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Structural and Functional Characterization of Pronyl-lysine, a Novel Protein Modification in Bread Crust Melanoidins Showing in Vitro Antioxidative and Phase I/II Enzyme Modulating Activity

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Application of an in vitro antioxidant assay to solvent fractions isolated from bread crust, bread crumb, and flour, respectively, revealed the highest antioxidative potential for the dark brown, ethanol solubles of the crust, whereas corresponding crumb and flour fractions showed only minor activities. To investigate whether these browning products may also act as antioxidants in biological systems, their modulating activity on detoxification enzymes was investigated as a functional parameter in intestinal Caco-2 cells. The bread crust and, in particular, the intensely brown, ethanolic crust fraction induced a significantly elevated glutathione *S*-transferase (GST) activity and a decreased phase I NADPH−cytochrome *c* reductase (CCR) activity compared to crumb-exposed cells. Antioxidant screening of Maillard-type model mixtures, followed by structure determination, revealed the pyrrolinone reductones **1** and **2** as the key antioxidants formed from the hexose-derived acetylformoin and N_α-acetyl-L-lysine methyl ester or glycine methyl ester, chosen as model substances to mimic nonenzymatic browning reactions with the lysine side chain or the N terminus of proteins, respectively. Quantitation of protein-bound pyrrolinone reductonyl-lysine, abbreviated pronyl-lysine, revealed high amounts in the bread crust (62.2 mg/kg), low amounts in the crumb (8.0 mg/kg), and the absence of this compound in untreated flour. Exposing Caco-2 cells for 48 h to either synthetically pronylated albumin or purified pronyl-glycine (**3**) significantly increased phase II GST activity by 12 or 34%, respectively, thus demonstrating for the first time that "pronylated" proteins as part of bread crust melanoidins act as monofunctional inducers of GST, serving as a functional parameter of an antioxidant, chemopreventive activity in vitro.

KEYWORDS: Antioxidants; detoxication enzymes NADPH−cytochrome *c* reductase; glutathione *S*-transferase; melanoidins; pronyl-lysine; pronyl-glycine

INTRODUCTION

The Maillard reaction between reducing carbohydrates and amino acids or proteins is chiefly responsible for the development of the brown color that occurs during baking, roasting, or frying of foods. This browning of thermally processed foods, for example, the crust of a freshly baked bread, is highly desirable and is intimately associated in consumers' minds with a delicious, high-grade product. Depending on their molecular weight, the colored components affecting this nonenzymatic browning may be divided into two classes, namely, the low molecular weight colored compounds and the melanoidins, which are assumed to be nitrogen-containing, high molecular weight colored compounds with masses up to 100000 Da (*1*).

Although numerous attempts have been undertaken to isolate and purify melanoidins from foods, for example, from coffee (*2*−*4*), soy sauce (*5*), malt (*6*), and dark beer (*7*), it has as yet not been possible to isolate and characterize partial structures of complex food melanoidins. Very recently, EPR and LC/MS spectroscopy as well as carefully planned synthetic and quantitative model experiments have given first insights into melanoidin formation in toasted wheat bread crust and roasted coffee and demonstrated the previously unknown protein-bound 1,4-bis(5-amino-5-carboxy-1-pentyl)pyrazinium radical cation (CROSSPY), showing protein cross-linking ability and high browning potential, as a key intermediate in roasting-induced melanoidin genesis (*8*). These findings suggested that the lysine side chains of proteins are the active sites in the formation of melanoproteins as part of food melanoidins (*9*, *10*).

Besides the sensory impact of the browning products, little is known so far about the physiological relevance of these Maillard-type melanoidins. Maillard reaction products with

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3(2*H*)-furanone structures have been reported to possess DNA-damaging properties (*11*, *12*). However, potentially beneficial effects were also reported, for example, the intensely colored 3-hydroxy-4-[(*E*)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione was found to potently inhibit the growth of human tumor cells in vitro by effectively shutting down the activity of the MAP kinase cascade (*13*). By far the most studies in the past three decades, however, addressed the antioxidant function of Maillard products. With a few exceptions, most investigations were focused on carbohydrate/amino acid model mixtures only (*14*, *15*), rather than on performing systematic studies on authentic food melanoidins. Iwansky and Franzke (*16*) were the first to show that fat spoilage in cookies could be significantly inhibited by the addition of melanoidins to the dough. Although total antioxidative activity was measured several times in browned foods such as roasted coffee (*17*, *18*), roasted malts (*19*), Asian soy sauce (*20*), or tomato powder (*17*), most investigations provided only cumulative information on the physiological effects of the particular browned food, rather than trying to correlate these effects on a molecular level with distinct chemical structures of the antioxidatively active sites present in these melanoidins.

In addition, it is as yet not clear how nonenzymatic browning products influence xenobiotic enzymes in the human body. Most chemopreventive, nonendogenously formed agents act through enzyme systems by modulating phase I and phase II enzymes. Phase I metabolic transformations include reduction, oxidation, and hydrolytic reactions, whereas phase II transformations generally act through conjugation reactions of the parent xenobiotics or of phase I metabolites. The conjugation reactions facilitate transport and enhance elimination of the inactive compounds via the renal and biliary routes. Therefore, the main determinant of whether exposure to xenobiotics will result in toxicity is the balance between the activities of phase I and phase II enzymes (*21*). Although phase II enzymes are hypothesized to facilitate the metabolic transit of food-derived Maillard reaction products formed in heat-treated proteins (*22*, *23*), it is still an open question whether nonenzymatic browning products formed in foods require specific detoxifying mechanisms or contribute to the chemopreventive potential of the organism via induction of phase II enzymes. Specifically, induction of the phase II glutathione *S*-transferase by antioxidants is proposed as a promising strategy for cancer prevention (*24*).

The objectives of the present study were, therefore, (i) to determine the in vitro antioxidant activity of fractions isolated from bread crust chosen as an example of an intensely browned food, (ii) to identify the chemical structure of an antioxidant site of bread crust melanoidins, and (iii) to investigate whether this structural domain of the crust melanoidins showing antioxidant activity in vitro may also modulate detoxification enzyme activities in biological systems using a human intestinal cell culture model.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: starch, glucose, hydrogen peroxide, iron(II) sulfate, EDTA, dimethylformamide, ethanol, 2-propanol, and 1-chloro-2,4-dinitrobenzene (Merck, Darmstadt, Germany); glycine methyl ester and N_α-acetyl-L-lysine methyl ester (Nova Biochem, Schwabach/Ts., Germany); albumin bovine fraction V and Tween 20 (Serva, Heidelberg, Germany); linolenic acid, Triton X-100, hemoglobin, fetal bovine serum, L-glutamine, penicillin, streptomycin, porcine trypsin, and dithiothreitol (Sigma, Deisenhofen, Germany); benzoyl leucomethylene blue and *N*-methylpyridone (Aldrich, Deisenhofen, Germany); Trolox (Fluka, Deisenhofen, Germany); and cytochrome *c* (Boehringer, Mannheim, Germany).

Table 1. Fractionation of Flour, Bread Crumb, and Crust by Sequential Solvent Extraction[a]

	yield, g/100 g of		
fraction[a]	flour	bread crumb	bread crust
I	26.2	28.6	20.7
II	2.8	2.1	2.0
III	0.7	0.4	0.2
IV	70.3	69.0	77.2

[a] Fractions were obtained by extracting the material with water (fraction I) or ethanol (fraction II), followed by 2-propanol (fraction III) and removing the solvent in vacuo or by freeze-drying. Fraction IV contains the nonsoluble materials.

The following compounds were synthesized as reported recently: 3-deoxy-1-hexosulose (*25*), acetylformoin (*26*), and 1-deoxy-2,3-hexodiulose (unpublished results).

