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The role of free amino acids present in yeast as precursors of the odorants 2-acetyl-1-pyrroline and 2-acetyltetrahydropyridine in wheat bread crust

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Zur Rolle freier Aminosäuren der Hefe als Vorläufer der Weißbrotkrustenaromastoffe 2-Acetyl-1-pyrrolin und 2-Acetyltetrahydropyridin

Zusammenfassung. Wir konnten kürzlich zeigen, daß die Bäckerhefe eine entscheidende Quelle von Vorläufern zur Bildung der Röstaromastoffe 2-Acetyl-1-pyrrolin (ACPY) und 2-Acetyltetrahydropyridin (ACTPY) in der Weißbrotkruste ist. Um die Rolle freier Aminosäuren der Hefe für die Bildung beider Geruchsstoffe zu untersuchen, wurden die Konzentrationen der in Hefe vorliegenden Aminosäuren bestimmt. Elf Aminosäuren, deren Konzentrationen 60 mg/100 g Trockenhefe überstiegen, wurden in Modellversuchen mit 2-Oxopropanal umgesetzt und die freigesetzten Mengen von ACPY und ACTPY über eine Isotopenverdünnungsanalyse bestimmt. ACPY wurde sowohl aus Prolin als auch aus Ornithin freigesetzt, während ACTPY ausschließlich aus Prolin entstand. Aus den übrigen Aminosäuren wurden die beiden Aromastoffe nicht gebildet. Weitere Versuche ergaben, daß die Bildung von ACPY aus Ornithin über 4-Aminobutyraldehyd und 1-Pyrrolin als Intermediate erfolgt. Die Menge an freiem Ornithin in der Hefe war mehr als dreimal so groß wie die des freien Prolins. Weiterhin erhöhten Zusätze von Prolin bzw. Ornithin zu Weizenteigen die Konzentrationen von ACPY in der Kruste um den Faktor 2 bzw. 4. Die Daten ließen den Schluß zu, daß Ornithin der wichtigste Vorläufer zur Bildung von ACPY beim Backen ist.

Summary. Recently we established bakers' yeast as a potent source of precursors for the roast-smelling odorants 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) in wheat bread crust. To reveal their role in the formation of both odorants, the concentrations of free amino acids occurring in baker's yeast were determined. The 11 amino acids present in concentrations above 60 mg/100 g dry yeast were separately reacted with 2-oxopropanal in model solutions and the amounts of ACPY and ACTPY formed, determined by a stable isotope dilution assay (SIDA). ACPY was formed from proline and ornithine, while ACPTY was exclusively liberated from proline. The remaining amino acids were ineffective. Further experiments revealed that the formation of ACPY from ornithine proceeds via 4-aminobutyraldehyde and 1-pyrroline as intermediates. The amount of free ornithine in yeast was more than three times the amount of free proline. Furthermore, additions of either proline or ornithine to wheat doughs enhanced the amounts of ACPY in the bread crust by a factor of two or four, respectively. The data led to the conclusion that ornithine is the most important precursor for the formation of ACPY during baking.

Introduction

In previous investigations [1–3] 2-acetyl-1-pyrroline (ACPY) was established as the most important roast-smelling odorant of wheat bread crust. Recently it was shown (unpublished results) that 2-acetyltetrahydropyridine (ACTPY), identified in wheat bread crust by Hunter et al. [4], also contributed with a somewhat lower odor unit (ratio of concentration to odor threshold) to the roasting odor of wheat bread crust. In the crust of a model bread prepared from a dough without yeast addition [5], the odor unit of both flavor compounds was significantly lowered compared to the crust from a yeasted dough. Further experiments [5] underlined the important role of bakers' yeast as a source of the precursors in the formation of ACPY and ACTPY: boiling a fraction of water-soluble, low molecular mass compounds prepared from disrupted bakers' yeast resulted in significant amounts of both odorants.

