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Detection and Quantitation of 2,5-Diketopiperazines in Wheat Sourdough and Bread

Liam A. M. Ryan,[†,‡,§] Fabio Dal Bello,[†,‡] Elke K. Arendt,*[,†] and Peter Koehler[§]

[†]Department of Food Science, Food Technology and Nutrition, National University of Ireland, Cork, Ireland, [‡]National Food Biotechnology Centre, National University of Ireland, Cork, Ireland, and [§]Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany

Liquid chromatography mass spectrometry (LC−MS) was used to quantify the levels of the 2,5-diketopiperazines *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) in acidified dough and bread. Dough acidification led to a significant increase in the level of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) over 48 h compared to a nonacidified dough. However, no differences were found between chemically (mix of lactic and acetic acid in the presence of antibiotics) and biologically acidified doughs. On examination of the levels of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) in bread crumb and crust, it was found that temperature is the main causative agent of 2,5-diketopiperazine formation during the baking process. Bread crumb and crust contained almost 100 and 2000 times respectively the levels found in dough prior to baking. *cis*-Cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) were also found to be at sensorally active levels in bread crust, however both 2,5-diketopiperazines were found to be below the minimum inhibitory concentration for antifungal activity in bread.

KEYWORDS: Diketopiperazines; wheat sourdough; bread; antifungal activity

INTRODUCTION

2,5-Diketopiperazines are cyclic dipeptides, and are among the most common peptide derivatives in nature. They are found in a range of foods and beverages including beer and coffee (*1*−*3*). In food systems 2,5-diketopiperazines have been shown to be important sensory compounds (*3*), contributing to the taste of the final products and being perceived as astringent, salty, grainy, metallic or bitter (*2*). 2,5-Diketopiperazines are also found in protein and peptide hydrolysates as well as culture broths of yeasts, lichens and fungi (*4*−*6*). They have been shown to accumulate *in vitro* during thermal and chemical manipulations as well as storage of proteins and peptides (*7*). Formation can also be catalyzed by acids (*8*) and bases (*9*), or by enzymes produced by members of the protist and plant kingdoms (*7*). Furthermore, they have been isolated from broth fermented by lactic acid bacteria, and identified as compounds contributing to the antifungal activity of these bacteria (*12*, *13*). 2,5-Diketopiperazines have been shown to have a variety of effects on the growth and metabolism of fungi, such as inhibition of family C_{18} chitinases (*10*) or aflatoxin production in *Aspergillus parasiticus* (*11*).

We have recently isolated the 2,5-diketopiperazines *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) from the broth of the antifungal strain *Lactobacillus plantarum* FST 1.7 (*14*). This strain was successfully used to produce sourdough which improves the shelf life of wheat bread. The improvement in shelf life was linked to the production/association of 2,5-diketopiperazines by *L. plantarum* FST 1.7, even though the presence of these compounds in the sourdough was not determined. It has also previously been shown that thermal treatment and acidification (*7*), both of which occur during sourdough fermentations and baking, play a role in the formation of 2,5-diketopiperazines. The aim of the present study was to quantify both *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) in acidified wheat dough and bread. Furthermore, LC−MS/MS was used to determine the effects of chemical or biological acidification, presence of divalent metal ions, and thermal treatment on the accumulation of these compounds in dough and bread.

*Corresponding author: Elke Arendt Tel.: +35321 4902064; Fax: +353 214270213 Email: e.arendt @ucc.ie.

MATERIALS AND METHODS

Chemicals. *cis*-Cyclo(L-Leu-L-Pro) (**1**) and *cis*-cyclo(L-Phe-L-Pro) (**2**) (**Figure 1**) were obtained from Bachem (Weil am Rhein, Germany). The linear dipeptides L-leucyl-L-proline (L-Leu-L-Pro) (**4**) and L-phenylalanyl-L-proline (L-Phe-L-Pro) (**5**) (**Figure 1**), as well as lactic acid, acetic acid, pentane, dichloromethane, Dowex 50, carbobenzyloxyglycyl-L-norleucine methyl ester (*Z*-Gly-L-Nle-OCH_3) (**3**) and glycyl-L-norleucine (Gly-L-Nle) (**6**) (**Figure 1**), were all purchased (Sigma Aldrich Steinheim, Germany). Acetonitrile was obtained from (Merck Darmstadt, Germany). The quality of all chemicals was "pro analysi" unless stated otherwise.