Preparation of Rye/Wheat Mixed Bread. A preferment was prepared from rye flour (type 1150, 108 g), starter cultures (Böcker mother, 12 g), and water (96 g), which were kneaded and incubated for 16−20 h at 26 °C. A sourdough (1500 g) was prepared by incubating a dough of rye flour (type 1150, 674 g), preferment (75 g), and water (750 g) for 16 h at 26 °C. For preparation of the rye/wheat mixed bread, rye flour (type 1150, 1117 g), wheat flour (type 812, 745 g), water (1650 g), sourdough (1241 g), baker's yeast (43 g), and NaCl (43 g) were kneaded (dough weight = 4839 g), filled into two baking tins (5 cm high), and baked using the following procedure: dough temperature (28−29 °C), dough resting (15 min), dough fermentation (45 min), water vaporization (10 s before and 5 s after the bread was introduced into the oven; after 1 min, the water vapor was removed through the discharge pipe for 1 min); baking was done for 24 min at 260 °C, followed by 186 min at 220 °C.

Fractionation of Bread Crumb and Crust. The brown bread crust was carefully separated from the bread crumb with a kitchen knife; crust and crumb were frozen in liquid nitrogen and then ground in a mill. The powder obtained from crust and crumb (300 g each) as well as the flour (300 g) were defatted by stirring with chloroform (3 × 300 mL), and, after filtration, solvent residues were removed in vacuo. The defatted powders were extracted twice with tap water (800 mL) upon stirring for 3 h at 50 °C. After filtration, the aqueous filtrates were combined to give fraction I. The nonsoluble residue was then extracted twice for 3 h at room temperature with aqueous ethanol (60% EtOH in water, 800 mL), yielding fraction II upon filtration and solvent evaporation and a residue, which was finally extracted for 24 h with aqueous 2-propanol (50% 2-propanol in water, 1000 mL) to give fraction III after centrifugation and solvent evaporation. Trace amounts of solvents were removed from fractions I−III as well as the nonsoluble materials (fraction IV) in high vacuum. After freeze-drying, the yields of the individual fractions I−IV were determined by weight (**Table 1**).

Measurement of Antioxidative Activity in Vitro. Following a procedure reported in the literature (*19*) with some modifications, the antioxidative activity of melanoidin fractions was determined in vitro by measuring their inhibitory effect on linoleic acid peroxidation. First, the linoleic acid substrate was prepared by dropwise adding linoleic acid (0.125 mL) to a mixture of oxygen-free borate buffer (2.5 mL; 50 mmol/L; pH 9.0) and Tween 20 (0.125 mL). Then aqueous sodium hydroxide solution (1 mol/L) was added until a clear solution was obtained, and the mixture was diluted with oxygen-free borate buffer (50 mmol/L; pH 9.0) to 25 mL and made up to 50 mL with oxygen-free, distilled water. An aliquot (60 μL) of a solution of the melanoidin sample (10 mg/mL in 50% aqueous ethanol) or a purified compound (1 mmol/mL in 50% aqueous ethanol) was added to a solution of oxygen-saturated phosphate buffer (3 mL; 0.2 mol/L; pH 6.75), hydrogen peroxide (100 μL; 16 mmol/L), iron(II) sulfate (100 μL; 16 mmol/L; containing 15 mM EDTA), and linolenic acid substrate (1.0 mL). After incubation of the mixture for 10 min at room temperature, an aliquot (1.0 mL) of that solution was pipetted into disposable cuvettes (1 cm i.d.) containing a solution (2.0 mL) of the color reagent consisting of dimethylformamide (8%), Triton X-100 (1.4%), hemoglobin (56

Table 2. Yields of Molecular Weight Fractions Obtained by Subfractionation of the Ethanolic Fraction II Using Multistep Ultrafiltration

		yield (%)		
fraction	MW (kDa)	flour	bread crumb	bread crust
II/5	≥100	26.3	4.6	1.8
II/4	≥30	16.1	2.5	2.5
II/3	≥10	6.6	4.5	1.9
II/2	≥1	5.5	14.8	21.2
II/1	<1	40.3	73.6	68.9
	Σ (I/1–VII/5)	94.8	100.0	96.3

mg/L), and benzoyl leucomethylene blue (130 μM) in phosphate buffer (0.2 mol/L; pH 5.0). After an additional incubation time of 30 min, the absorbance was measured at $\lambda = 666$ nm against the buffer/color reagent blank. The results were related to the absorption of an aqueous standard solution of Trolox (1 mmol/L) and were expressed as Trolox equivalents (TE values). Each of the experiments was performed in triplicate.

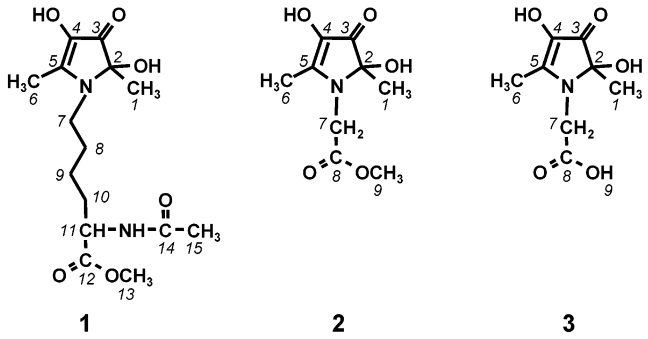
Ultrafiltration. A solution of fraction II (400 mg in 100 mL 50% EtOH) was fractionated sequentially by multistep ultrafiltration (Amicon, Witten, Germany) starting with a filter having a cutoff of 100000 Da (Diaflo YM 100), followed by 30000 Da (Diaflo YM 30), 10 kDa (Diaflo YM 10), and, finally, 1 kDa (Diaflo YM 1). The separation was performed under a nitrogen pressure of 1.5 bar for the filter YM 100 or under 4.0 bar for the filters YM 1, YM 10, and YM 30. The single fractions were freeze-dried, and the residues were weighed and stored in a desiccator. The weight content of each fraction from the total fraction II was calculated in percent (**Table 2**).

Antioxidant Screening of Maillard Reaction Mixtures Prepared from N_α-Acetyl-L-lysine Methyl Ester and Carbohydrates. Binary solutions of N_α-acetyl-L-lysine methyl ester (1 mmol) and starch (162 mg), glucose (1 mmol), 3-deoxy-1-hexosulose (1 mmol), 1-deoxy-2,3-diulose (1 mmol), or acetylformoin (1 mmol), respectively, were heated in phosphate buffer (2.5 mL; 0.1 mol/L; pH 5.5) for 25 min at 100 °C. After cooling to room temperature, the reaction mixtures were freeze-dried, the residues were taken up in aqueous ethanol (4 mL, 50% EtOH in water), and aliquots (60 μL) were then used to measure the antioxidative potential using the in vitro assay reported above. As the control, the antioxidative potential of the corresponding nonheated solutions was determined.

Antioxidant Screening of Maillard Reaction Products Formed from Acetylformoin and Glycine Methyl Ester or N_α-Acetyl-L-lysine Methyl Ester, Respectively. Solutions of acetylformoin (1 mmol) and glycine methyl ester (1 mmol) or N_α-acetyl-L-lysine methyl ester (1 mmol), respectively, in phosphate buffer (5 mL; 0.1 mol/L; pH 5.5) were heated at 100 °C for 25 min. After cooling to room temperature, the reaction mixtures were separated by RP-HPLC, and the effluent was separated into 26 fractions, which were separately collected in glass vials. The corresponding fractions obtained from six HPLC runs were collected, combined, and freeze-dried. The residues obtained from these pooled HPLC fractions were taken up in water/etanol (4 mL; 1:1, v/v) and were then used to measure the antioxidative potential using the in vitro assay reported above.