ACPY and ACTPY were identified as minor reaction products in thermally degraded proline/carbohydrate model mixtures [6]. In quantitative studies on ACPY and ACTPY formation, we recently corroborated [5, 7, 8] the outstanding role of proline as precursor of both flavor

compounds in bread crust. The data revealed that the reaction between proline and dihydroxyacetone phosphate (or its thermal degradation product 2-oxopropanal [5] inside the yeast cells is a key step in the formation of ACPY and ACTPY during baking. ACPY was shown to be formed via 1-pyrroline as intermediate [8].

Relatively high levels of free amino acids are present in bakers' yeast [9]. Besides proline, the rare amino acids 4-aminobutyric acid and ornithine were identified [9]. Recently it was shown [10] that cultivation of yeast in the presence of sodium chloride significantly increased the amounts of intracellular free basic amino acids (e.g. ornithine, citrulline, arginine).

The following investigations were undertaken to reveal the role of free amino acids present in yeast in the formation of ACPY and ACTPY during baking.

Experimental procedures

Materials. Bakers' yeast (water content 69%) and wheat flour (type 550) were purchased from a local bakery. The following compounds were obtained commercially: L-ornithine HCl, L-arginine, L-lysine and L-proline (Sigma, Munich, FRG). 2-oxopropanal (methylglyoxal; 40% solution in water) and 4-aminobutyraldehyde diethylacetal (Aldrich, Steinheim, FRG). 4-Aminobutyraldehyde was liberated from its diethylacetal by boiling in 5% aqueous H_2SO_4 and, after neutralization with 20% aqueous sodium hydroxide, used immediately. Deuterated 2-acetyl-1-pyrroline ([^{2}H]-ACPY) was synthesized as described in [2]; the synthesis of deuterated 2-acetyltetrahydropyridine ([^{2}H]-ACTPY) will be reported shortly (unpublished results). Solvents were purified according to [11].

Preparation of 1-pyrroline. 1-Pyrroline was prepared as described in [12] using some modifications: proline (2.5 g), dissolved in 50 ml water, was added to 70 ml of an aqueous solution of sodium metaperiodate (0.3 mol/L) and the mixture stirred for 2 h in the dark at room temperature. The pH was adjusted to 10.5 with aqueous sodium hydroxide (80 g/L) and the solution, after addition of 25 g NaCl, extracted repeatedly with a total volume of 120 ml diethyl ether. The extract was washed with 50 ml of a saturated aqueous solution of NaCl and, after drying over anhydrous Na_2SO_4, concentrated to 2 ml on a Vigreux column (60 cm × 1 cm) at 37° C. The concentrate was placed on the top of two water-cooled glass columns (20 cm × 1 cm), packed with a slurry of neutral alumina (deactivated with 10% water, by mass) in pentane. After flushing with 50 ml pentane, the 1-pyrroline was eluted with 100 ml pentane/diethyl ether (1+1, by vol.). The pyrroline was characterized by its MS/EI [*m/z* (%): 41 (100); 69 (68); 42 (40); 68 (30)] which is in agreement with data reported in the literature [13]. The amount of 1-pyrroline was calculated gas chromatographically using pyrimidine as internal standard.

Determination of free amino acids in yeast. A slurry of 30 g yeast, 110 g glass powder (0.15–0.30 mm, Roth, Karlsruhe, FRG) and 7 ml phosphate buffer (0.1 mol/L; pH 7.0) was treated for 15 min in a cell mill (Bühler, Switzerland). The resulting suspension was filtered (black ribbon paper filter; Schleicher-Schüll, Dassel, FRG), centrifuged for 30 min (4° C) at 18000 rpm (40000 × *g*) and the supernatant filtered over an ultrafiltration membrane (Diaflo ultrafilter type YM 2:*M* cut off 1000; Amicon, Witten, FRG). In the filtrate the amino acids were determined using post-column derivatization with ninhydrin (amino acid analyzer type 5001; Biotronic, Munich, FRG). Identification and calibration was performed using reference amino acids.

