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Quantitation of Dityrosine in Wheat Flour and Dough by Liquid Chromatography–Tandem Mass Spectrometry

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A method for the quantitation of dityrosine in wheat flour and dough by high-performance liquid chromatography/tandem mass spectrometry (HPLC−MS/MS) using an isotope dilution assay with the internal standard 3,3′-$^{13}C_2$-dityrosine in the single-reaction monitoring mode was developed. The method consisted of the release of protein-bound dityrosine by hydrolysis in 4 mol/L hydrochloric acid/8.9 mol/L propionic acid for 24 h at 110 °C after addition of the internal standard, cleanup by C_{18} solid-phase extraction, and HPLC−MS/MS. The limit of detection of dityrosine was 80 ng/g of sample (0.22 nmol/g), and the limit of quantitation was 270 ng/g of sample (0.75 nmol/g). The method was sensitive enough to analyze wheat flour and dough and to study the effect of flour improvers on the dityrosine content. Furthermore, the effect of the mixing time was studied. The dityrosine concentration in the flour was 0.66 nmol/g. After we mixed a dough to peak consistency, the dityrosine concentration doubled and remained constant on further mixing. Overdoses of hydrogen peroxide and hexose oxidase (HOX, E.C. 1.1.3.5) resulted in a strongly increased dityrosine content, whereas no increase of the dityrosine concentration was found after the addition of ascorbic acid and potassium bromate. Calculation of the percentage of dimeric tyrosine showed that less than 0.1% of the tyrosine residues of wheat protein were cross-linked. Therefore, dityrosine residues seem to play only a very minor role in the structure of wheat gluten.

KEYWORDS: Wheat; dough; flour; tyrosine; dityrosine; cross-link; quantitation; HPLC; mass spectrometry; glucose oxidase; hexose oxidase

INTRODUCTION

Dityrosine (2-amino-3-[5′-(2-amino-2-carboxyethyl)-6,2′-dihydroxybiphenyl-propionic acid) was at first identified by Gross and Sizer (*1*) who oxidized L-tyrosine with a peroxide−peroxidase system *in vitro*. They postulated a radical mechanism for dityrosine formation (**Figure 1**). This mechanism could be approved by theoretical calculations with different models (*2*) and by detecting the intermediate tyrosyl radicals with electron spin resonance (ESR) spectroscopy (*3*). *In vivo* dityrosine was discovered for the first time in some elastic ligaments of the rubber-like resilin protein in arthropods by Anderson in 1964 (*4*). Since then, it was found in many different natural occurring proteins such as the adhesive discs of sea mussels (*5*), wool keratin (*6*) or the human eye lens (*7*). Because dityrosine has been found predominantly in tissues providing structural integrity, it was recognized as a stabilizing cross-link in structural proteins.

In medical analysis, dityrosine is a marker for oxidative stress and pathogenic changes, because oxidative damage is considered to be involved in many diseases. Increased levels of dityrosine have been found in both atherosclerotic plaques (*8*) and in Alzheimer-affected brain tissue (*9*). Free dityrosine in plasma and urine is an often used noninvasive marker for oxidative damage caused by radical oxygen species (ROS) (*10*−*13*).

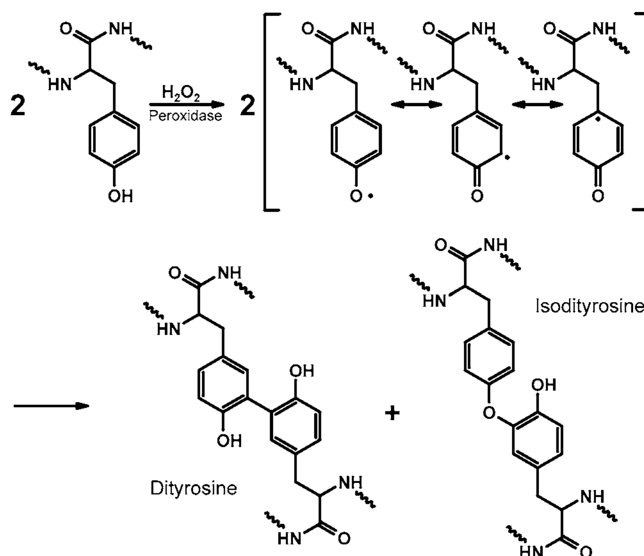

Figure 1. Formation of protein-bound tyrosine and dityrosine by a radical mechanism (modified according to ref *2*).