Isolation of 2,4-Dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2*H*-pyrrole (1) from a Model Mixture of Acetylformoin and N_α-Acetyl-L-lysine Methyl Ester. A solution of acetylformoin (4 mmol) and N_α-acetyl-L-lysine methyl ester (4 mmol) in phosphate buffer (20 mL; 0.1 mol/L; pH 5.5) was heated at 100 °C for 25 min. After cooling to room temperature, the reaction mixtures were separated by semipreparative RP-HPLC. When the effluent was monitored at λ 360 nm, a peak was detected and collected between 19 and 20 min. After freeze-drying, the target compound was obtained as a pale yellow powder. On the basis of UV−vis, LC/MS, and ^{1}H NMR data the lysine derivative was identified as 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonylpentyl)-3-oxo-2*H*-pyrrole (**1** in **Chart 1**): UV−vis (water) λ_{max} 363 nm; LC/MS(APCI$^+$), *m*/*z* 311 (100, [M + 1 − H_2O]$^+$), 329 (86, [M + 1]$^+$); ^{1}H NMR (400 MHz, MeOD-d_3; arbitrary numbering of the carbon atoms refers to structure **1** in **Chart 1**) δ 1.34 [s, 3H, H−C(1)], 1.36−1.86 [3 × m, 6H, H−C(8−10)], 1.98 [s, 3H, H−C(15)], 2.22 [s, 3H, H−C(6)], 3.31 [m, 2H, H−C(7)], 3.72 [s, 3H, H−C(13)]; 4.4 [m, 1H, H−C(11)].

Chart 1. Structures of 2,4-Dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2*H*-pyrrole (**1**) and 2,4-Dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (**2**) Isolated as the Key Antioxidants from Heated Solutions of Acetylformoin and N_α-Acetyl-L-lysine Methyl Ester or Glycine Methyl Ester, Respectively, and Structure of 2,4-Dihydroxy-2,5-dimethyl-1-carboxymethyl-3-oxo-2*H*-pyrrole (**3**) Liberated from **2** by Enzyme Treatment



1 **2** **3**

Isolation of 2,4-Dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (2) from a Model Mixture of Acetylformoin and Glycine Methylester. A solution of acetylformoin (20 mmol) and glycine methyl ester (20 mmol) in phosphate buffer (120 mL; 0.1 mol/L; pH 5.5) was heated at 100 °C for 25 min. After cooling to room temperature, a small aliquot of the reaction mixture was separated by RP-HPLC. When the effluent was monitored at $\lambda = 360$ nm, a peak was detected and collected between 15 and 16 min. For a preparative isolation of the target compound, the major aliquot of the reaction mixture was extracted with methylene chloride (5 × 50 mL); the aqueous phase was mixed with silica gel (20 g) and then freeze-dried. The residue was applied onto the top of a column filled with a slurry of silica gel (150 g) in ethyl acetate. Chromatography was performed with ethyl acetate (300 mL), followed by ethyl acetate/methanol (90:10, v/v; 300 mL), affording an orange fraction. Thin-layer chromatography on RP-18 material using water/methanol (70:30) as the eluent revealed a yellow, fluorescent compound at R_f 0.61. The solvent was removed in vacuo and the residue taken up in methanol (3 mL). Upon addition of ethyl acetate, the target compound was obtained as an yellow powder. Recrystallization from methanol/ethyl acetate (80:20, v/v) afforded compound **2** as yellow crystals (4.5 mmol; 15% yield): UV−vis (H_2O) λ_{max} 363 nm; MS(EI), *m*/*z* 43 (100), 56 (89), 215 (78, M$^+$), 172 (86, M + −CH_3CO), 84 (55), 149 (53), 115 (52), 86 (47), 156 (35), 140 (32), 144 (25), 199 (20); ^{1}H NMR (360 MHz, MeOD-d_3; arbitrary numbering of the carbon atoms refers to structure **2** in **Chart 1**) δ 1.26 [s, 3H, H−C(1)], 2.16 [s, 3H, H−C(6)], 3.73 [s, 3H, H−C(9)], 4.15 [d, 1H, $^2J_{7a,7b} = 18.13$ Hz, H_a-C(7)], 4.23 [d, 1H, $^2J_{7a,7b} = 18.13$ Hz, H_a-C(7)];^{13}C NMR (360 MHz, MeOD-d_3, HMQC, HMBC, DEPT; arbitrary numbering of the carbon atoms refers to structure **2** in **Chart 1**) δ 11.5 [CH_3, C(1)], 22.0 [CH_3, C(6)], 43.3 [CH_3, C(9)], 53.4 [CH_2, C(7)], 88.2 [C, C(2)], 128.2 [C, C(5)], 169.1 [C, C(4)], 172.7 [C, C(8)], 193.1 [C, C(3)].

Isolation of 2,4-Dihydroxy-2,5-dimethyl-1-carboxymethyl-3-oxo-2*H*-pyrrole (Pronyl-glycine, 3). Following a procedure reported recently (*26*) with some modifications, a solution of 2,4-dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (**2**; 1.0 mmol) and porcine liver esterase (2000 units) in phosphate buffer (10 mL; 0.2 mol/L; pH 7.5) was stored overnight at 37 °C. The mixture was concentrated by partial freeze-drying to 1 mL, and the target compound was purified by chromatography on RP-18 material (15.0 g; Lichroprep 25−40 μm, Merck, Darmstadt, Germany) using a mixture (20:80, v/v) of methanol and trifluoracetic acid (0.1% TFA in water) as the mobile phase. After application of the crude material, chromatography with the same eluent afforded compound **3** in the effluent >80 mL. The combined eluates were freeze-dried, yielding the colored compound as an orange powder (0.35 mmol, ∼35% yield): LC/MS(APCI$^-$), *m*/*z*

200 (100, $[M + 1 - H_2]^-$); ^{1}H NMR (360 MHz, MeOD-d_3; arbitrary numbering of the carbon atoms refers to structure **3** in **Chart 1**) δ 1.28 [s, 3H, H−C(1)], 2.17 [s, 3H, H−C(6)], 4.17 [d, 1H, $^2J_{7a,7b}$ = 18.2 Hz, H_a-C(7)], 4.25 [d, 1H, $^2J_{7a,7b}$ = 18.2 Hz, H_a-C(7)].

Synthesis of 5-Acetyl-4-hydroxy-1,3-dimethylpyrazole. A solution of methyl hydrazine (1.5 mmol) in ethanol (5 mL) was adjusted to pH 4 with concentrated hydrochloric acid, pronyl-glycine (**3**, 1 mmol) was added, and the mixture was refluxed for 5 min. The solution was neutralized with an aqueous sodium hydroxide solution (1 mmol/L), concentrated, and then separated by semipreparative HPLC using RP-18 as the stationary phase. Two peaks were detected in a ratio of 3:1; the effluents were collected and freeze-dried affording 5-acetyl-4-hydroxy-1,3-dimethylpyrazole (0.48 mmol, 48% yield) as the major reaction product and 3-acetyl-4-hydroxy-1,5-dimethylpyrazole as a trace compound in a purity of >99%. Spectroscopic data of 5-acetyl-4-hydroxy-1,3-dimethyl-pyrazole: GC/MS(EI), *m*/*z* 154 (78), 139 (54), 111 (5), 97 (5), 56 (100), 42 (46); ^{1}H NMR (400 MHz, $CDCl_3$, DQF-COSY) δ 2.20 (s, 3H, CH_3), 2.52 (s, 3H, CO−CH_3), 3.78 (s, 3H, N−CH_3); ^{13}C NMR (360 MHz, $CDCl_3$, HMQC, HMBC) δ 8.2 (CH_3), 25.5 (CH_3), 37.6 (N−CH_3), 123.5 (N=C), 135.5 (N−C=), 143.2 (C−OH), 198.1 (CO). Spectroscopic data of 3-acetyl-4-hydroxy-1,5-dimethylpyrazole: GC/MS(EI), *m*/*z* 154 (100), 139 (39), 85 (34), 70 (21), 56 (10), 42 (88); ^{1}H NMR (400 MHz, $CDCl_3$, DQF-COSY) δ 2.20 (s, 3H, CH_3), 2.56 (s, 3H, CO−CH_3), 4.04 (s, 3H, N−CH_3).

