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Influence of Baking Conditions and Precursor Supplementation on the Amounts of the Antioxidant Pronyl-L-lysine in Bakery Products

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The influence of baking conditions and dough supplements on the amounts of the antioxidant and Phase II-Enzyme modulating, protein-bound 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2*H*-pyrrol (pronyl-L-lysine) in bakery products was investigated in quantitative studies. These studies revealed high amounts of the antioxidant in bread crust, only low amounts in the crumb, and the absence of this compound in untreated flour. The amounts of pronyl-L-lysine were found to be strongly influenced by the intensity of the thermal treatment. For example, increasing the baking time from 70 to 210 min or increasing the baking temperature from 220 to 260 °C led to a 5- or 3-fold increase in the concentrations of this antioxidant in the crust, respectively. In addition, modifications in the recipe showed to have a major impact on pronyl-L-lysine formation. For example, substituting 5% of the flour with the lysine-rich protein casein or with 10% of glucose increased the amounts of the antioxidant by more than 200%. Quantitative analyses of commercial bread samples collected from German bakeries revealed the highest amount of 43 mg/kg for a full grain bread, followed by a rye/wheat bread, both of which have been sourdough fermented. A mixed-grain bread as well as pale wheat bread, both prepared without sourdough fermentation, contained significantly lower amounts of pronyl-L-lysine, and German pretzels, which are treated with a dilute sodium hydroxide solution prior to baking, contained only trace amounts of pronyl-L-lysine (e.g., less than 5 mg/kg were detectable in pretzels). Systematic studies revealed that the decrease of the pH value induced by microbial acid formation during sourdough fermentation is the clue for producing high amounts of pronyl-L-lysine in baking products. These data clearly demonstrate for the first time that the amounts of the antioxidant and chemopreventive compound pronyl-L-lysine in bakery products is strongly dependent on the manufacturing conditions as well as the recipe.

KEYWORDS: Pronyl-L-lysine; Maillard reaction; protein glycation; chemoprevention; antioxidants; bread; sourdough fermentation; bakery products

INTRODUCTION

During baking of bread dough, a complex cascade of nonenzymatic reactions, the so-called Maillard reaction, is taking place between reducing carbohydrates and proteins and is chiefly responsible for the development of the attractive aroma and the typical brown coloration of the bread crust developing. These flavor attributes are highly desirable and are intimately associated in consumers' minds with a delicious, high-grade product.

Aside from the sensory impact of the browning products, little is known so far about the physiological relevance of these Maillard-type compounds. Maillard products with 3(2*H*)-furanone structures have been reported to possess DNA-damaging properties (*1*, *2*), and very recently, acrylamide, classified as a potential carcinogen, has been shown to be formed by Maillard reactions (*3*, *4*). However, potentially beneficial effects were also reported (e.g., the Maillard-type chromophore 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) (*5*).

Very recently, application of an in vitro antioxidant assay to fractions isolated from bread crust, crumb, and flour revealed the highest antioxidative potential for the dark brown, ethanol solubles of the crust, whereas corresponding crumb and flour fractions showed only minor activity (*6*). In addition, the bread crust, and in particular, the intensely brown colored ethanol solubles of the crust, induced a significantly elevated glutathion-S-transferase (GST) activity and a decreased Phase I NADPH-