Model reactions. Each of the amino acids given in Table 1 was dissolved in 100 ml phosphate buffer (0.1 mol/L; pH 7.0) containing 2-oxopropanal (0.1 mmol) and then boiled at backflush for 2 h. After cooling, each solution was spiked with 20 µg [^{2}H]-2-acetyl-1-pyrroline and 30 µg [^{2}H]-2-acetyl-tetrahydropyridine, dissolved in 1 ml diethyl ether, and extracted repeatedly with a total volume of 140 ml diethyl ether. The organic layer was washed with 100 ml saturated NaCl solution and, after drying over anhydrous Na_2SO_4, concentrated to 250 µl by microdistillation [11]. In another series of experiments (cf. Table 2) 250 µmol 1-pyrroline, 4-aminobutyraldehyde or 4-aminobutyric acid were reacted with 250 µmol 2-oxopropanal, and the ACPY formed was isolated as described above.

Baking experiments. A dough was prepared from 500 g wheat flour, 30 g yeast, 20 g sucrose, 10 g NaCl and 270 ml water using a kneading machine (type UM 12, Stephan, Hameln, FRG). Mixing time was 60 s at 1500 rpm. After dough fermentation (60 min), additions were made as follows: a small piece of dough (about 80 g) was taken off, spiked with a solution of 1 mmol amino acid and 1 mmol fructose in 5 ml phosphate buffer (pH 7.0; 0.1 mol/L; cf. Table 4) and then extended into a thin plate. The remaining dough material was made up to a loaf, covered with the thin dough plate and baked for 10 min at 190° C followed by 20 min at 160° C (baking oven type B4; Wiesheu, Affalterbach, FRG). Immediately after baking, the amino-acid-enriched part of the crust (about 120 g) was peeled off, frozen in liquid nitrogen, then ground in a commercial blendor and extracted with dichloromethane [2]. The extract obtained was spiked with 25 µg [^{2}H]-2-acetyl-1-pyrroline and the volatile material isolated by sublimation in vacuo [2].

Quantitative measurements. The amounts of ACPY and ACTPY were determined by a stable isotope dilution assay (SIDA) using deuterated internal standards as detailed for ACPY in [2]. Bread extracts were separated by column chromatography prior to mass chromatography [2]. The ACPY was measured in combined fractions D and E, the ACTPY was determined in fraction C. Extracts from model experiments were analyzed without separation by column chromatography.

Capillary gas chromatography (HRGC) mass spectrometry (MS). Mass spectrometry was performed using an ion trap detector (ITD 800; Fa. Finnigan, Bremen, FRG) in the chemical ionization mode with methanol as reactant gas. The following ions were selected for quantification (cf. [2]): ACPY (*m/z* 112), [^{2}H]-ACPY (*m/z* 114–118), ACTPY (*m/z* 126), [^{2}H]-ACTPY (*m/z* 128–132). The samples (0.5 µl) were applied by the on-column injection technique at 35° C on a fused silica capillary (25 cm × 0.25 mm, CP-wax-51 for amines; Fa. Chrompack, Müllheim, FRG) coupled to the ITD. The temperature was raised by 40° C/min to 60° C, 2 min isothermally, and then raised by 6° C/min to 220° C. Helium (2 ml/min) was used as carrier gas.

Results

In a yeast extract which was previously shown [5] to liberate ACPY and ACTPY after heat treatment, the concentrations of free amino acids were determined. As shown in Table 1, 11 amino acids amounted to concentrations above 60 mg/100 g dry yeast. Proline was present in a concentration of 89 mg/100 g dry yeast. Each of the amino acids was separately reacted with 2-oxopropanal and the amounts of ACPY and ACTPY determined. As detailed in Table 2, the reactions of proline and ornithine with 2-oxopropanal resulted in nearly identical amounts of ACPY (cf. expts 1 and 2, Table 2). Replacement of 2-

Table 1. Concentrations of free amino acids in an aqueous yeast extract[a]