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In food chemistry, dityrosine is rarely mentioned. Oestdal et al. used dityrosine as marker for the effect of antioxidative additives in milk (*14*). Dityrosine was also discovered in wheat flour, dough, and bread, where it is suggested to be a new kind of stabilizing cross-link in the wheat gluten structure in addition to disulfide bonds (*15*). However, no quantitative data of dityrosine in flour and dough have been provided, and no estimation of the portion of tyrosine residues involved in dityrosine cross-links has been given. Because no standard compounds are commercially available, no method for quantitation of dityrosine in wheat flour and dough are available up to now. Especially, the release of protein-bound tyrosine by acid hydrolysis and the extensive sample preparation lead to dityrosine losses, which have to be compensated by the use of a suitable internal standard.

In previous studies on dityrosine synthesis, analysis of dityrosine was primarily performed by thin-layer chromatography, as well as UV and fluorescence spectroscopy (*1*, *16*, *17*, *18*). Later, liquid chromatography in combination with fluorescence detection was established as a method for quantitation (*10*, *14*, *15*). To lower the detection limits and for definite identification, gas chromatogrophy with mass detection (GC−MS) and liquid chromatography with tandem mass spectrometry (LC−MS/MS) have been used, especially in combination with stable isotope dilution assays (*10*, *12*, *13*).

The aim of this study was to develop a method for the quantitative determination of protein-bound dityrosine in wheat flour and dough and to determine the effects of different oxidative dough conditioners on dityrosine formation during dough mixing.

MATERIALS AND METHODS

Chemicals. L-Tyrosine was obtained from Aldrich (Sigma−Aldrich, Taufkirchen, Germany); 3-^{13}C-tyrosine (99%) was from CIL (Andover, MA); wheat starch was from Roth (Karlsruhe, Germany); trifluoroacetic acid (TFA) and 3-*tert*-butyl-4-hydroxyanisole (BHA) were from Fluka (Taufkirchen, Germany); liquid nitrogen was from Linde (Hoellriegelskreuth, Germany); and all other chemicals were from VWR International (formerly Merck, Darmstadt, Germany). The quality was "pro analysi" or better. Hexose oxidase (HOX, E.C. 1.1.3.5; TS-E 662, approximately 0.1 unit/mg; 1 unit corresponds to a glucose consumption of 1 μmol/min) was from Danisco (Brabrand, Denmark); glucose oxidase (GOX, E.C. 1.1.3.4; approximately 10 units/mg; 1 unit corresponds to a glucose consumption of 1 μmol/min) was from Stern-Enzym (Ahrensburg, Germany); and horseradish peroxidase (931 units/mg) was from Sigma (Sigma−Aldrich, Taufkirchen, Germany).

Flour. Kernels of the German wheat cultivar Flair from the 2003 harvest were milled into flour at 14% moisture by means of a Quadrumat Junior mill (Brabender, Duisburg, Germany), sieved (∅ = 0.2 mm), and stored for at least 2 weeks prior to use. The moisture of the flour was determined according to ICC method 110/1 (*19*); the ash content was determined according to ICC method 104/1 (*20*); and the nitrogen content was determined on a FP328 nitrogen analyzer (Leco, Moenchengladbach, Germany). The protein content was calculated from the nitrogen content by multiplication with the conversion factor of 5.7. The moisture content of the flour was 13.7%; the ash content (dry mass) was 0.596%; and the protein content was 11.8%.

Dough Preparation. Flour (10 g; 8.6 g of dry mass) and sodium chloride (0.2 g) were mixed with water (5.9 mL) in a microfarinograph (Brabender, Duisburg, Germany) at 22 °C and 60 rpm. After 1, 4, 7 (peak consistency), 10, and 20 min, the dough was removed from the farinograph, immediately frozen in liquid nitrogen, lyophilized, and milled in an ZM 1 ultra centrifugal mill at 6000 rpm (∅ = 0.2 mm, Retsch, Haan, Germany). Additives (HOX, GOX, ascorbic acid, hydrogen peroxide, and potassium bromate) were dissolved in the water for mixing. Glucose was added directly to the flour. BHA was dissolved in petroleum ether (40−60 °C) and dropped onto the flour. After evaporation of the solvent for 5 min at room temperature, the mixing was started.