Dough Production. The cereal isolate *L. plantarum* FST 1.7 was routinely grown on MRS5 medium (*15*) at 30 °C under microaerophilic conditions. The strain was used to ferment wheat sourdough as described previously (*16*). Briefly, a fresh *L. plantarum* culture was used to inoculate (at 1% level) 80 mL of MRS5 broth and was incubated overnight at 30 °C. Cells were harvested by centrifugation at 4000*g* for 10 min, washed twice and resuspended in 40 mL of sterile tap water (this suspension contained ca. 5×10^9 colony forming units (CFU)/mL). Wheat flour (600 g), sterile

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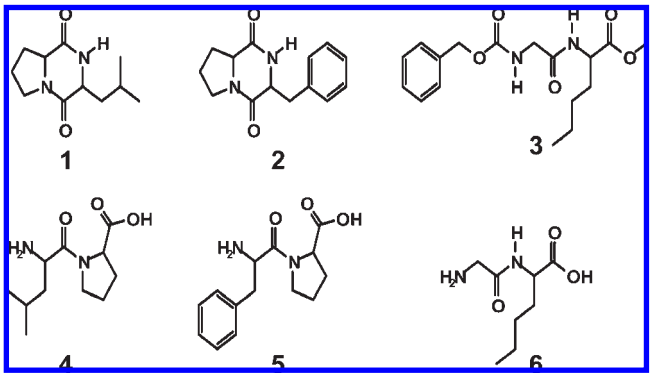

Figure 1. Chemical structures of *cis*-cyclo(L-Leu-L-Pro) (**1**), *cis*-cyclo(L-Phe-L-Pro) (**2**), Z-Gly-L-Nle-OCH_3 (**3**), L-Leu-L-Pro (**4**), L-Phe-L-Pro (**5**), and Gly-L-Nle (**6**).

tap water (599 mL) and cellular suspension (1 mL), giving a dough yield of 200, were mixed to homogeneity for 1 min with a Kenwood mixer (Kenwood, Japan). The dough was incubated at 30 °C for 48 h. Bacterial cell counts, pH and total titrable acid (TTA) were determined as previously described (*16*). Chemically acidified dough was prepared with the addition of antibiotics as well as lactic and acetic acids as previously described (*16*). Acids were added to achieve the same pH and TTA of those measured in the sourdough fermented by *L. plantarum* FST 1.7. To ensure that the antibiotics did not contribute to the formation of 2,5-diketopiperazines, controls were performed in which antibiotic aqueous solutions were set at pH 3.5, 4, 4.5 as well as 5 and incubated for 48 h and the level of diketopiperazines was determined. It has previously been reported that the addition of acidified doughs can result in the release of divalent metal ions from a complex with phytate. In order to evaluate the effect of divalent cations on the formation of 2,5-diketopiperazines, 1 g/kg dough of Ca^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+} in the form of chlorides were added to dough containing antibiotics. After resting for 24 h at 30 °C, the 2,5-diketopiperazines were extracted and quantified as described below. All doughs were added at the 20% (w/w) level to a base bread formulation and bread was baked as previously described by Ryan et al. (*16*).

Isolation of Dipeptides from Dough and Bread. The levels of **1** and **2** as well as of the linear dipeptides **4** and **5** were determined using two internal standards displaying structural and behavioral homology to these dipeptides. The compound **6** was chosen as the internal standard for the linear dipeptides **4** and **5**, whereas **3** was chosen for **1** and **2**.