Amino acid	Concentration (mg/100 g dry yeast)
Glutamic acid	1290
Alanine	457
Ornithine	318
Histidine + 4-aminobutyric acid	277
Aspartic acid	223
Threonine	156
Serine	140
Lysine	129
Arginine	111
Valine	97
Proline	89

[a] Concentrations >60 mg/100 g dry yeast are reported

Table 2. Amounts of 2-acetyl-1-pyrroline and 2-acetyltetrahydropyridine formed in amino acid/2-oxopropanal model solutions

Expt	Amino acid	2-Acetyl-1-pyrroline (μg)	2-Acetyltetrahydropyridine (μg)
1	Ornithine	43	< 0.3
2	Proline	41	160
3	Ornithine	53	< 0.3
4	Proline	< 0.3	478
5	Ornithine	0.5	< 0.3
6	Lysine	< 0.3	< 0.3

In expts 1, 2, and 6, the amino acid (4 mmol), dissolved in 100 ml phosphate buffer (0.1 mol/L; pH 7.0), was reacted with 2-oxopropanal (0.1 mmol). In expts 3, 4, and 5, 2-oxopropanal was replaced by fructose (2 mmol); in expt 5, the phosphate buffer was replaced by malonate buffer (0.1 mol/L, pH 7.0)

Table 3. Amounts of 2-acetyl-1-pyrroline formed from 4-aminobutyraldehyde, 1-pyrroline or 4-aminobutyric acid in the presence of 2-oxopropanal

Expt	Nitrogen compound	2-Acetyl-1-pyrroline (μg)
1	4-Aminobutyraldehyde	125
2	1-Pyrroline	562
3	4-Aminobutyric acid	< 0.3

The nitrogen compound (0.25 mmol), dissolved in 100 ml phosphate buffer (0.1 mol/L; pH 7.0), was boiled for 2 h after addition of 2-oxopropanal (0.25 mmol)

oxopropanal by fructose slightly enhanced the amounts of ACPY formed from ornithine (cf. expts 1 and 3). In contrast, in the proline/fructose solution, no ACPY was formed (expt 4). The presence of phosphate was essential for ACPY formation in the ornithine/fructose solution. Replacement of phosphate by malonate drastically lowered the amounts of ACPY to 1% (cf. expts 3 and 5, Table 2).

From proline ACTPY also was formed (expts 2 and 4, Table 2). Its concentration was four times that of ACPY when proline was reacted with 2-oxopropanal (expt 2, Table 2). In contrast, ornithine/2-oxopropanal did not produce ACTPY (cf. expts 1 and 2, Table 2). The formation of ACTPY from proline was enhanced by a

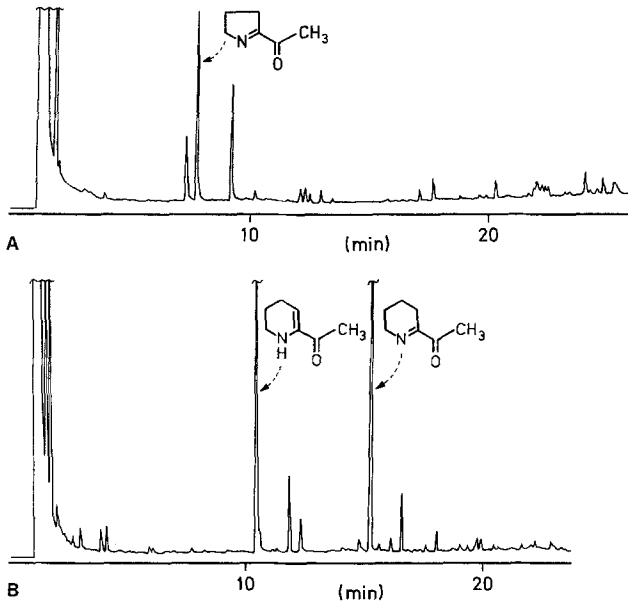

Fig. 1 A, B. HRGC chromatograms of the volatile fractions isolated from aqueous mixtures of ornithine/fructose (**A**) and proline/fructose (**B**) boiled for 2 h in the presence of phosphate ions

factor of three when 2-oxopropanal was replaced by fructose (cf. expts 2 and 4, Table 2).