Enzymatic Synthesis of Dityrosine and 3,3′-^{13}C$_2$-Dityrosine. L-Tyrosine or 3-^{13}C-tyrosine (100 mg, respectively) was dissolved in ammonium formate buffer (120 mL, 0.1 mol/L at pH 9.2), and hydrogen peroxide (0.03 mmol) was added. Horseradish peroxidase (3 mg) was added, and the solution was incubated at 40 °C without stirring. After 1 h, hydrogen peroxide (0.03 mmol) was added. After another hour, the solution was acidified to pH 2−3 with concentrated formic acid and filtered with an Amicon Diaflo stirred ultrafiltration cell equipped with a PM10 membrane (cutoff 10.000; Amicon, Danvers, MA) and the filtrate was lyophilized. Only traces of isodityrosine were formed by using the enzymatic pathway in contrast to chemical methods under acidic conditions as described by Miller and Fry (*21*). The residue was purified using gel filtration on a Sephadex G-10 column (60 × 1.5 cm; Amersham Biosciences, Freiburg, Germany). Elution was carried out by formic acid (0.1%, v/v) with a flow rate of 6.4 mL/h. The column effluent was monitored continuously at 280 nm using an LKB 4071A Uvicord spectrophotometer (Bromma, Sweden), and fractions were collected in 15-min steps. All fractions were checked for dityrosine by using a Kontron liquid chromatograph (Bio-Tek Kontron, Neufahrn, Germany) equipped with a Synergi Hydro-RP reversed-phase column (250 × 4.6 mm, 4 μm, 8 nm; Phenomenex, Aschaffenburg, Germany). Fractions were analyzed under the following conditions: injection volume of 100 μL, flow rate of 1 mL/min, temperature of 30 °C, detection UV absorbance at 280 nm, solvent A with 0.1% (v/v) TFA in water, solvent B with 0.1% (v/v) TFA in acetonitrile, and a linear gradient for 0−35 min with 3−10% solvent B. All fractions containing more dityrosine than tyrosine were lyophilized. The residue was dissolved in formic acid (3 mL; 0.1%, v/v). Semipreparative isolation of dityrosine was performed on an Kontron liquid chromatograph (Bio-Tek Kontron, Neufahrn, Germany) equipped with a Hyper-Clone ODS reversed-phase column (250 × 10 mm, 5 μm, 12 nm; Phenomenex, Aschaffenburg, Germany) under the following conditions: flow rate of 3 mL/min, temperature of 35 °C, detection UV absorbance at 280 nm, solvent A with 0.1% (v/v) TFA in water, solvent B with 0.1% TFA in acetonitrile (v/v), and a linear gradient for 0−30 min with 4−7% solvent B. The peak at a 20 min elution time was collected. The eluate was frozen and lyophilized. The overall yield of dityrosine was 11.6%. The purity was checked by HPLC−UV and ^{1}H NMR.

Dityrosine: ^{1}H NMR (400 MHz, D_2O, calibrated on HDO 4.65 nm) δ 7.19 (2 H, dd, J = 2.4, 8.3 Hz), 7.10 (2 H, d, J = 2.4 Hz), 6.96 (2 H, d, J = 8.3 Hz), 4.03 (2 H, dd, J = 5.4 and 7.4 Hz), 3.21 (2 H, dd, J = 5.5 and 14.6 Hz), 3.10 (2 H, dd, J = 7.5 and 14.6 Hz).

3,3′-^{13}C$_2$-Dityrosine: ^{1}H NMR (400 MHz, D_2O, calibrated on HDO 4.65 nm) δ 7.18 (2 H, m), 7.10 (2 H, m), 6.95 (2 H, d, J = 8.3 Hz), 4.03 (2 H, m), 3.21 (2 H, ddd, J = 5.2, 14.7, and 131.6 Hz), 3.10 (2 H, ddd, J = 7.5, 14.9, and 130.5 Hz).