The extraction from sourdough and bread was performed according to Stark and Hoffmann (*3*), with the following modifications. Sourdough samples (100 g) were collected after 0, 11, 24, 30, 35, and 48 h of fermentation. Samples were frozen using liquid nitrogen, freeze-dried and then crushed using a pestle and mortar. Bread samples were separated into crust and crumb before being analyzed because a substantial variation of the dipeptide levels was expected. After lyophilization the crumb and crust samples (50 g each) were blitzed in a food processor (Kenwood, Japan) to reduce the particle size and increase the efficiency of extraction. The pretreated dough/bread (50 g of dough and 30 g of bread) samples were spiked with 50 μg of **3** and **6** (previously dissolved in 100 μL of 50% acetonitrile) and allowed to equilibrate for 1 h. The mixture was defatted with *n*-pentane (5 × 300 mL) at room temperature for 30 min. After centrifugation, the residual material was extracted 5 times with acetone/water (70/30, v/v; 300 mL each) for 45 min at room temperature, while stirring. The samples were centrifuged, and the combined supernatants were dried under reduced pressure at 30 °C. The residue was dissolved in 50% (v/v) acetonitrile (150 mL), and 50 g of anhydrous sodium sulfate was added. After filtration, the acetonitrile was removed under reduced pressure at 30 °C, and the aqueous solution obtained was extracted 5 times with 150 mL of dichloromethane. The dichloromethane phases were then dried under reduced pressure to give the dichloromethane extractables. The dichloromethane extract was suspended in aqueous hydrochloric acid (0.001 mol/L in water; 5 mL) and applied on top of a glass column filled with a slurry of Dowex 50WX 8, 50−100 mesh, which was preconditioned with 0.1 mol/L aqueous hydrochloric acid (500 mL), followed by 0.001 mol/L aqueous hydrochloric acid (500 mL). Elution was carried out using 0.001 mol/L aqueous hydrochloric acid, and the eluate was collected and freeze-dried. The residue was then dissolved in 10 mL of 50% (v/v) acetonitrile and analyzed by means of LC−MS/MS.

Table 1. *m/z* Signals for the Protonated Molecular Ions and the Most Intensive Fragments after MS/MS Used for the Quantitation of Dipeptides in Dough and Bread

compound	*m/z* of $(M + H)^+$	*m/z* of mass transitions used for single reaction monitoring
L-Leu-L-Pro (**4**)	229	70, 183
L-Phe-L-Pro (**5**)	263	116, 120
cis-cyclo(L-Leu-L-Pro) (**1**)	211	70, 183
cis-cyclo(L-Phe-L-Pro) (**2**)	245	70, 120
Gly-L-Nle[a] (**6**)	189	132, 143
Z-Gly-L-Nle-OCH_3[a] (**3**)	337	120, 146

[a] Internal standards.

Quantitation of 2,5-Diketopiperazines Using LC−MS/MS. Mass spectra were recorded by means of a triple-quadrupole tandem mass spectrometer (TSQ Quantum Discovery, Thermo Electron, Dreieich, Germany) coupled to a Surveyor high performance liquid chromatography system (Thermo Electron) equipped with a thermostated (20 °C) autosampler and a 150 × 2.0 mm i.d., 4 μm, 8 nm Synergi Hydro RP-HPLC column (Phenomenex, Aschaffenburg, Germany) kept at 30 °C connected to a 4 × 2.0 mm i.d. guard column of the same material (Phenomenex). The solvent system was composed of formic acid in water (0.1%, v/v; A) and formic acid in acetonitrile (0.1%, v/v; B). Starting with aqueous formic acid (0.1% in water, pH 2.5) at a flow rate of 0.2 mL/min for 10 min, the acetonitrile content was then increased to 60% within 40 min, and again increased to 100% within 10 min, and finally, held at 100% for 10 min. The mass spectrometer was operated in the positive electrospray ionization mode (ESI^+) with a spray needle voltage of 4.0 kV and a spray current of 5 μA. The temperature of the capillary was 300 °C, and the sheath and auxiliary gas (nitrogen) were adjusted to 35 and 10 arbitrary units, respectively. The collision cell was operated at a collision gas (argon) pressure of 0.13 Pa.

The dipeptides **1**, **2**, **4**, and **5**, as well as the internal standards **3** and **6**, were first characterized by means of their molecular masses obtained in the full scan mode. The molecular ions obtained as base peaks ($M^+ + 1$) are shown in **Table 1**. These were then subjected to MS/MS (collision energy = 20 V). Initially, the most intense transitions of the precursor ions were determined (**Table 1**). The yields of the product ions were optimized by performing a series of runs with different collision energies and flow rates of the sheath and auxiliary gas. The mass transitions given in **Table 1** were selected for quantitation. An example of the molecular weight and fragmentation patterns for compounds **1** and **2** is given in **Figure 2**. On both mass filter quadrupoles, resolution settings were 0.7 full width at half-maximum, scan time for each transition and single reaction monitoring (SRM) was 0.50 s, scan width was 0.7 amu, and source CID was 12 V.

The response factor (mass to signal intensity ratio) of all compounds quantified was calculated by analyzing mixtures of the dipeptides and internal standards in defined molar ratios (0.133:1 to 1:1) (**Figure 3**). The response factor was calculated as previously described (*17*). To ensure that both internal standards and dipeptides were extracted to the same extent in dough and bread, known levels of each compound were added to previously extracted dough and analyzed as described above.