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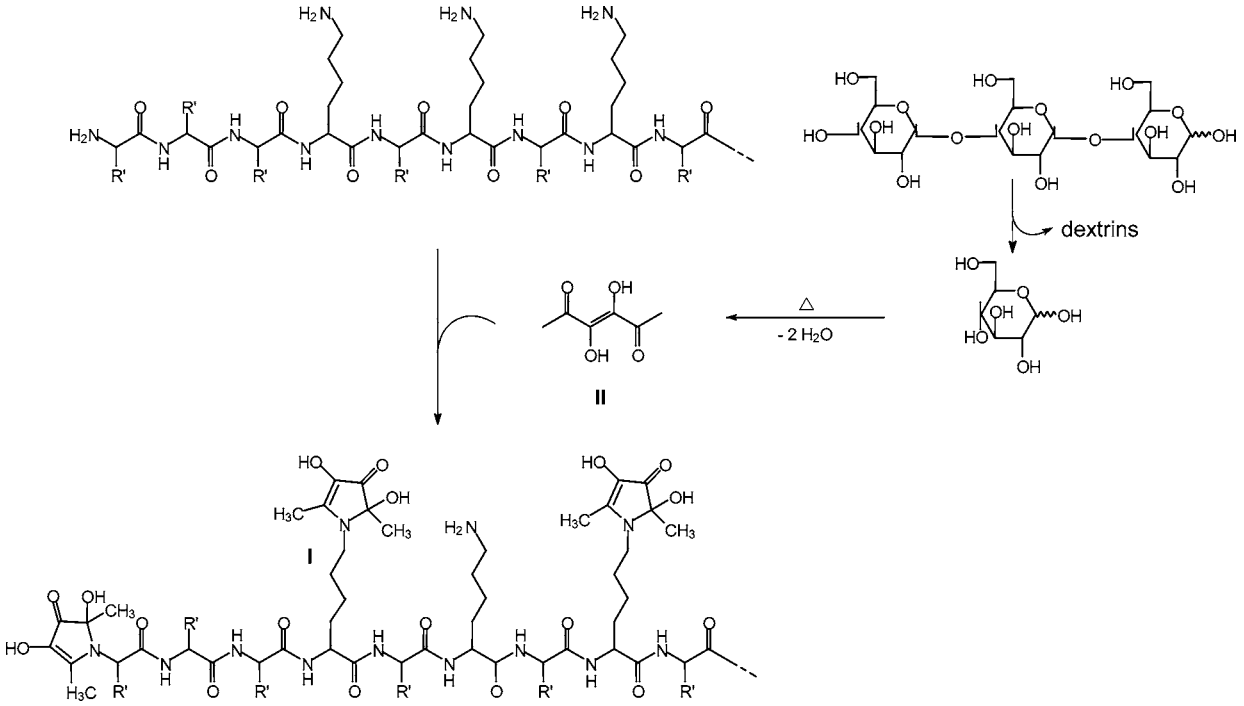

Figure 1. Reaction scheme on the formation of protein-bound pronyl-L-lysine (I) from proteins and starch via the key intermediate acetylformoin (II).

cytochrome *c* reductase activity in intestinal Caco-2 cells. Activity-guided fractionation of bread crust as well as suitable Maillard-type model systems led to the identification of the protein-bound 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2*H*-pyrrol, named pronyl-L-lysine (**I** in **Figure 1**), as a key antioxidant in bread crust (*6*). During baking, glucose is thermally liberated from starch and was found to produce the antioxidant by Maillard reactions via the transient intermediate and penultimate precursor acetylformoin (**II** in **Figure 1**) and lysine side chains or the N-terminus of the flour proteins. These data demonstrated 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. However, neither the amounts of pronyl-L-lysine in various bread products, nor the influence of processing parameters on antioxidant content are known so far. This information is, however, the necessary prerequisite to technologically increasing the amounts of bioactive compounds in foods.

The objectives of the present investigation were, therefore, (i) to quantify the amounts of pronyl-L-lysine in breads produced by modifying baking time and temperature, (ii) to quantitatively study the influence of food-grade supplements on pronyl-L-lysine formation, and (iii) to analyze a selection of commercial bakery products for their pronyl-L-lysine content.

MATERIALS AND METHODS

Materials. The following compounds were obtained commercially: glucose, casein, (Merck, Darmstadt, Germany), trolox (Fluka, Deisenhofen, Germany), and *N*-methylpyridone (Aldrich, Deisenhofen, Germany). All solvents were HPLC grade. The bread samples were purchased from local bakeries.

The antioxidant activity of the melanoidin fractions was determined in vitro by measuring their inhibitory effect on linoleic acid peroxidation closely following the procedure reported recently (*6*).