Protein Hydrolysis and Sample Preparation Procedure. Dry dough sample or flour (25−100 mg) was mixed with hydrochloric acid (1 mL, 6 mol/L, freshly distilled), propionic acid (0.5 mL), and internal standard (20 ng; 3,3′-^{13}C$_2$-dityrosine). This mixture was heated to 110 °C for 24 h and cooled to room temperature. The solvent was removed under a stream of nitrogen, and the residue was dissolved in formic acid (1.5 mL; 0.1%, v/v) by ultrasonication. For purification, a solid-phase extraction (SPE) on a C_{18} column (3 mL, Strata C-18 T, Phenomenex, Aschaffenburg, Germany) was carried out. The SPE column was conditioned with methanol (2 mL), $NaHPO_4$ (6 mL, 50 mmol/L at pH 7.4), and finally with TFA (6 mL, 0.1%, v/v). After a short mixing, the hydrolysate was loaded onto the column, and the column was washed with TFA (6 mL, 0.1%, v/v). Dityrosine was eluted with 0.1% (v/v) formic acid/methanol (3 mL, 85:15, v/v) and concentrated to dryness under nitrogen flow. The residue was then dissolved in formic acid (250 μL, 0.1%, v/v) by ultrasonication, and the solution was directly applied to HPLC−MS/MS.

HPLC−MS/MS. The determination of dityrosine was performed using a Thermo Finnigan Surveyor liquid chromatograph with a triple quadrupole tandem mass spectrometer (TSQ Quantum Discovery 1.1, Thermo Electron, Dreieich, Germany) equipped with a Synergi Hydro-

Table 1. Most Intense Mass Transitions and Collision Energies for the MS/MS Detection of Dityrosine and 3,3′-$^{13}C_2$-Dityrosine

	ion transition	collision energy (%)[a]
dityrosine	*m/z* 361 → *m/z* 315	20
	m/z 361 → *m/z* 254	28
	m/z 361 → *m/z* 237	32
3,3′-$^{13}C_2$-dityrosine	*m/z* 363 → *m/z* 317	20
	m/z 363 → *m/z* 256	28
	m/z 363 → *m/z* 239	32

[a] Arbitrary percentage.

RP reversed-phase column (150 × 2 mm, 4 μm, 80 Å; Phenomenex, Aschaffenburg, Germany). Sample solution (20 μL) was analyzed using the following conditions: flow rate of 0.2 mL/min, temperature of 30 °C, UV detection at 280 nm, solvent A with 0.1% (v/v) formic acid, solvent B with methanol, and a linear gradient at 0−20 min with 0−60% solvent B. The effluent between 9 and 16 min was directed into the electrospray interface. The mass spectrometer was operated in the positive electrospray ionization mode (ESI^+) with a spray needle voltage of 3.5 kV. The temperature of the capillary was 300 °C, and the capillary voltage was 35 V. The sheath and auxiliary gas was adjusted to 40 and 10 arbitrary units, respectively. The collision cell was operated at a collision gas (argon) pressure of 6.7×10^{-5} kPa. On both mass filter quadrupoles, the resolution settings were 0.7, the scan time for each transition and single-reaction monitoring (SRM) was 0.2 s, and the scan width was 0.6 amu.

The synthetics were first characterized by means of their molecular masses obtained in the full scan mode. These were then subjected to MS/MS to optimize the yield of the fragment ions for SRM by performing a series of runs with different collision energies. **Table 1** shows the results of the optimization. For quantitation, the mass transitions *m/z* 361 → *m/z* 315 for dityrosine and *m/z* 363 → *m/z* 317 for 3,3′-$^{13}C_2$-dityrosine were chosen.

To determine the response factor, solutions of dityrosine and 3,3′-$^{13}C_2$-dityrosine (1 μg/mL) in 0.1% formic acid were mixed in six mass ratios between 0.2 and 9 to give a total dityrosine content of 200 μg/mL. This determination had to be repeated every day. The obtained regression curves were used for the quantitation.

Calibration. For standard calibration, solutions of 3,3′-$^{13}C_2$-dityrosine (80 ng/mL) and dityrosine (5 concentrations between 40 and 500 ng/mL) were analyzed in triplicates. For matrix calibration, a flour matrix free of tyrosine was mixed from different amino acids (13.15%), cellulose (4%), and wheat starch (82.85%). Dityrosine (10, 25, 50, 75, and 100 ng) was added to the matrix (100 mg) and analyzed in triplicates as described for the samples. To determine the response factor, solutions of dityrosine and 3,3′-$^{13}C_2$-dityrosine (1 μg/mL) in 0.1% (v/v) formic acid were mixed in 10 mass ratios between 0.1 and 9 to give a total dityrosine content of 200 μg/mL. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to Haedrich and Vogelgesang (*22*).

Amino Acid Analysis. This was carried out according to ref *23* on an Amino Acid Analyzer LC 3000 (Laborservice Onken, Gründau, Germany) using postcolumn derivatization with ninhydrin.