Screening for Further 2,5-Diketopiperazines in Sourdough. Besides the compounds **1** and **2**, the wheat dough was screened for the presence of additional 2,5-diketopiperazines. Screening of a range of different 2,5-diketopiperazines was performed using the molecular weights and fragmentation patterns previously reported (*3*). To ensure the accuracy of detection and identification, the compatibility of the published data with that generated in our study was confirmed with respect to molecular weight and fragmentation behavior. This involved the direct injection and detection of the molecular weight and fragmentation patterns of **1** and **2** and comparison of these values with those previously reported (*3*). Once the compatibility of both LC−MS/MS methods was established, the dough was screened for the presence of an additional 26 diketopiperazines (**Table 2**).

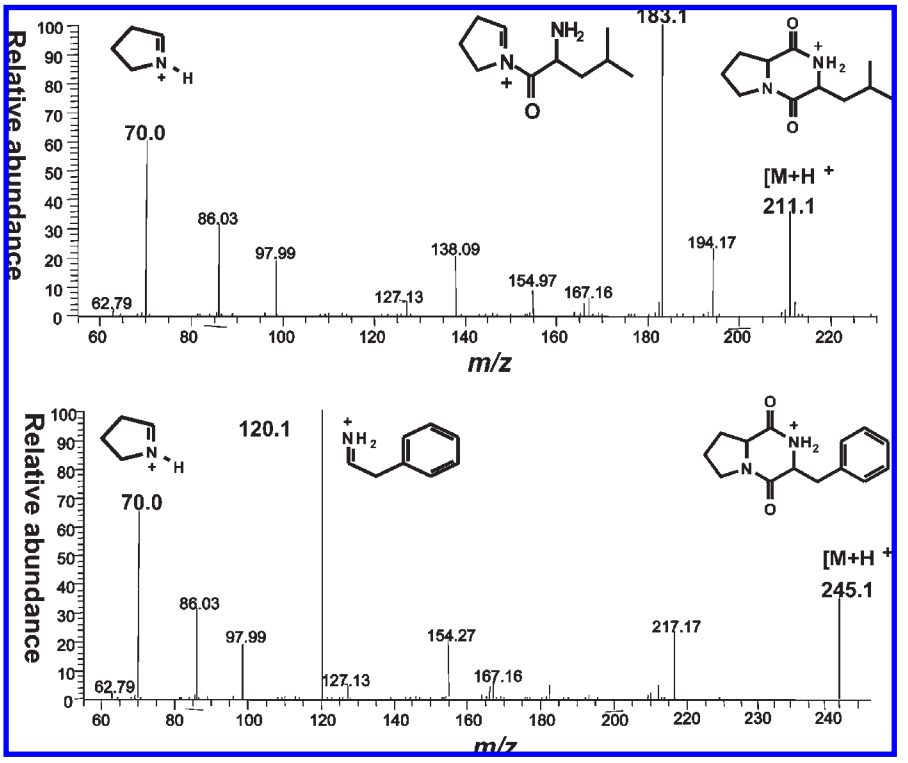

Figure 2. ESI-MS/MS spectrum of *cis*-cyclo(L-Leu-L-Pro) (top) and *cis*-cyclo(L-Phe-L-Pro) (bottom).

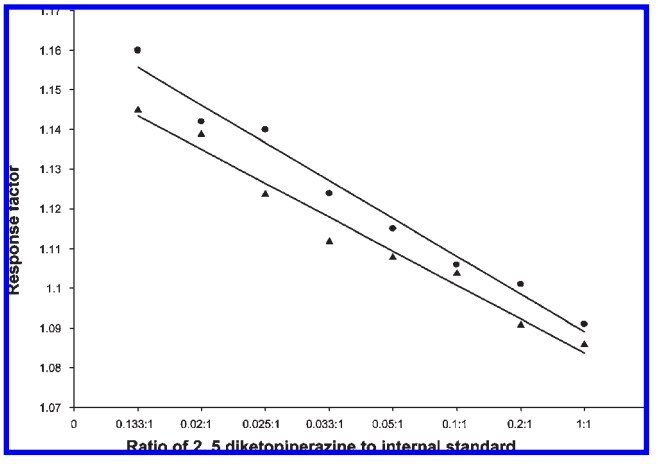

Figure 3. Calibration curve of mixtures of *cis*-cyclo(L-Leu-L-Pro) (circles) and *cis*-cyclo(L-Phe-L-Pro) (triangles). The R^2 value for both lines was approximately 97%.