Quantitation of Pronylation Degree of Proteins. Ground bread crust, crumb, or flour (each 50 g) was defatted with chloroform (3 × 100 mL) and suspended in water (250 mL); after addition of methyl hydrazine (5 g), the pH was adjusted to 4.0 using concentrated hydrochloric acid. After the mixture had been incubated for 45 min at 80 °C, it was cooled to room temperature, the pH was adjusted to 7 using aqueous sodium hydroxide (1 mmol/L), and the solution was extracted with methylene chloride (3 × 200 mL). The combined organic layers were extracted with aqueous sodium hydroxide solution (0.1 mmol/L, 150 mL), and the aqueous phase was adjusted to pH 3.0 with concentrated hydrochloric acid and was then again extracted with methylene chloride (3 × 50 mL). A defined amount of 1-methylpyrrolidone in methanol was added as the internal standard, and, after concentration, the extract was analyzed by HRGC/MS(CI). The amount of pyrrolinone reductone was calculated from a calibration curve determined from aqueous solutions containing defined amounts of pronyl-glycine. Each of the experiments was performed in triplicate.

Preparation of Model Melanoidins. Gluten/starch and gluten/glucose melanoidins were prepared by heating doughs prepared from wheat gluten (40 g), water (150 mL), and starch (160 g) or glucose (160 g), respectively, in a baking oven for 1 h at 220 °C. Gluten/acetylformoin melanoidins were prepared by heating a mixture of acetylformoin (500 mg), gluten (500 mg), and water (1.5 mL) for 30 min at 150 °C in an baking oven. For isolation of the high molecular weight melanoidins, the thermally treated mixtures were suspended in water (40 mL) and were freed from low molecular weight compounds by ultrafiltration (Amicon, Witten, Germany) with a molecular weight cutoff of 10 kDa (Diaflo YM 10). The low molecular weight fraction was freeze-dried and then used for the quantitation experiments.

Preparation of Pronylated Bovine Serum Albumin (Pronyl-BSA). A suspension of BSA (10.24 g) and acetylformoin (5.76 g) in phosphate buffer (100 mL; 0.1 mol/L; pH 6.5) was incubated for 1 h at 80 °C, with stirring. After cooling to room temperature, the mixture was placed in a dialysis tubing (Sigma, Deisenhofen, Germany) with a molecular weight cutoff of 12 kDa, and distilled water was added until the height of the solution in the tube was ∼50 cm. The tubing was closed and submerged in distilled water (4 L) at 4 °C. After 12 h, the water surrounding the dialysis tubing was replaced with fresh water (4 L) and dialysis continued for another 12 h at 4 °C. After this step had been repeated twice, the content of the tubing was freeze-dried, yielding pronyl-BSA (13.8 g) as a yellow powder, which was stored in a desiccator. Quantitation of the content of BSA-linked pyrrolinone reductone revealed an amount of 270 μg/g calculated as pronyl-lysine corresponding to a lysine substitution of 1.2 nmol/mmol.

Cell Culture Experiments. Caco-2 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DMSZ, Braunschweig, Germany). Caco-2 cells (passages 11, 12, 15, and 16) were maintained at 37 °C in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 2% L-glutamine (200 mM), and 2% penicillin−streptomycin (5000 units of penicillin and 5 mg of streptomycin/mL of 0.9% NaCl) in an atmosphere of CO_2/air (1:20, v/v). Cells grown in 75 cm^2 culture flasks were supplied on culture medium (30 mL), which was exchanged (50%) twice a week. Cells were seeded at a density of 20000 cells/cm^2 to achieve confluency at day 6 after seeding. The fractions isolated from bread crust, BSA, and the synthesized model compounds pronyl-BSA and pronyl-glycine (**3**) were dissolved in the medium and were exposed to the cells for 48 h. Each of the experiments was performed in triplicate. After exposure to the different bread fractions, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS), harvested with trypsin−EDTA (0.5 g of porcine trypsin and 0.2 g of NaEDTA/L of Hank's balanced salt solution; incubation time = 10 min), centrifuged (5 min, 1000*g*), washed (DPBS), and centrifuged (5 min, 1000*g*) again. Cells were diluted 1:5 in Tris-HCl buffer (20 mM, pH 7.4) containing sucrose (0.25 M) and dithiothreitol (1.4 mM), subsequently homogenized in a glass/Teflon homogenizer (*27*), and centrifuged (60 min, 105000*g*). The cytosolic 105000*g* supernatant was used for the GST analysis. The pellet containing the microsomal fraction was resuspended in 5 mL of 0.9% NaCl. Microsomal NADPH−cytochrome *c* reductase activity was determined in the remaining pellet according to a method reported in the literature (*28*). GST activity was determined using 1-chloro-2,4-dinitrobenzene as the substrate as described in the literature (*29*), and protein content was measured as reported earlier (*30*).

Data obtained from triplicate experiments are given as means and standard deviations in relation to the basal activity of nonexposed control cells (basal activity analyzed for CCR and GST was 3.85 ± 0.40 nmol of cytochrome *c*/mg of protein/min and 350 ± 42 nmol of CDNB/mg of protein/min, respectively). Means of each treatment were compared with untreated control cells by Student's *t* test. The level of significance was set at $P < 0.05$ (*).

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (BIO-TEK Instruments, Eching, Germany) consisted of two pumps (type 422), a gradient mixer (M 800), a Rheodyne injector (100 μL loop), and a diode array detector (DAD type 540+) monitoring the effluent in a wavelength range between 210 and 500 nm. Separations were performed on a stainless steel column packed with RP-18 (C-18 Nucleosil 300 nm, 5 μm) either in an analytical (4.6 × 240 mm, 1.6 mL/min) or in a semipreparative scale (10 × 250 mm, 3.0 mL/min). For the analytical runs, a gradient was used starting with a 100% aqueous TFA (0.1% TFA in water) and increasing the methanol content to 100% within 40 min. For the semipreparative runs, a gradient was used starting with a 100% aqueous formic acid (0.1% in water) and increasing the methanol content to 62.5% within 25 min and then to 100% within 28 min.

High-Resolution Gas Chromatography/Mass Spectrometry (HRGC/MS). HRGC was performed with a GC 3800 gas chromatograph (Varian, Darmstadt, Germany) equipped with a 30 m × 0.32 mm i.d., 0.25 μm, fused silica capillary CP SIL 19CB (Chrompack, Frankfurt, Germany) by on-column injection at 40 °C. After 1 min, the temperature of the oven was raised at 15 °C/min to 100 °C, then raised at 6 °C/min to 160 °C, and finally raised at 10 °C/min to 230 °C and held for 5 min. The flow of the carrier gas, helium, was 2.5 mL/min. MS analysis was performed with a Saturn GC MS/MS 2000 (Varian) in tandem with the HRGC. Mass chromatography in the electron impact mode (MS/EI) was performed at 70 eV and in the chemical ionization mode (MS/CI) at 115 eV with methanol as the reactant gas.

Liquid Chromatography/Mass Spectrometry (LC/MS). An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel, Dürren, Germany) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI). After injection of the sample (2−20 μL), analysis was performed using a gradient starting with 100% aqueous TFA (0.1% TFA in water) and increasing the methanol content to 100% within 40 min.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, DQF-COSY, HMQC, and HMBC spectroscopies were performed on AMD 360 and AMX 400 spectrometers (Bruker, Rheinstetten, Germany), respectively.