Self-Made Bread. For preparation of the breads A−E (**Table 1**), a preferment, prepared from rye flour (type 1150, 108 g), starter cultures (Böcker mother, 12 g), and water (96 g), was 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), bakers yeast (43 g), and NaCl (43 g) were kneaded (dough weight, 4839 g), filled into two baking tins (5 cm high), and were 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 introducing the bread into the oven; after 1 min, the water vapor was removed through the discharge pipe for 1 min), the heating regimes used for the baking the breads A−E are detailed in **Table 1**.

Table 1. Baking Conditions Chosen for the Manufacturing of the Self-Prepared Breads A–E and CIE-Lab Data Measured for the Bread Crusts

bread type	baking regime (temp., deg C (time, min))	color values		
		*L**	*a**	*b**
A	260 (8) → 220 (62)	44.5	+14.9	+25.2
B	260 (16) → 220 (124)	32.8	+11.9	+11.0
C	260 (24) → 220 (186)	24.7	+2.6	−0.1
D	280 (8) → 240 (62)	40.9	+15.9	+21.5
E	300 (8) → 260 (62)	31.4	+11.6	+10.6

In addition, the following breads were prepared using the procedure detailed above for bread A, but differed in their recipes: bread I, yeast fermented wheat bread was prepared from wheat flour (type 812, 950 g), water (512 g), bakers yeast (19 g), and NaCl (19 g); bread II, yeast fermented rye/wheat bread was prepared from a dough (pH 5.5) made from rye flour (type 1150, 425 g), wheat flour (type 812, 425 g), water (512 g), bakers yeast (19 g), and NaCl (19 g); bread III, artificially acidified, yeast-fermented rye/wheat bread was prepared from the same dough as reported for bread II, but the pH value was adjusted to 4.0 with an aqueous mixture (9/1) of lactic acid and acetic acid prior to baking; bread IV, sourdough fermented rye/wheat mixed bread was prepared from a dough (pH 4.0) consisting of rye flour (type 1150, 290 g), wheat flour (type 812, 290 g), water (510 g), sourdough (385 g), bakers yeast (12 g), and NaCl (12 g).

Measurement of the CIE-Lab Color Space. Color of bread crusts was measured in CIE Lab space (*Y*) by reflection spectrometry using a spectro color pen (Dr. Lange, Germany). Data are reported as *L**, uniform lightness, and the chromaticness coordinates *a** (+red to −green) and *b** (+yellow to −blue). To achieve this, the spectro color

pen was placed on different places on the bread crusts, and the CIA-Lab color space was analyzed. The results are given as the average of the measurements performed at 10 different places on the crust, and the single measurements were performed in triplicates.

Toasting of Wheat Bread Crumb Slices. Commercial wheat bread was cut into slices (1.5 cm i.d.), and the slices were then toasted using a kitchen toaster (Braun, Germany) to give a medium toasted, golden colored bread slice after 50 s and an intensely roasted, brown colored bread slice after 100 s of thermal treatment.

Precursor Supplemented Bread. Following the procedure described above, various types of bread dough were prepared after substituting 5 and 10% of the rye/wheat flour (1862 g) with casein, respectively, or with 5 and 10% glucose. After introducing the dough into the oven, baking was done for 16 min at 260 °C, followed by 124 min at 220 °C.

Isolation of Ethanol-Soluble Compounds. To separate the brown bread crust from the pale bread crumb, the crust was carefully cut off from the crumb with a kitchen knife. The separated crusts and crumbs were frozen in liquid nitrogen and then ground in a mill. To isolate the ethanol soluble material from breads A−E, the powder obtained from crust and crumb (300 g each) as well as the flour (300 g) were de-fatted by stirring with chloroform (3 × 300 mL), and after filtration, solvent residues were removed in vacuo. After stirring the de-fatted powders with tap water (800 mL) for 3h and filtration, the nonsoluble residue was extracted twice for 3 h at room temperature with an aqueous ethanol solution (60% EtOH in water, 800 mL), yielding the ethanol solubles upon filtration and solvent evaporation in vacuo.