Baking Tests. Baking tests were carried out on a microscale using 10 g of flour according to ref *24*.

RESULTS AND DISCUSSION

Sample Preparation. Dityrosine was released from the protein by acid hydrolysis in a mixture of hydrochloric acid and propionic acid. The latter was chosen as additional cleaving agent, because it has been found to be efficient for hydrolysis of hydrophobic peptide bonds as is the case for dityrosine bridges in the wheat proteins (*25*). In initial studies, the hydrolyzates were directly analyzed by HPLC−MS/MS. However, a high noise background in the mass detection was obtained, and in some cases, a clear peak identification was not possible. Therefore, the solutions were purified by solid-phase extraction on C18 columns prior to HPLC−MS/MS (*11*). After washing with 0.1% TFA, most of the highly polar components were removed. Elution with 15% methanol provided the dityrosine-containing fraction, and more nonpolar substances remained on the column. This additional cleanup step resulted in clear peaks in mass detection. To compensate for the potential loss of dityrosine during hydrolysis and sample cleanup, a stable isotope-labeled internal standard (3,3′-$^{13}C_2$-dityrosine) was added prior to hydrolysis. Because of the structural similarity to the analyte, it can be assumed that the analyte and the internal standard showed the same loss during the process of sample preparation.

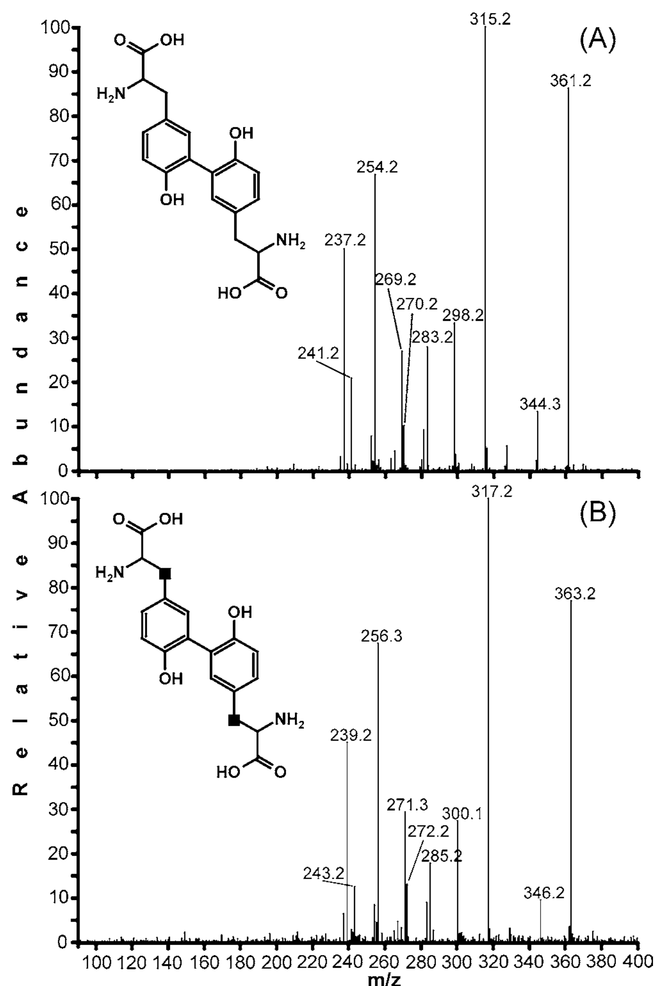

Figure 2. MS/MS spectrum (ESI positive) of dityrosine (A) and 3,3′-$^{13}C_2$-dityrosine (B) after fragmentation of the protonated molecules (■, ^{13}C label).

Mass Detection. For unequivocal identification and quantitation, MS/MS was applied. After fragmentation of the protonated molecule ions, MS/MS spectra of dityrosine and 3,3′-$^{13}C_2$-dityrosine were obtained (**Figure 2**). From each spectrum, the most abundant ion transitions were selected and used for quantitation. Apart from the mass difference of *m/z* 2, both substances showed the same fragmentation pattern. Ion transitions *m/z* 361 → *m/z* 315, *m/z* 361 → *m/z* 254, and *m/z* 361 → *m/z* 237 were recorded for the analyte. Ion transitions *m/z* 363 → *m/z* 317, *m/z* 363 → *m/z* 256, and *m/z* 361 → *m/z* 239 were recorded for the internal standard. **Figure 3** depicts a chromatogram of the analysis of an untreated wheat flour dough with all mass transition traces. For quantitation, only the most intense mass transitions (*m/z* 361 → *m/z* 315 and *m/z* 363 → *m/z* 317) were used.