Statistical Analysis. Statistical analyses were performed using SPSS 12.0 for Windows computerized statistical analysis package (SPSS Inc., Chicago, IL). Data were examined using one-way analysis of variance (ANOVA). Where an *F*-test showed significant differences ($p < 0.05$), Tukey's honesty significant difference (HSD) test was used for multiple comparisons. Each result is the average of 3 separate experiments with 3 independent samples from each batch.

RESULTS AND DISCUSSION

Recent advances in analytical chemistry have allowed the detection of previously unknown compounds or compounds present at very low levels (*18*, *19*), which might affect functional and sensory properties of food systems. By using the LC−MS/MS protocol described in this study we have detected and quantified the 2,5-diketopiperazines **1** and **2** in wheat dough and bread. We have shown that both compounds are present in low levels in wheat dough, but that acidification and/or the presence of divalent metal ions significantly increase their formation. Furthermore, thermal treatment occurring during baking dramatically increases the levels of both 2,5-diketopiperazines.

Table 2. Molecular Weight and Most Intense Fragments after MS/MS Used for the Screening of 2,5-Diketopiperazines in Dough and Bread

2,5-diketopiperazines	*m/z* of $(M + H)^+$	*m/z* of most intensive fragments
cis-cyclo(L-Leu-L-Pro)[a] (**1**)	211	70, 183
cis-cyclo(L-Phe-L-Pro)[a] (**2**)	245	70, 120
cis-cyclo(L-Val-L-Pro)	197	70, 124
cis-cyclo(L-Ala-Gly)	129	72, 84
cis-cyclo(L-Ala-L-Ala)	143	72, 98
cis-cyclo(L-Ala-L-Pro)	169	70, 169
cis-cyclo(Gly-L-Pro)	155	70, 155
cis-cyclo(L-Pro-L-Thr)	199	70, 153
cis-cyclo(L-Val-L-Val)	199	72, 126
cis-cyclo(L-Phe-L-Ser)	235	120, 162
cis-cyclo(L-Ala-L-Tyr)	235	107, 135
cis-cyclo(L-Asp-L-Phe)	263	175, 203
cis-cyclo(L-Val-L-Tyr)	263	136, 235
cis-cyclo(L-Gly-L-Phe)	205	120, 132
cis-cyclo(L-Val-L-Leu)	213	72, 86
cis-cyclo(L-Val-L-Phe)	247	120, 174
cis-cyclo(L-Ala-L-Leu)	185	86, 140
cis-cyclo(L-Ala-L-Phe)	219	120, 146
cis-cyclo(L-Leu-L-Phe)	261	86, 120
cis-cyclo(L-Leu-Gly)	171	72, 86
cis-cyclo(L-Gly-Gly)	115	58, 87
cis-cyclo(L-Ala-L-Val)	171	72, 98
cis-cyclo(L-Pro-L-Tyr)	261	46, 136
cis-cyclo(L-Asn-L-Phe)	262	203, 245
cis-cyclo(L-Ala-L-Ile)	185	86, 140
cis-cyclo(L-Ile-L-Phe)	261	86, 120
cis-cyclo(L-Ile-L-Pro)	211	70, 183
cis-cyclo(L-Pro-L-Pro)	195	70, 98

[a] Used to confirm that LC−MS/MS molecular weight and fragmentation patterns were consistent with those previously reported by Stark and Hoffmann (*3*).

Screening for an additional 26 food-relevant 2,5-diketopiperazines revealed that the number and type of these compounds present in dough and bread is higher than previously recognized.