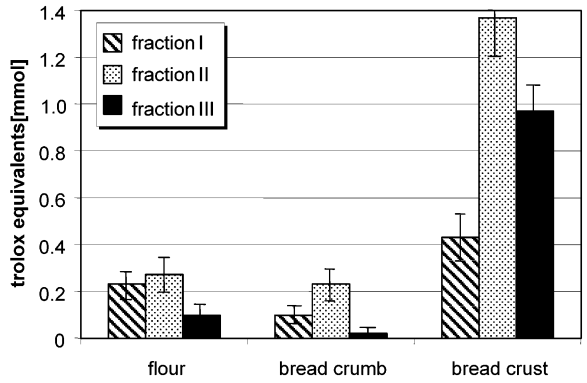


Figure 1. In vitro antioxidative activity of soluble fractions I–III isolated from flour, bread crumb, and bread crust.

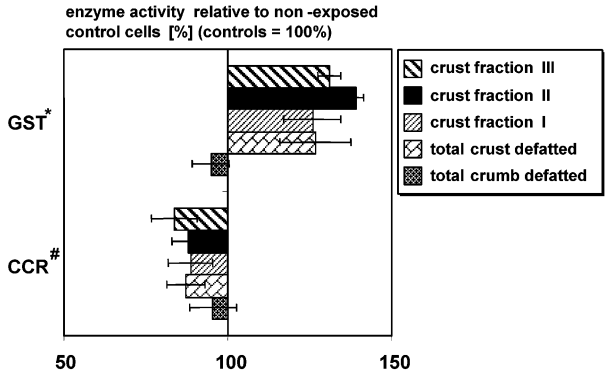


Figure 2. Effect of bread crumb and different fractions of bread crust on the enzyme activity of NADPH–cytochrome *c* reductase (CCR) and glutathione *S*-transferase (GST) in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 0.5 g of the individual bread crust or bread crust fraction per 100 mL of cell culture medium for 48 h. GST*/CCR#: Enzyme activity of cells exposed to bread crust fractions was significantly different from that of nonexposed control cells ($p < 0.05$).

RESULTS AND DISCUSSION

To gain first insights into the antioxidative potential of bread crust, a sourdough bread was freshly baked to a pleasant dark brown crust, the crust was then carefully separated from the crumb, and both materials were frozen in liquid nitrogen and ground in a kitchen mill. The powder obtained from crumb and crust, respectively, was then defatted by chloroform extraction and then sequentially extracted with water, 60% aqueous ethanol, and 50% aqueous 2-propanol, affording the corresponding solvent fractions I−III. These fractions containing the soluble compounds as well as the nonsoluble materials (fraction IV) were freed from solvent in vacuo and then freeze-dried. In comparison, the wheat/rye flour used for bread preparation was fractionated following this procedure. The yields obtained for the fractions I−IV of flour, bread crumb, and crust are given in **Table 1**.

Antioxidant Activity of Bread Crust Fractions. To compare their antioxidant potentials, the efficiencies of the soluble fractions I−III, isolated from flour, crumb, and crust, in inhibiting the peroxidation of linolenic acid were measured following the procedure reported recently (*19*). Using Trolox as the reference for a highly active antioxidant, the antioxidative potential was calculated as Trolox equivalents (TE values). As given in **Figure 1**, the antioxidative potential of all the fractions isolated from bread crust exceeded the activities measured for the flour and crumb isolates. In particular, the dark brown ethanol solubles (fraction II) of the crust showed the highest activity; for example, 6-fold higher TE values were measured in comparison to the corresponding fraction isolated from flour and the bread crumb, respectively. Also, the crust fraction III showed a high antioxidative activity of ∼1.0 TE value. In comparison, the pale fractions isolated from the bread crumb did not show higher activities that those isolated from the flour (**Table 1**). These data clearly indicate that during bread baking, nonenzymatic browning reactions, which are obviously favored in the bread crust, lead to the formation of reaction products with antioxidative potential in vitro.

To investigate whether these reaction products showing a significant antioxidant activity in vitro may also act as antioxidants in biological systems, their enzyme modulating activity on detoxification enzymes was investigated as a functional parameter in intestinal Caco-2 cells. In general, detoxification enzymes protect cells from a wide variety of nonendogenously formed xenobiotics and endogenous toxins. Current data suggest that the balance between the phase I carcinogen-activating enzymes and the phase II detoxifying enzymes is critical to determining an individual's risk for cancer. Human deficiencies in phase II enzyme activity, specifically glutathione *S*-transferase (GST), have been identified and associated with increased risk for colon cancer (*21*). On the other hand, induction of phase II enzymes by antioxidants represents a promising strategy for cancer prevention. Although the molecular mechanism by which antioxidants bind to an antioxidant response element resulting in the specific, monofunctional induction of phase II enzymes has been intensively studied so far (*24*), it is still an open question as to whether a compound showing an antioxidant activity in vitro may function as a phase II inducer in biological systems. Among all of the biological systems suitable for in vitro studies, the intestinal Caco-2 cell line is widely used to investigate the effects of dietary compounds on xenobiotic enzymes as the colon is clearly one of the most likely sites for the development of different types of dietary-induced cancers (*27*, *31*).

In the present study, intestinal Caco-2 cells exposed to bread crust and the respective fractions I−III showed a significantly elevated GST activity compared to nonexposed control cells and compared to those cells exposed to bread crumb. In contrast, phase I NADPH−cytochrome *c* reductase (CCR) activity was inhibited by all of the fractions that activated the GST enzyme (**Figure 2**). Thus, CCR and GST activities in intestinal Caco-2 cells exposed to bread crust and its isolated fractions were modulated in such a way as to be interpreted as a protective functional parameter of an antioxidant activity in vitro.

To gain further insights into the molecular weight of those crust compounds exhibiting the highest antioxidative potential, the most active, ethanolic fraction II was further fractionated using multistep ultrafiltration. Using membrane filters with stepwise decreasing molecular weight cutoffs, five fractions were obtained containing ethanol-soluble crust compounds with molecular weights of <1 kDa (fraction II/1), 1−10 kDa (fraction II/2), 10−30 kDa (fraction II/3), 30−100 kDa (fraction II/4), and >100 kDa (fraction II/5). From fraction II/1 to II/5 a significant increase in browning intensity was observable, thus demonstrating that the browning is due to high molecular weight melanoidins. As a reference, the corresponding fraction II isolated from the flour and showing only a very low antioxidative potential (**Figure 1**) was fractionated by ultrafiltration. As summarized in **Table 2**, nearly 69% of the ethanol-soluble crust compounds were obtained in fraction II/1 and exhibited molecular weights of <1 kDa. In addition, 21.2% of these compounds showed molecular weights between 1 and 10 kDa,

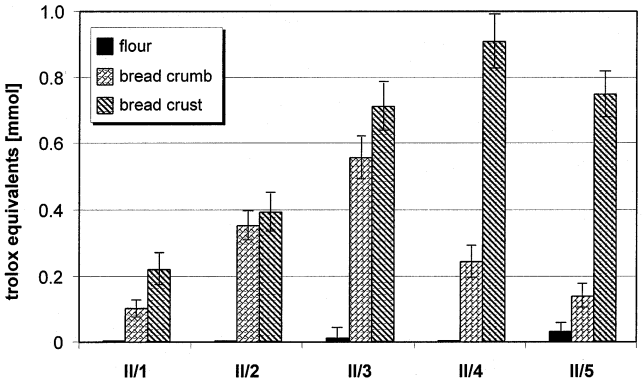


Figure 3. In vitro antioxidative activity of molecular weight fractions obtained by multistep ultrafiltration of fraction II isolated from flour, bread crumb, and crust, respectively.

whereas ~6.2% consisted of macromolecules with molecular weights of >10 kDa. Also, the crumb fraction II consisted mainly of low molecular weight compounds; for example, 73.6% was present in fraction II/1. Comparing these data with those obtained from the flour fraction II clearly showed major differences (**Table 2**); for example, only 40.3% of the flour components showed molecular weights of <1 kDa, whereas 42.4% consisted of macromolecules with molecular weights of >30 kDa. These differences clearly demonstrate that the baking process induced major changes in the chemical composition of the flour.