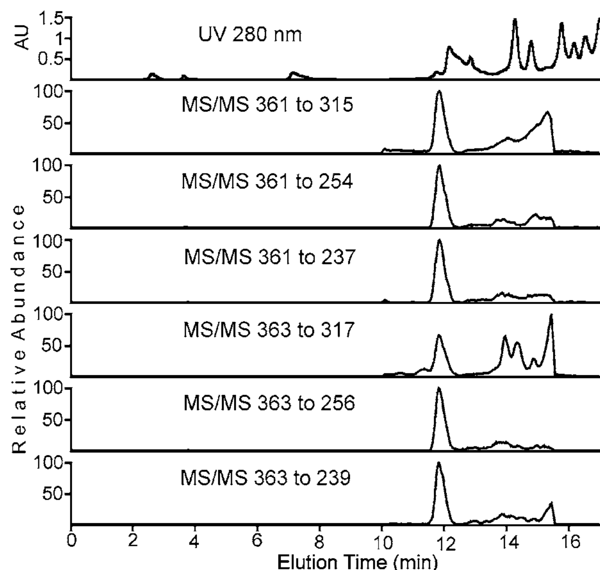

Figure 3. HPLC–MS/MS chromatograms obtained from the analysis of an amino acid hydrolyzate of wheat flour dough mixed to peak consistency. Mass traces for the most intense ion transitions.

For calibration solutions of dityrosine and 3,3′-$^{13}C_2$-dityrosine (1 μg/mL) in 0.1% (v/v) formic acid, six mass ratios were mixed between 0.2 and 9 and analyzed with LC−MS/MS. From the data obtained, a calibration curve of area ratio/mass ratio was calculated. The calibration curve was not linear because (a) the internal standard contained 1% nonlabeled analyte and (b) the mass difference of 2 between the internal standard and the analyte, and the $[M + H]^+$ signal of the internal standard was “contaminated” with the signal $[M + 2H]^+$ caused by the naturally occurring carbon isotopes in the analyte. Because the response calibration showed a strong day−day variance, it had to be repeated with every series of measurements.

Evaluation and Recovery. In the calibration range (40−500 ng/mL), the standard calibration showed a standard deviation of 6.47 ng/mL, whereas the standard deviation of the matrix calibration was 16.10 ng/mL, because of the additional sample preparation steps. The LOD and LOQ were calculated according to Haedrich and Vogelgesang (*22*). The calculation of the LOD is based on the highest value for the standard deviation of multiple blank runs. In a calibration curve ($y = a + bx$), this maximum value is used as the y value and the corresponding concentration x is calculated, which is the lowest concentration that is different from the blank (=LOD). After further statistical calculations, the LOQ can be obtained, which is apprimately 3-fold higher that the LOD. With a calculated LOD of 79.6 ng/g of sample (0.22 nmol/g) and a LOQ of 269.1 ng/g of sample (0.75 nmol/g), the method was sensitive enough for the analysis of dityrosine in dough and flour samples. Considering the detection and identification with three mass transitions, the LOD would have been even lower. The area of the analyte peak in the matrix calibration was only 11−18% of the area of equal concentrated standards in standard calibration. This was not only due to a possible loss in the sample preparation but also to problems in ionization in ESI−MS detection. The highly ionized matrix obviously suppressed extensive analyte ionization. Both effects were compensated by using the internal standard 3,3′-$^{13}C_2$-dityrosine. The recovery with the internal standard in the matrix calibration was 116.3% with a coefficient of variation (CV) of 22.0%. Experiments in which the lowest concentration was omitted gave much better values for recovery and CV.

Concentration of Dityrosine in Wheat Flour and Dough. Wheat flour and doughs with different mixing times were analyzed for dityrosine content. The results are shown in **Figure 4**. The value for a mixing time of 0 min represents the dityrosine content of the flour, which was in the range of the LOQ. Although the variation of the results was relatively high, a clear correlation between mixing time and dityrosine content was established. During mixing, the dityrosine content increased until peak consistency (7 min) as far as 1.2−1.4 nmol/g dry mass. Beyond peak consistency, the dityrosine content remained constant. Overmixing (20 min) did not change the dityrosine content of the dough.