Quantitation of 2,5-Diketopiperazines Using LC−MS/MS. The levels of **1** and **2** in sourdough and bread were determined using LC−MS/MS. The compounds **3** and **6** were chosen as internal standards because of their structural similarity to the selected dipeptides, and because they are not naturally present in wheat dough. Furthermore, these compounds were found to be extracted from sourdough and bread to the same extent as the targeted dipeptides, and their levels were below the limit of detection in the residual dough after the extraction procedure (data not shown). The elution times of the linear peptides **4**, **5** and **6** were 16.8, 2.8, and 2.9 min, respectively. The 2,5-diketopiperazines **1** and **2** as well as the internal standard **3** eluted after 18.0, 18.5, and 21.5 min, respectively. The molecular weights and most intensive ions after MS/MS fragmentation of the precursor ions are shown in **Table 1** and **Figure 2**. The 2,5-diketopiperazines showed similar fragmentation patterns, but different intensities of the fragments. Compound **1** yielded intense fragments with m/z 70 and m/z 183, the latter being the product of a fragmentation of one peptide bond with subsequent elimination of CO. A possible structure of this ion is given in **Figure 2**. The fragment at m/z 70 represented the immonium ion of the proline part of **1** after formation of an ion and subsequent CO and H_2 elimination. This ion had also been formed after fragmentation of the molecular ion of **2**. In addition, **2** yielded a second immonium ion at m/z 120 in high intensity originating from the phenylalanine part of the molecule.

The ions given in **Table 1** were used for the quantitation of **1** and **2**. The response factors (mass to area ratios) obtained with distinct dipeptide/internal standard mixtures were determined (**Figure 3**) and used to calculate the amounts of the compounds from their area counts.

Concentration of Linear and Cyclic Dipeptides in Wheat Dough. The 2,5-diketopiperazines **1** and **2** were recently isolated from broth fermented by the antifungal strain *L. plantarum* FST 1.7 (*14*). As addition of sourdough fermented by this strain increased the shelf life of bread, it was speculated that these compounds may be produced by the *Lactobacillus* strain during fermentation, and thus contribute to the antifungal activity of the sourdough. An LC−MS/MS protocol was used to determine the level of accumulation of the selected dipeptides during biological or chemical acidification of wheat dough as well as in nonacidified (control) dough (**Figure 4**). Over the 48 h of fermentation, the amounts of **1** and **2** did not significantly differ between biologically or chemically acidified samples (data not shown). The levels of **1** and **2** increased significantly ($p < 0.05$) from approximately 1 to 35 and 25 μg/kg of dough, respectively, after 48 h of incubation. Compared to the level measured in the control dough, acidification resulted in an increase by a factor of 17 and 8 in the accumulation of **1** and **2**, respectively (**Figure 4**). In the control dough, incubation for 48 h resulted only in a slight increase in the 2,5-diketopiperazine levels. The presence of antibiotics in the dough did not significantly contribute to the accumulation of the 2,5-diketopiperazines (data not shown). Additionally, the levels of the linear dipeptides **4** and **5** were also determined. Biological or chemical acidification did not significantly ($p < 0.05$) affect the level of linear dipeptides in dough compared to the control dough, and the levels of both **4** and **5** remained constant over time, and were found to be around 0.2 μg/kg of dough in all investigated doughs.

By using the developed LC−MS/MS protocol, we showed that these compounds are indeed present in sourdough fermented by *L. plantarum* FST 1.7. However, comparable amounts of these 2,5-diketopiperazines were detected in chemical acidified doughs, thus indicating that the acidification itself, and not the metabolic activity of *L. plantarum*, is the key factor responsible for the formation of these compounds. These results suggest that these compounds could be produced in sourdough fermented by a range of LAB as well as during traditional sourdough and sourdough bread production.

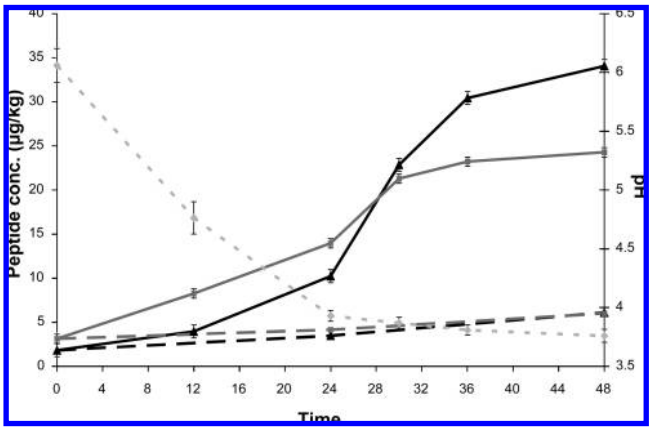

Figure 4. Accumulation of *cis*-cyclo(L-Leu-L-Pro) (black line) and *cis*-cyclo(L-Phe-L-Pro) (gray line) in biological acidified dough (continuous line) and nonacidified dough (dashed line) over 48 h of incubation. The pH curve in the acidified dough is represented by a light gray dashed line.