These fractions obtained by ultrafiltration were then used to estimate the molecular weights of the antioxidants in bread crust, crumb, and flour (**Figure 3**). Crust fraction II/4, containing compounds with a molecular weight between 30 and 100 kDa, was found to be most efficient in inhibiting linolenic acid peroxidation, closely followed by fractions II/3 and II/5. However, the low molecular weight fraction II/1 showed only low activity (**Figure 3**). In comparison, the highest antioxidative activity in the crumb was detectable in fractions II/2 and II/3 containing compounds with a molecular weight between 1 and 30 kDa. The crumb fractions II/4 and II/5 were significantly less active than the corresponding crust fractions containing the dark brown melanoidins. None of the molecular weight fractions isolated from the ethanolic flour extract showed any significant antioxidative potential. These results indicate that macromolecules exhibiting antioxidant potential are formed nonenzymatically from inactive precursors during the baking process.

Structure of an Antioxidant Site of Melanoidins. Because brown melanoidins were shown to be formed upon carbohydrate-induced modifications and/or cross-linking of reactive amino acid side chains in food proteins (*9*, *10*) and acidic hydrolysis of the crust fractions II/3−II/5 revealed all of the proteinogenic amino acids, it was hypothesized that the antioxidatively active sites of the crust macromolecules might be due to Maillard-type modifications of flour proteins. It is well-known from the literature that, besides arginine residues, in particular, the ϵ-amino group of lysine side chains as well as the amino group of the N terminus are the primary targets for nonenzymatic browning reactions with carbohydrate degradation products (*9*). To gain first insights into the efficiency of carbohydrates and carbohydrate degradation products in transforming the ϵ-amino group of protein-bound lysine into reactions products with antioxidative activity, starch, glucose, and the hexose degradation products 3-deoxy-2-hexosulose and 1-deoxy-2,3-hexodiulose, as well as acetylformoin, a well-known dehydration product formed from 1-deoxy-2,3-hexodiulose, were reacted

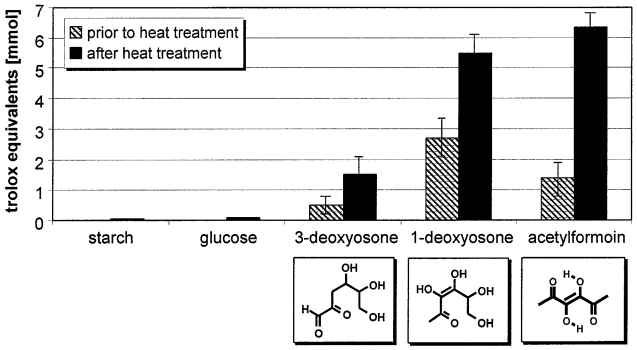


Figure 4. In vitro antioxidative activity of binary mixtures of carbohydrate precursors and N_α-acetyl-L-lysine methyl ester prior to and after thermal treatment, respectively.

with N_α-acetyl-L-lysine methyl ester, chosen as a suitable model substance for the ϵ-amino group of protein-bound lysine. The individual binary reaction mixtures were analyzed for antioxidative activity in vitro prior to and after thermal treatment. The results, summarized in **Figure 4**, show that under the mild reaction conditions applied, neither starch nor glucose is able to produce high amounts of antioxidants. Experiments with 3-deoxyosone as well as 1-deoxyosone revealed that, in particular, the 1-deoxyosone-derived reaction products showed high antioxidative potential; for example,e.g. 5.4 TE values were measured for the experiment including 1-deoxyosone (**Figure 4**). In comparison, 3-deoxyosone showed only 25% of the antioxidative potential found for 1-deoxyosone. The highest activity was, however, found for the reaction mixture containing acetylformoin, a major dehydration product formed from 1-deoxyosone. Measuring the TE values of these mixtures prior to thermal treatment clearly showed that all of the hexose-derived intermediates show antioxidative potential per se, among which 1-deoxyosone and acetylformoin were the most active due to their reductone-type structures. Comparing these data with those found after heating, however, clearly showed that the reaction of, in particular, 1-deoxyosone and acetylformoin with the lysine derivative led to the formation of reaction products with high antioxidative potential.

On the basis of these findings, the most active precursor mixture, consisting of acetylformoin and N_α-acetyl-L-lysine methyl ester, was selected to elucidate the active key compounds by a screening procedure. To achieve this, the reaction mixture was separated by RP-HPLC, and the effluent was monitored using either a diode array detector (DAD) operating at wavelengths between 210 and 500 nm or an LC/MS. The effluent was separated into 26 fractions, which were separately collected in glass vials (**Figure 5A**). After freeze-drying, the residues obtained from these HPLC fractions were taken up in the same amount of water and were then used to measure the antioxidative activity using the in vitro assay (**Figure 5B**). The results clearly showed by far the highest antioxidative activity for the fraction 14; for example, 4.5 TE values were determined. With the exception of fraction 7, the antioxidative potential of all the other HPLC fractions did not exceed the activity of 1.0 TE value, thus indicating the active principle in fraction 14 to be the key compound responsible for the high antioxidative potential of the heated Maillard mixture.

The following identification experiments were, therefore, focused on the main reaction product present in fraction 14. After chromatographic isolation, the determination of its chemical structure (**1** in **Chart 1**) was performed by ^{1}H NMR, LC/MS, and UV−vis spectroscopy. LC/MS measurements of

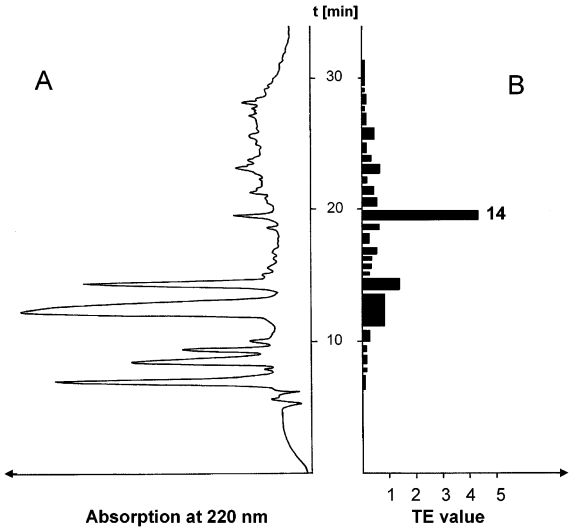


Figure 5. HPLC chromatogram (A) and in vitro antioxidative potential of fractions collected (B) from a heated solution of acetylformoin and N_α-acetyl-L-lysine methyl ester.