Quantitation of Pronyl-L-lysine. Following the procedure detailed recently (*6*), ground bread crust, crumb, or flour (each 50 g) was de-fatted with chloroform (3 × 100 mL), suspended in water (250 mL), and after addition of methyl hydrazine (5 g), the pH was adjusted to 4.0 using concentrated hydrochloric acid. After incubating the mixture for 45 min at 80 °C, the mixture 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), 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-L-lysine. Each of the experiments was performed in triplicate.

High-Resolution Gas Chromatography/Mass Spectrometry (HRGC/MS). HRGC was performed with a GC 3800 gas chromatograph (Varian, Darmstadt, Germany) equipped with a fused silica capillary CP SIL 19CB (30 m × 0.32 mm, 0.25 μm, 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, Darmstadt, Germany) 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.

RESULTS AND DISCUSSION

To gain first insights into the influence of the baking conditions on the antioxidant potential of rye/wheat bread, five sourdough breads (breads A−E) were freshly baked with variations in temperature and time of thermal treatment (**Table 1**). Breads A to C differed in the baking time (e.g. for bread C, the baking time was three times longer than that used to prepare bread A). In contrast, breads A, D, and E varied in the baking temperature (e.g., the baking temperature of bread E was 40 °C above the temperature used for baking bread A) (**Table 1**). Measurement of CIE lab lightness (L^*) and chroma (a^*, b^*) was performed to monitor the progress in color development during baking. Bread A showed the highest value for lightness, but comparatively high chroma values. Increasing the baking temperature induced a decrease in lightness (L^*) from 44.5 (bread A) to 24.7 (bread C) and a drastic decrease in the chroma values. The same tendency was observed when the baking temperature was increased. It is interesting to note that bread B and bread E, although differing strongly in the baking conditions, showed nearly identical lightness as well as chroma values (**Table 1**).

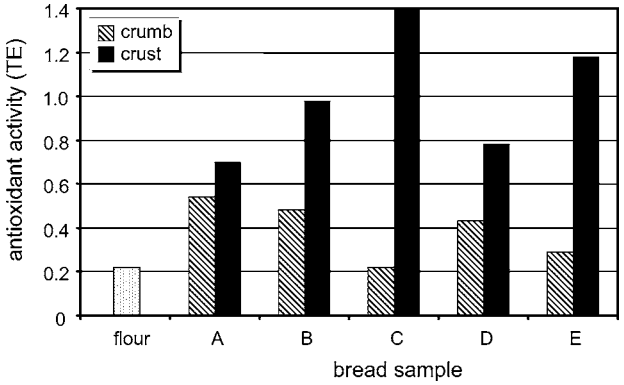

Figure 2. In vitro antioxidative activity of the ethanol fraction isolated from rye/wheat flour as well as crumb and crust of breads A–E.

Aimed at characterizing the antioxidant activity of these bread crusts, the ethanol soluble compounds, which were recently reported as the key contributors to the overall antioxidant activity of bread crust (*6*), were isolated and used for the antioxidant assay. To achieve this, bread crust and crumb have been carefully separated, both materials were frozen in liquid nitrogen, ground in a kitchen mill, and then defatted by chloroform extraction. To remove water-soluble compounds of low antioxidant activity, the powders were extracted with water, and the ethanol soluble browning products were then isolated from the residue by extraction with 60% aqueous ethanol. The ethanolic fractions obtained were freed from solvent in vacuo and were then freeze-dried.

Antioxidant Activity of Ethanol Solubles. To compare their antioxidant potential, the efficiency of the ethanol solubles, isolated from flour, crumb, and crust of the breads A−E, in inhibiting the peroxidation of linoleic acid was measured by means of the antioxidant assay reported recently (*6*). Using Trolox as the reference for a highly active antioxidant, the antioxidative potential was calculated as Trolox equivalents (TE values). As given in **Figure 2**, the antioxidant potential of the ethanolic fractions isolated from the bread crusts exceeded the activities measured for the flour and the corresponding crumb isolates. In particular, the dark brown colored ethanol solubles isolated from the crust of bread C showed the highest activity (e.g., 6-fold higher TE values were measured in comparison to the fraction isolated from flour and the corresponding bread crumb). In addition, these data show a close relationship between the antioxidant activity of the crust and the baking time. For example, bread C, produced by baking the dough for 210 min, showed a TE value of 1.39, which is 2-fold above the antioxidant activity found for bread A, which was thermally treated for 70 min only. Also, the increase in baking temperature was found to accelerate antioxidant production. For example, increasing the temperature from 220 (bread A) to 260 °C (bread E) induced a 40% increase in antioxidant activity (**Figure 2**). It is interesting to note that, in contrast to the crust, the increase of either the baking time or the baking temperature showed a pronounced inhibitory effect on antioxidant formation in the bread crumb.