Interestingly the rate of formation of the 2,5-diketopiperazines was not constant throughout the incubation, and a significant increase in accumulation was observed when the pH of the dough dropped below 4 (**Figure 4**). Acidification of wheat dough to these pH values triggers a series of reactions, among which proteolytic activity by endogenous enzymes is one of the key processes (*20*, *21*). Peptidases with optimum activity at acidic pH may release dipeptides, which can be considered as precursors of 2,5-diketopiperazines. Taken together, these results indicate that the effect of acids on 2,5-diketopiperazine formation is 2-fold, providing substrate, in the form of dipeptides released by the increased enzymatic activity; and favoring cyclization as acidic conditions lead to the protonation of free carboxyl groups, thus, promoting the release of water and the formation of new intramolecular peptide bonds.

Therefore, as it was ruled out that the presence of these compounds is due to a specific activity, we hypothesized that other similar compounds could also be present in acidified doughs. Beside **1** and **2**, acidified dough and bread were screened for the presence of 26 additional 2,5-diketopiperazines, which have been previously reported in food systems (*3*). The finding that the fragmentation patterns of **1** and **2** obtained with our LC−MS protocol were identical to those previously reported (*3*) allowed us to screen for 26 additional 2,5-diketopiperazines using the mass transitions reported in **Table 2**. All of the different 2,5-diketopiperazines were present in acidified and nonacidified doughs and breads.

In addition, acidification of doughs causes the release of metal ions due to an increased activity of endogenous phytases (*22*, *23*). To determine if the presence of divalent metal ions can affect their formation, ions were added to wheat dough and the levels of 2,5-diketopiperazines accumulating were determined. Results confirmed that the presence of divalent metal ions in wheat dough contributes significantly to the increased accumulation of 2,5-diketopiperazines in acidified doughs. Independently from the considered 2,5-diketopiperazine, addition of ions to the dough resulted in a significant increase ($p < 0.05$) in the levels of accumulation. The effect of individual ions was, however, not determined. After addition of ions, the level of **1** increased significantly ($p < 0.05$) from 3.5 to 8.3 μg/kg dough, whereas **2** increased from 4.2 to 6.3 μg/kg dough. The presence of the ions

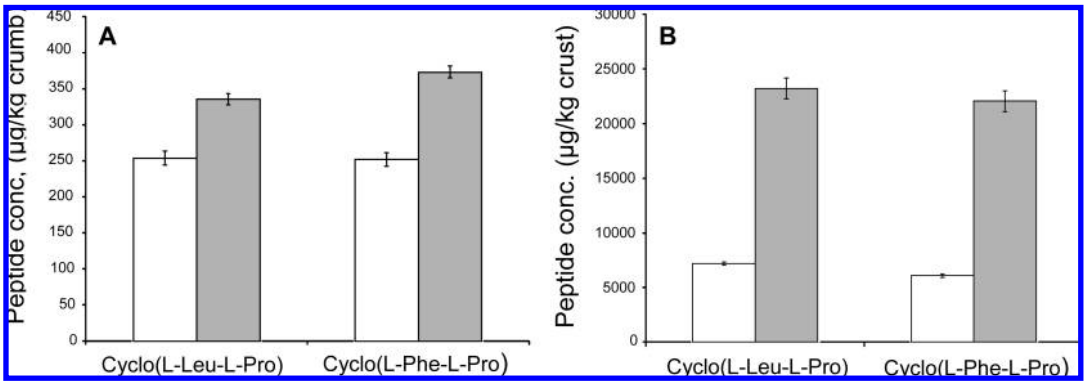

Figure 5. *cis*-Cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) levels quantified in bread crumb (**A**) and bread crust (**B**). Black bars, bread baked with nonacidified dough; gray bars, bread baked with 20% addition of dough acidified for 48 h by *L. plantarum* FST 1.7. Levels reported are those present in the fresh sample.

did not significantly affect the levels of the linear dipeptides **4** and **5** present in the samples.

Quantitation of Selected Dipeptides in Bread. Since the formation of 2,5-diketopiperazines is affected by heating processes (*7*), we investigated the levels of **1** and **2** in bread. Bread crumb was separated from the crust, and the levels of 2,5-diketopiperazines were determined individually. Bread was baked using nonacidified dough (control) as well as with the addition of 20% biologically acidified dough.