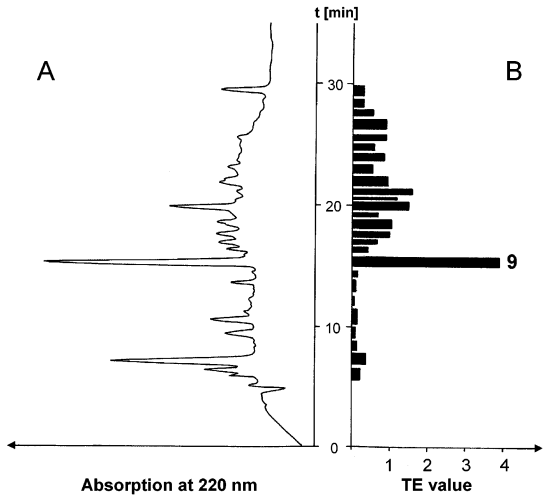


Figure 6. HPLC chromatogram (A) and in vitro antioxidative potential of fractions collected (B) from a heated solution of acetylformoin and glycine methyl ester.

the purified compound, exhibiting an absorption maximum at 363 nm, gave an intense $[M + 1]^+$ ion at *m*/*z* 329 (86%) with a loss of 18 to the base peak at *m*/*z* 311 (100%), most likely due to the cleavage of one molecule of water. In addition, LC-MS/MS experiments revealed the loss of 42 from *m*/*z* 311 to 269 and, in addition, the loss of 59 to *m*/*z* 251 corresponding most likely to the elimination of ketene from the acetylamino group and of the methoxycarbonyl group from the ester function, respectively. These data corroborate the *N*-acetyllysine methyl ester moiety in the proposed structure of 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonylpentyl)-3-oxo-2*H*-pyrrole (**1**) outlined in **Chart 1**.

The ^{1}H NMR spectrum of compound **1** showed nine resonance signals. Double-quantum-filtered δ,δ-correlation spectroscopy (DQF-COSY) revealed strong H,H-shift correlations between the hydrogens H−C(7)/H−C(8), H−C(8)/H−C(9), H−C(9)/H−C(10), and H−C(10)/H−C(11) and, in addition, the two singlets at 1.98 and 3.72 ppm, corresponding to an acetamide group and a methoxy group, respectively, thus confirming the presence of the N_α-acetyl-L-lysine methyl ester moiety in compound **1**. In addition, two singlets were detected resonating at 1.34 and 2.22 ppm and were assigned as the methyl groups C(1) and C(6) in structure **1**. Due to the instability of compound **1** during concentration, it was, however, not possible to isolate the target compound in sufficient amounts to perform ^{13}C NMR studies for the final structure confirmation.

With the expectation that probably another amino acid derivative might be more stable for doing ^{13}C NMR studies and to further confirm the proposed reductone structure of compound **1**, an additional set of experiments was performed in which the lysine derivative was substituted by glycine methyl ester as a model for the N terminus of a protein. An equimolar mixture of acetylformoin and glycine methyl ester was thermally treated, cooled to room temperature, and then screened for the most active antioxidant by using the HPLC screening assay reported above (**Figure 6**). From the 26 fractions isolated, fraction 9 was found to have by far the highest antioxidative potential. For this fraction, an activity of nearly 4 TE values were determined, whereas all of the other fractions did not exceed an activity of 1.5 TE values. Isolation and purification afforded the target compound as yellow crystals, which were stable enough to perform UV−vis, GC/MS, and ^{1}H and ^{13}C NMR studies. The absorption maximum at 363 nm and the molecular weight of 215 Da as well as the resonance signals in the ^{1}H NMR spectrum were very well in line with the structure of 2,4-dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (**2**), shown in **Chart 1**. The structure of the proposed pyrrolinone ring was unequivocally confirmed by ^{13}C NMR and heteronuclear chemical shift experiments. Although this compound was reported earlier as a Maillard reaction product formed from the Amadori product 1-deoxy-1-piperidino-D-fructose and glycine esters (*32*), its function as an antioxidant has as yet not been reported.

To confirm the antioxidative potential of these purified pyrrolinone reductones, compounds **1** and **2**, and, in comparison, ascorbic acid, were analyzed using the in vitro antioxidant assay. Both pyrrolinone reductones, **1** and **2**, showed high antioxidative activities of 0.53 and 0.49 TE, respectively, whereas the ascorbic acid showed a lower activity by a factor of 5.

Quantitation of Protein-Bound Pyrrolinone Reductones. Neither acidic hydrolysis nor enzymatic hydrolysis revealed high recoveries of pyrrolinone reductones (data not shown). Because the pyrrolinone reductonyl moiety, which we abbreviate "pronyl", is fixed to the protein backbone via the lysine side chain as pronyl-lysine and via the N terminus, respectively, this carbohydrate modification was cleaved from the protein and subsequently transformed into 5-acetyl-4-hydroxy-1,3-dimethylpyrazole upon hydrazinolyses using methyl hydrazine (**Figure 7**). After this derivatization, solvent extraction, and simple cleanup, the amount of pyrrolinone reductones could be quantified as the stable 5-acetyl-4-hydroxy-1,3-dimethylpyrazole by means of HRGC/MS(CI). In control expertiments, it could be shown that heating carbohydrates in the presence of methyl hydrazine does not generate 5-acetyl-4-hydroxy-1,3-dimethylpyrazole (data not shown). Thus, this method offered the possibility to quantitate the total degree of pronylation of the N terminus as well as the lysine side chains, calculated as pronyl-lysine.

To gain insights into the efficiency of carbohydrates and carbohydrate degradation products in pyrrolinone reductone formation during bread baking, binary model mixtures containing wheat gluten and either starch, glucose, or acetylformoin, respectively, were thermally treated in an baking oven, the high molecular weight melanoidins were isolated by ultrafiltration, and the amounts of pronyl-lysine formed were determined. The data, summarized in **Table 3**, showed that acetylformoin was most efficient in generating high mounts of pyrrolinone reduc-

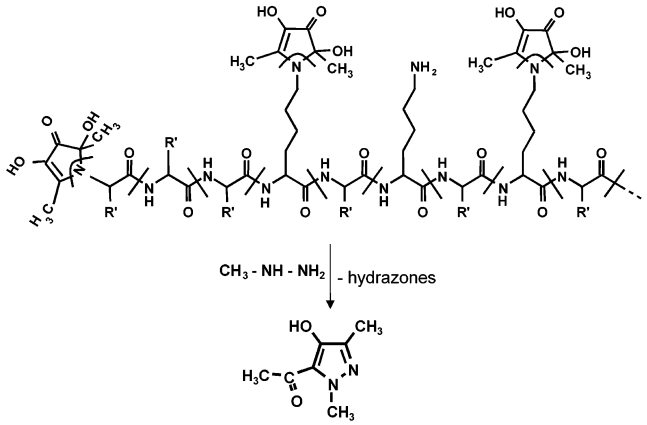


Figure 7. Cleavage of pronylated proteins by hydrazinolysis using methyl hydrazine and formation of 5-acetyl-4-hydroxy-1,3-dimethylpyrazole.

Table 3. Amounts of Protein-Bound Pyrrolinone Reductones (Calculated as Pronyl-lysine) in Model Melanoidins Generated by Heating Wheat Gluten in the Presence of Carbohydrate Sources under Baking Conditions

carbohydrate precursor	concn of pronyl-L-lysine[a] (mg/kg)
starch[b]	4.0 (± 3.4–4.6)
glucose[b]	18.9 (± 15.2–22.6)
glucose[c]	nd
acetylformoin[d]	7100.0 (± 6035.0–8165.0)

[a] Glucose (160 g) was heated in the absence of wheat gluten. nd, not detectable. [b] A mixture of acetylformoin (500 mg), wheat gluten (500 mg), and water (1.5 mL) was heated for 30 min at 150 °C in an baking oven. [c] The concentration is calculated as milligram per kilogram of the total reaction mixture. [d] A mixture of wheat gluten (40 g), water (150 mL), and starch (160 g) or glucose (160 g), respectively, was heated in a baking oven for 1 h at 220 °C.