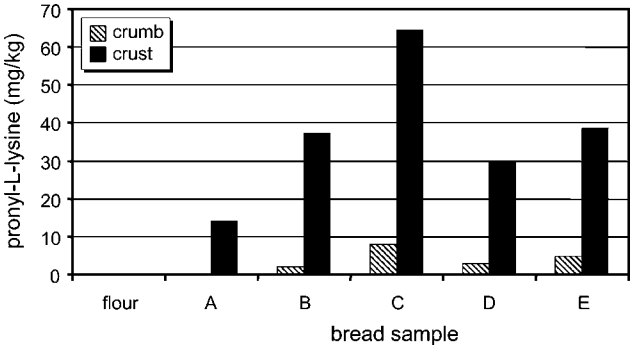

Figure 3. Amounts of protein-bound pyrrolinone reductone (calculated as pronyl-lysine) in flour, crumb, and crust of breads A–E varying in baking time and temperature.

For example, the antioxidant activity of the crumb accounted for 80% of the activity measured for the corresponding crust of bread A, but only about 50 or 15% for bread B and C, respectively (**Figure 2**). These data clearly demonstrate that, during bread baking, the Maillard reaction, occurring more pronounced in the crust, lead to the formation of reaction products with antioxidative potential in vitro.

Quantitation of Pronyl-L-lysine in Bread. To compare the data measured for the overall antioxidant activity of the breads A−E with the amounts of the pyrrolinone reductone pronyl-L-lysine, recently identified as a key antioxidant in bread crusts, we then quantitatively analyzed the amounts of protein-bound pyrrolinone reductone (calculated as pronyl-L-lysine) in flour as well as in crumb and crust of the breads A−E. As given in **Figure 3**, the highest amount of pronyl-L-lysine has been determined in the crust of bread C (e.g., more than 62 mg/kg have been found corresponding to a lysine substitution of 11.24 nmol/mmol protein). In comparison, the pale colored bread crumb contained the pyrrolinone reductone in 8-fold lower amounts, whereas the nontreated flour did not show any significant amounts of pronylated proteins. These data are well in line with the antioxidant activity measured for the crust and crumb of bread C (**Figure 2**). Fitting well with the results of the antioxidant assay, the intensity of thermal treatment has a major impact on the amounts of antioxidants. For example, the amounts of pronyl-L-lysine increased from bread A over B to bread C as well as from bread A over D to bread E (**Figure 3**). Interestingly, breads B and E, although varying strongly in the baking conditions, contained similar amounts of pronyl-L-lysine (**Figure 3**) and showed also identical lightness and chroma values (**Table 1**).

To study whether the pronyl-L-lysine is also formed during toasting processes, freshly cut white bread slices were toasted in a kitchen toaster for 50 or 100 s to give a yellowish-golden or brown coloration, respectively, and the amounts of pronyl-L-lysine were quantified and compared to those detected in the nontoasted control. As given in **Figure 4**, the amounts of the antioxidant increased with toasting time (e.g., the brown colored slices toasted for 100 s contained three times the amount of pronyl-L-lysine as the nonheated control).