The levels of 2,5-diketopiperazines measured in bread crumb are shown in **Figure 5A**. In the nonacidified bread crumb, **1** and **2** reached a level of approximately 250 μg/kg of bread crumb. Addition of biologically acidified dough resulted in a significant increase ($p < 0.05$) in the amount of 2,5-diketopiperazines, and **1** and **2** were detected at levels of 336 and 373 μg/kg of bread crumb, respectively. Addition of acidified dough did not significantly affect ($p < 0.05$) the levels of linear dipeptides, and in both acidified and nonacidified breads levels of 3.5 and 4.6 μg/kg of bread crumb were measured for **4** and **5**, respectively.

The levels of 2,5-diketopiperazines were also measured in bread crust (**Figure 5B**). Independently from the bread evaluated, significantly higher levels ($p < 0.05$) of 2,5-diketopiperazines were measured in bread crust, when compared to bread crumb. In the crust of nonacidified bread, the levels of **1** and **2** reached 7.2 and 6.1 mg/kg of bread crust, respectively. The levels of **1** and **2** in the crust of bread baked using the acidified dough increased to 23.0 and 22.1 mg/kg of bread crust, respectively. Baking significantly ($p < 0.05$) affected the level of linear dipeptides, and higher levels were measured in the crust when compared to the crumb. The levels of **4** in the crust of nonacidified and biologically acidified bread were 11.5 and 75.2 μg/kg, respectively. The linear dipeptide **5** was detected at higher levels, i.e. 24.7 and 325.0 μg/kg of bread crust in nonacidified and acidified bread, respectively.

A database search showed that both dipeptide motifs **4** and **5** occur in wheat gluten proteins with motif **5** occurring more frequently than motif **4** (**Table 3**). Thus, formation of linear dipeptides by suitable enzymatic activities is possible during fermentation as well as acid-catalyzed formation in the crust during baking. Assuming a gliadin/glutenin ratio of 1.5:1 (by weight) in typical wheat flour it is obvious that the gliadin fraction is the main source of **4** and **5**. Within the glutenin fraction a ratio of HMW/LMW subunits of approximately 1/4 is present indicating that HMW subunits are the type of gluten proteins that have the lowest contribution to the formation of these dipeptides. Interestingly both dipeptides have a C-terminal proline, with the liberation of such peptides by enzymatic proteolysis requiring proline-specific enzymes. A small number of prolyl peptidases have been identified in wheat so far, and their action requires prehydrolysis by other peptidases. However, even when only low enzyme activities are present in wheat flour, extensive hydrolysis of gluten proteins might occur during sourdough fermentation because of the long incubation time of 48 h providing the linear dipeptides as precursors of 2,5-diketopiperazines. However, the results suggest that the actions of peptidases are not the only contributor to the formation. It can be assumed that, in particular during heating, acidic pH caused hydrolysis and cyclization of dipeptides. As the cyclic forms are more stable than the linear forms, the latter are only transient intermediates, which are not accumulated during processing.

Table 3. Maximum Occurrences of L-Leu-L-Pro (**4**) and L-Phe-L-Pro (**5**) in the Amino Acid Sequence of Wheat Gluten Protein Types

	gluten protein type			
peptide	α-gliadins	γ-gliadins	LMW subunits	HMW subunits
L-Leu-L-Pro (**4**)	3	3	6	2
L-Phe-L-Pro (**5**)	5	12	3	1

The 2,5-diketopiperazines **1** and **2** have been reported as important antifungal and organoleptically active compounds (*3*). The minimal inhibitory concentration against common bread spoilage fungi for both compounds is approximately 20 mg/g (*13*, *16*). This value is about 1000-fold higher than the highest level detected in our study, i.e. acidified bread crust containing 25 mg/kg, thus indicating that both 2,5-diketopiperazines play only a marginal role in the preservation of bread. On the contrary, taste thresholds for the metallic and bitter flavors have been reported of about 100 and 1000 μmol/L, respectively (*3*). In our study, the highest levels of the 2,5-diketopiperazines detected in acidified bread crust correspond to ca. 100 μmol/kg. Thus, these results indicate that both compounds impart a metallic flavor, and contribute to the bitter flavor associated with the crust of bread. Based on the intensities of the signal detected during LC−MS/MS experiments, it was found that all other 2,5-dipetopiperazines are present at levels similar to those of **1** and **2**. In conclusion, at the levels and varieties in which they are present diketopiperazines may contribute to the sensory attributes, and possibly the shelf life of acidified breads.

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