ferulate and tyrosine.

With a concentration of about 80 μg ferulate/g and about 3 mg tyrosine/g in wheat flour, the formation of roughly 0.8 μg DFT/g during dough preparation would correspond to the linkage of 0.50% of the ferulate and about 0.013% of the tyrosine present in wheat flour. Assuming an arabinoxylan molecule with a molecular mass of 100.000 and a protein molecule with a molecular mass of 40.000, the identified amount of formed DFT during dough preparation would lead to about 300 μg newly cross-linked arabinoxylan-protein/g of flour. Although a final statement on the significance of the newly identified cross-link on the functional properties of the dough with the results obtained is not possible yet, the DFT cross-link seems to be of minor importance for the functionality of dough. However, with one ferulate-modification identified, it is not clear how many other reactions ferulate is capable of during dough and bread preparation. This assumption is confirmed by the results of **Table 1**, which shows that additional cross-links may be formed, as about 24% of the added ferulic acid radio tracer has reacted with prolamines in wheat bread. Nevertheless, for the first time, a chemical reaction was identified that may be discussed for the chemical properties of arbinoxylans and ferulate during wheat dough preparation.

ABBREVIATIONS USED

AX, arabinoxylan; DFT, dehydro-ferulic acid-tyrosine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; dpm, disintegration per minute; ESI, electrospray ionization; HPLC, high performance liquid chromatography; RP-, reversed-phase; SD, standard deviation; SDS, sodium dodecyl sulfate; SPE, solid-phase extraction; THF, tetrahydrofuran; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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