Precursor-Spiking Experiments. Recent studies identified protein-bound lysine as one of the precursors in the formation of pronyl-L-lysine (*6*). It might be, therefore, possible that the rather low amount of protein-bound lysine in the gluten (3−4%) is a crucial factor limiting pronyl-L-lysine formation during bread baking. To investigate whether the amount of that antioxidant in bread crust can be increased in the presence of some lysine- rich proteins, we substituted 5 and 10% of the flour by casein containing 8% lysine and quantified pronyl-L-lysine in the corresponding bread crusts. Although the visual appearance of the bread crust differed not significantly from the casein-free control bread, the pronyl-L-lysine content was significantly increased (e.g., the addition of only 5% casein increased the amounts of the antioxidant by more than 200%) (**Figure 5**). Increasing the casein supplement to 10%, however, did not result in a major further acceleration of pronyl-L-lysine production.

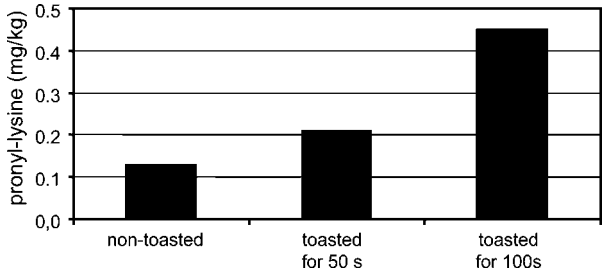

Figure 4. Influence of toasting time on the amounts of pyrrolinone reductone (calculated as pronyl-lysine) in wheat bread slices.

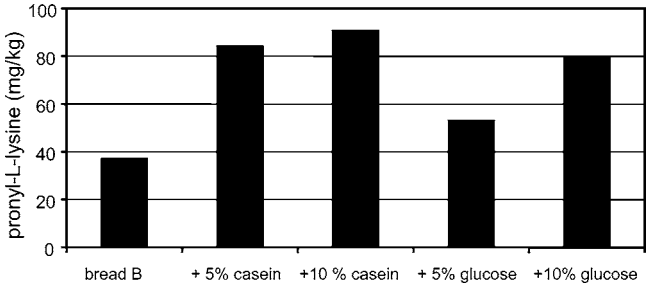

Figure 5. Influence of the supplementation of the dough with casein and glucose, respectively, on the amounts of pyrrolinone reductone (calculated as pronyl-lysine) in bread crust.

In addition to the protein-bound lysine, hexoses thermally liberated from starch were identified to make up the heterocyclic ring of the pronyl-L-lysine during baking. As the amount of free glucose is rather low in the dough, it was studied whether the yields of the antioxidant can be further increased upon supplementing the dough with glucose prior to baking. Therefore, we substituted 5 and 10% of the flour by glucose and quantified the amount of pronyl-L-lysine in the bread crust produced during baking (**Figure 5**). The data clearly demonstrate that the glucose supplement significantly favored pronyl-L-lysine formation (e.g., in the presence of 10% glucose, a similar amount of the antioxidant was detectable as found in the bread containing 5% casein). These experiments unequivocally demonstrate that, in addition to the baking parameters, modifications of the recipe are also helpful to increase the amounts of pronyl-L-lysine in bakery products.

Pronyl-L-lysine in commercial breads. To gain some insight into the amounts of pronyl-L-lysine present in commercial bread samples, the antioxidant was quantified in the crusts of a selection of breads collected from German bakeries. The data, summarized in **Figure 6**, show that the highest amount of 43 mg/kg was found in full grain bread, followed by the rye/wheat bread, both of which are sourdough fermented breads. In comparison, the pumpernickel, which is a crustless full grain bread baked at a low temperature for 20 h, contained about 12 mg/kg pronyl-L-lysine only. Somewhat lower amounts were quantified in a mixed-grain bread as well as in white wheat bread, both of which have not been sourdough fermented (**Figure 6**). Although German pretzels are rich in brown crust color, they contained less than 5 mg/kg, by far the lowest amounts of pronyl-L-lysine (**Figure 6**). As the surface of these