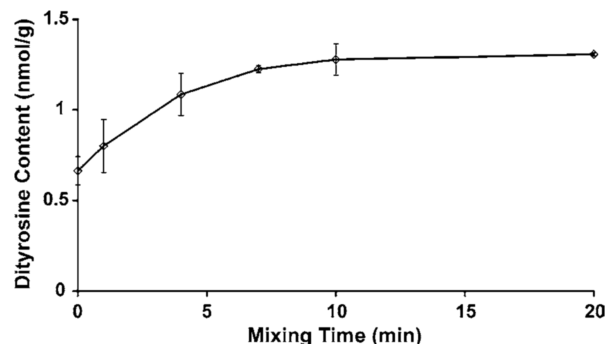

Figure 4. Formation of dityrosine in wheat flour and wheat dough in relation to mixing time. Number of experiments ≥ 2.

Table 2. Dityrosine Content in Wheat Flour and Doughs as Affected by the Addition of Oxidizing Reagents

sample	dityrosine content (nmol/g)	n^a
flour	0.664 ± 0.078	2
dough mixed to peak, no additive	1.225 ± 0.020	2
dough from flour with low peroxidase activity[b]	1.313 ± 0.125	2
shredded whole-wheat grains	2.497 ± 0.388	2
dough with ascorbic acid (100 mg/kg) added	0.954 ± 0.068	2
dough with potassium bromate (100 mg/kg) added	1.152 ± 0.078	2
dough with hydrogen peroxide (6420 mg/kg) added	3.952 ± 0.083	2

[a] n = number of experiments. [b] Flour (50 g) was extracted twice with water (100 mL) and lyophilized.

Effect of Flour Improvers on Dityrosine Concentration. Because the formation of dityrosine is a result of oxidative reactions, the effects of oxidizing flour improvers were tested. The results for chemical oxidants and oxidizing enzymes are summarized in **Tables 2** and **3**, respectively. In comparison to dough containing no additive, the addition of the classical flour improvers ascorbic acid and potassium bromate led to lower dityrosine concentrations in the dough (**Table 2**). Ascorbic acid acted as an antioxidant in the dough, possibly as a radical scavenger, and, therefore, caused a significantly decreased dityrosine content. The effect of potassium bromate was not so definite, because its reactions are more pronounced at higher temperatures (baking) or longer reaction times (*26*). Because potassium bromate is prohibited in Europe, other oxidizing additives, for example, oxidative enzymes, are used as alternate bread improvers. Glucose oxidase (GOX, E.C. 1.1.3.4) and hexose oxidase (HOX, E.C. 1.1.3.5) produce hydrogen peroxide by oxidizing glucose and other hexoses (*27*, *28*). The hydrogen peroxide is supposed to be the active agent of these enzymes. The ideal dosage of enzyme was 30−40 units/kg of flour in baking. In this concentration, the enzymes showed no effect on

Table 3. Dityrosine Content in Wheat Flour and Dough as Affected by the Addition of Enzymes[a]

flour/dough with addition of	dityrosine content (nmol/g)	*n*
flour	0.664 ± 0.078	2
dough mixed to peak, no additive	1.225 ± 0.020	2
GOX (3.2 units/kg)	1.206 ± 0.089	2
GOX (37 units/kg)	1.230 ± 0.258	4
GOX + Glc (3.2 units/kg and 1 g/kg)	1.405 ± 0.221	2
GOX + Glc (37 units/kg and 1 g/kg)	1.829 ± 0.185	2
HOX (40 units/kg)	1.300 ± 0.375	4
HOX + Glc (35 units/kg and 1 g/kg)	1.839 ± 0.202	3
HOX + Glc (116.5 units/kg and 1 g/kg)	7.016 ± 0.318	2
HOX + Glc + BHA (116.1 units/kg, 1 g/kg, and 424 mg/kg)	1.635 ± 0.437	4

[a] GOX, glucose oxidase; HOX, hexose oxidase; Glc, glucose; BHA, 3-*tert*-butyl-4-hydroxyanisole; *n*, number of experiments.