Table 4. Amounts of Protein-Bound Pyrrolinone Reductone (Calculated as Pronyl-lysine) in Flour, Bread Crumb, and Crust

sample	concn of pronyl-L-lysine[a] (mg/kg)	lysine substitution (nmol/mmol)
flour	nd	nd
bread crumb	8.0 (± 6.8–9.2)	1.40
bread crust	62.2 (± 52.9–71.5)	11.24

[a] Degree of pronylation was calculated as pronyl-L-lysine. nd, not detectable.

tones under baking conditions, for example, 7100 mg/kg pronyl-lysine was formed. Also, glucose and starch were precursor active in the presence of gluten but produced significantly lower amounts of pronyl-lysine when compared to acetylformoin. A control experiment, in which glucose was heated in the absence of gluten, did not show any amounts of the pronyl-lysine. These data clearly indicate that the reaction of lysine side chains and the N termini of flour proteins, respectively, with starch or hexoses is efficient in generating protein-bound pyrrolinone reductones under baking conditions via acetylformoin as the penultimate precursor.

To gain insights into the degree of protein pronylation in the flour proteins, the pyrrolinone reductone, calculated as pronyl-lysine, was determined in flour, bread crumb, and bread crust. The results given in **Table 4** reveal by far the highest amounts in the dark bread crust; for example, ~62.2 mg/kg pronyl-lysine was present in bread crust, corresponding to a lysine substitution of 11.24 nmol/mmol. In comparison, the pale bread crumb contained these pyrrolinone reductones in 8-fold lower amounts, whereas the nonthermally treated flour did not show any significant amounts of pronylated proteins (**Table 4**).

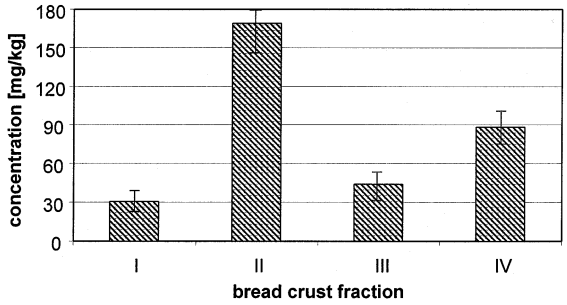


Figure 8. Concentrations of pyrrolinone reductones (calculated as pronyl-lysine) in individual bread crust fractions.

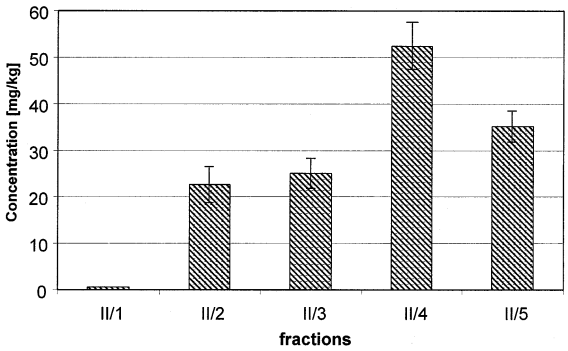


Figure 9. Influence of the molecular weight on the concentrations of pyrrolinone reductones (calculated as pronyl-lysine) in crust fraction II. Molecular weights of fractions II/1–II/5 are given in **Table 2**.

To gain further insights into the distribution of these protein-bound antioxidants in the crust fractions, the amount of pyrrolinone reductones, calculated as pronyl-lysine, was determined in fractions I−IV (**Figure 8**). All of the fractions analyzed contained the pyrrolinone reductone; however, by far the highest amounts were found in the ethanolic fraction II. The high amount of pronyl-lysine, determined to be 169.3 mg/kg in fraction II, is well in line with the high antioxidative activity of this fraction (**Figure 1**). In comparison, fractions III and I contained only 42.9 and 30.6 mg/kg pronyl-lysine, respectively, thus corresponding well with the lower antioxidative activity of the crust compounds extracted by water or 2-propanol. It is interesting to note that the nonsoluble crust materials (fraction IV) also contain rather high amounts of pyrrolinone reductones, for example, 88.5 mg/kg pronyl-lysine. As the insolubility of this fraction does not allow the measurement of the antioxidative activity in vitro, the quantitation of pronyl-lysine as an indicator for thermally generated, protein-linked antioxidants might be a suitable tool to gain some insights into their antioxidative status.

To study the influence of the molecular weight of the ethanol-soluble crust browning products on their amount of pyrrolinone reductone, pronyl-lysine was quantitated in the molecular weight fractions II/1−II/5 obtained by ultrafiltration of the ethanolic crust extract. The quantitative data, given in **Figure 9**, clearly show that the highest amounts of pyrrolinone reductones were present in the macromolecular browning products; for example, 52.3 and 35.2 mg/kg pronyl-lysine were determined in fractions II/4 and II/5, respectively. In comparison, the low molecular weight fraction II/1 contained only very low concentrations. Comparison of the amounts of pronyl-lysine (**Figure 9**) and the antioxidative activities (**Figure 3**) of these molecular weight fractions showed a close correlation between the antioxidative potential and the concentration of the pronyl-lysine, again demonstrating pronyl-lysine as a suitable indicator substance

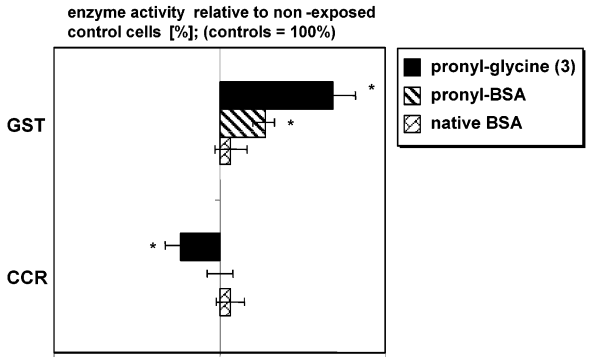


Figure 10. Effect of pronyl-BSA and pronyl-glycine (**3**) on the enzyme activity of NADPH–cytochrome *c* reductase (CCR) and glutathione *S*-transferase (GST) in Caco-2 cells relative to nonexposed controls (=100%). During the exposure time of 48 h, Caco-2 cells were exposed to the different compounds dissolved into the cell culture medium at final concentrations corresponding to the content of 0.03 mg of pyrrolinone reductone (calculated as pronyl-lysine) per 0.5 g of bread crust: 0.11 g of pronyl-BSA/100 mL, 0.11 g of BSA/100 mL, and 0.03 mg of pronyl-glycine/100 mL of medium for 48 h. *: Enzyme activity of exposed cells was significantly different from that of nonexposed control cells ($p < 0.05$).

to quantitatively monitor the thermal generation of antioxidants during baking processes.

Degree of pronylation is, however, tightly connected to the higher molecular weight crust melanoidins. To get closer to the real food system, "pronylated" protein, namely, BSA, was prepared by reacting acetylformoin and purified BSA under mild, aqueous conditions. Model experiments showed that under these mild conditions used, the lysine side chain was the only amino acid rapidly reacting with acetylformoin to give a lysine substitution of 1.2 nmol/mmol.

To study the effect of pronylated amino acids on the activity of detoxification enzymes, in comparison to the results obtained for the bread crust fractions (**Figure 1**), Caco-2 cells were exposed to either pronyl-BSA or purified pronyl-glycine (**3** in **Chart 1**), liberated from the corresponding methyl ester **2** by esterase treatment. After an exposure time of 48 h, phase II GST activity of cells exposed to pronyl-BSA and to pronyl-glycine was increased by 12 and 34%, respectively (**Figure 10**). A significant decrease in the CCR enzyme activity of ~12% compared to controls was analyzed for those cells exposed to pronyl-glycine. These results clearly show that the most effective compound in modulating the activity of phase II GST enzyme is the pronylated amino acid; its protein-linked form, as present in crust melanoidins, also shows significant activity. In intestinal Caco-2 cells at least, pronylated proteins act as a monofunctional inducer of GST, serving as a functional parameter of an antioxidant, chemopreventive activity in vitro.

The question of whether dietary pronylated proteins present in bread crust indeed are physiologically active antioxidants, being absorbed in the intestine and metabolized endogenously, is currently under investigation in an animal feeding study.

ACKNOWLEDGMENT

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