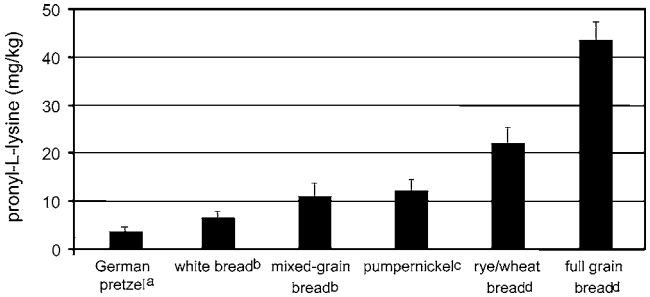

Figure 6. Concentrations of pyrrolinone reductone (calculated as pronyl-lysine) in a selection of commercial breads obtained from the German baking industry: (a) surface was treated with sodium hydroxide solution prior to baking; (b) not sourdough fermented; (c) crustless full grain bread baked for 20 h at low temperatures; (d) sourdough fermented.

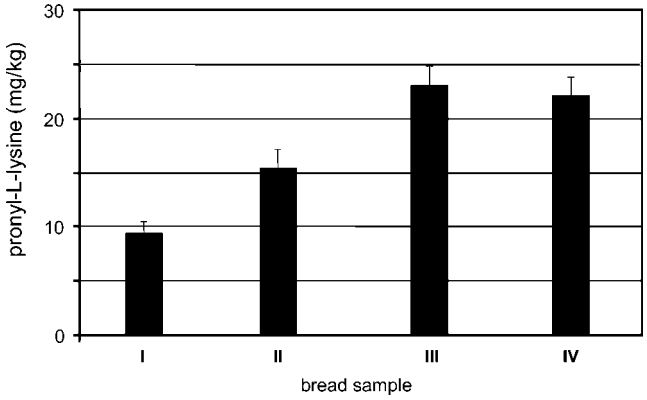

Figure 7. Influence of recipe and dough fermentation on the concentrations of pyrrolinone reductone (calculated as pronyl-lysine) in bread samples: (I) wheat bread, yeast fermented (pH 5.5); (II) rye/wheat bread (1:1), yeast fermented (pH 5.5); (III) rye/wheat bread (1:1), yeast fermented, pH adjusted to 4.0 with a mixture (9:1, v/v) of lactic acid and acetic acid prior to baking, (IV) rye bread, sourdough fermented (pH 4.0).

prezels is treated with a dilute sodium hydroxide solution, these alkaline conditions might be not favorable for the generation of high amounts of pronyl-L-lysine in the brown crust.

To answer the question of why pronyl-L-lysine is favorably produced in sourdough fermented, rye-containing bread, the following set of baking experiments was performed. To compare the precursor activity of wheat and rye flour, first, two yeast-fermented breads were prepared; one containing only wheat flour (bread I in **Figure 7**), the other containing a mixture (1/1, v/v) of wheat and rye flour (bread II in **Figure 7**). Quantification of pronyl-L-lysine revealed that baking of rye/wheat bread II generated 1.7-fold higher amounts of the antioxidant when compared to the bread I containing wheat flour only. Being well in line with the higher concentration of the precursor amino acid L-lysine in rye flour (3.1% of protein) compared to wheat flour (1.8% of protein), these data indicate somewhat higher pronyl-L-lysine generating activity of rye flour compared to wheat flour. In a second set of experiments, an artificially acidified rye/wheat bread (bread III in **Figure 7**) was prepared by adjusting the pH value of the yeast-fermented wheat/rye dough (pH 5.5), which was used for preparing bread II, to the pH value (pH 4.0) of a sourdough fermented rye bread (bread IV in **Figure 7**) using an aqueous mixture (9/1, v/v) of lactic acid and acidic acid. Quantitative analysis of the amounts of pronyl-L-lysine in bread III and IV showed that both samples were nearly equally efficient in producing the antioxidant in the highest concentration, thus demonstrating that the decrease in pH value by microbial acid formation is the clue for the favored pronyl-L-lysine production in sourdough fermented breads.

Taking all these data into consideration, it can be concluded that the amount of the antioxidant and chemopreventive compound pronyl-L-lysine in bakery products is strongly dependent on the manufacturing conditions as well as the recipe. Due to their lower pH-value, sourdough fermented breads were found to contain higher amounts of pronyl-L-lysine than breads that have been yeast fermented only.

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