dityrosine formation (**Table 3**), whereas the functional properties of the doughs were positively affected. Addition of the enzyme together with glucose increased the dityrosine content for all enzyme concentrations, because the enzymes lack a substrate in the dough. A too high dosage of HOX resulted in an extremely high dityrosine content (**Table 3**). All doughs with dityrosine concentrations of 1.405 nmol/g and higher showed poor dough characteristics: they were not moldable, not elastic, and had a hard consistency. Because hydrogen peroxide is the active reagent produced by the enzymes, a high concentration of hydrogen peroxide showed nearly the same effects as a high level of enzyme. To prove the idea of a radical mechanism for dityrosine formation during dough mixing, the radical scavenger BHA was used in combination with a high amount of HOX. This combination resulted in a 78% decrease of the dityrosine content in the dough, compared to the dough without BHA (**Table 3**). To test the dependence of dityrosine formation from endogenous wheat peroxidase, a flour with 90% less peroxidase activity than a native flour (extracted twice with water) was used for dough mixing and dityrosine analysis. Contrary to the expected decrease in the dityrosine content, the extraction of peroxidase from flour showed no effect on the dityrosine formation (**Table 2**). Either the remaining 10% of peroxidase activity was sufficient for dityrosine formation or there is an alternate peroxidase-independent pathway for dityrosine formation in wheat flour. The 3.8-fold higher dityrosine content of shredded whole-wheat grain compared to the flour indicates that in wheat grain dityrosine occurs mainly in the outer layers of the kernel, where it probably acts as a stabilizing cross-link (**Table 2**).

Significance of Dityrosine in Wheat Dough. Although the method used in this study did not reveal whether any of the dityrosine linkages are formed between glutenin polymer subunits, this can be assumed, because approximately 40% of the flour protein is glutenin polymer. To get an indication on the significance of dityrosine cross-links in wheat dough, the proportion of dimeric tyrosine in flour and dough was calculated. From the amino acid analysis of the flour, a total tyrosine content of 18.6 ± 0.5 μmol/g of flour was obtained. For dityrosine, concentrations between 0.66 and 7 nmol/g of flour were present. This corresponded to a degree of dimerization of 0.01 in the flour and up to 0.1% in the dough depending on the additive, meaning that only 1 of 1000 to 10 000 tyrosine residues were cross-linked. This makes it likely that dityrosine cross-links do not play a significant role in the structure of wheat proteins, especially in the structure of gluten. However, when disulfide linkages and low-molecular thiol compounds are considered, the opposite conclusion could be drawn. Glutathione and total cysteine are present in proportions comparable to dityrosine and tyrosine but on a higher concentration level. The concentration of glutathione is in the range of 50 nmol/g of flour (*29*, *30*), whereas cysteine is present in concentrations that are about 500 times higher [20−25 μmol/g of flour (*31*)]. This means, that glutathione amounts to only 0.2% of the total cysteine in the dough, but it is evident that these low levels of glutathione have a substantial effect on the rheological properties of the dough. The same might be true for dityrosine linkages. Only a very small amount of cross-links might contribute to an increase of the molecular mass of the gluten polymer corresponding to a stronger consistency, but this has to be proven experimentally.

Because of the data obtained in this study, the number of dityrosine and cystine linkages in the flour protein can roughly be compared. In a flour that is 12% protein, of which approximately 40% was glutenin polymer, an amount of 48 mg of polymer per gram of flour protein can be calculated. These polymers are roughly composed of 1 part high-molecular-weight glutenin subunits to 4 parts of low-molecular-weight glutenin subunits. An average molecular mass of 80 000 for the further and 40 000 for the latter or an average molecular mass of 48 000 per random subunits can be assumed. There is presumably a ratio of one cystine linkage per subunit. If so, there would be 1 μmol of subunits and 1 μmol of cystine linkages per gram of flour, compared to the maximum of 7 nmol of dityrosine cross-links per gram of flour dough, i.e., 1 dityrosine linkage per 143 cystine linkages.

Concluding Remarks. Oxidizing flour improvers have an effect on the formation of dityrosine in wheat dough only when high levels are applied. However, the very low percentage of dimeric tyrosine present in dough makes it likely that dityrosine cross-links do not play a significant role in the structure of wheat gluten. Because radical scavengers such as ascorbic acid and BHA inhibit dityrosine formation, a radical mechanism is supported. The peroxidase−peroxide reaction with endogenous wheat peroxidase can form the proposed tyrosyl radicals necessary for dityrosine formation. However, this might not be the only mechanism of dityrosine formation during dough mixing, because reduced levels of endogenous peroxidase do not affect the dityrosine content.

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