The reaction of lysine and 2-oxopropanal produced neither ACPY nor ACTPY (cf. expt 6, Table 2). The remaining yeast amino acids (cf. Table 1) were also ineffective in forming ACPY or ACTPY (data not shown).

Volatile fractions isolated from either the boiled ornithine/fructose or proline/fructose solutions showed a very pleasant, roasting odor. HRGC separation revealed ACPY as the main volatile reaction product from ornithine (Fig. 1 A), while both tautomers of ACTPY (Fig. 1 B) predominated in the volatile fraction from the proline/2-oxopropanal solution. Sniffing of the eluates revealed no further primary odorants in either extract. Strecker degradation [14] of ornithine would lead to 4-aminobutyraldehyde. To test whether this aldehyde was an intermediate in ACPY formation from ornithine, 4-aminobutyraldehyde was reacted in the presence of 2-oxopropanal. As shown in Table 3 (expt 1), large amounts of this flavor compound were formed. Substitution of 4-aminobutyraldehyde by 1-pyrroline enhanced the amounts of ACPY by a factor of four (cf. expts 1 und 2, Table 3). In contrast, 4-aminobutyric acid did not produce ACPY (expt 3, Table 3).

In previous studies we could show [7] that additions of proline to wheat dough enhanced the amounts of ACPY formed during crust formation. To compare their effectiveness as enhancers of the roasty crust flavor note, the surface layer of a dough piece was spiked with equimolar amounts of either proline or ornithine and the amounts of ACPY formed after baking compared to a control bread crust without amino acid addition. As shown in Table 4, both amino acids led to an increase in the concentration of ACPY compared to the control crust without amino acid addition (compare expts 2 and 4 with expt 1). Ornithine was more effective than proline. The latter amino acid enhanced the amount of ACPY

Table 4. Influence of additions of ornithine, proline and arginine to wheat doughs on the amounts of 2-acetyl-1-pyrroline formed during baking

Expt	Added amino acid	2-Acetyl-1-pyrroline in the crust (μg/100 g)
1	None	1.9
2	Ornithine (1 mmol)	9.4
3	Ornithine (10 mmol)	43
4	Proline (1 mmol)	5.9
5	Arginine (1 mmol)	1.7

by a factor of two, while in the ornithine-enriched crust the amount of the odorant was nearly four times that in the control crust.

Although arginine was not effective as a precursor of ACPY in the model system (data not shown), its structure suggests the possibility of thermal degradation to 4-aminobutyraldehyde. Therefore, the crust from an arginine enriched dough was also analyzed. As in the model system, arginine was not effective in the formation of ACPY during baking (expt 5, Table 4).

Discussion

The data revealed that yeast contained relatively large amounts of the free amino acids ornithine and proline. Furthermore, recent results indicated [5] that 2-oxopropanal was liberated from a boiled yeast extract. The reaction of ornithine or proline with 2-oxopropanal led to significant amounts of the crust odorant 2-acetyl-1-pyrroline, while the further roast-smelling odorant 2-acetyltetrahydropyridine was specifically formed from proline.

As recently demonstrated [8], the key intermediate in the formation of ACPY from proline and 2-oxopropanal was 1-pyrroline, which was formed by an oxidative decarboxylation (Strecker reaction) of proline [15]. 1-Pyrroline may also be generated from ornithine as outlined in Fig. 2. An oxidative decarboxylation of ornithine initiated by 2-oxopropanal leads to 4-aminobutyraldehyde as intermediate. The reaction between 4-aminobutyraldehyde and 2-oxopropanal was also shown to liberate ACPY. Therefore it could be assumed that the aminoaldehyde rapidly cyclized to 1-pyrroline, as proposed in Fig. 2.

1-Pyrroline could not be formed from 4-aminobutyric acid. The latter compound did not produce ACPY in the presence of 2-oxopropanal. Further details regarding ACPY formation from 1-pyrroline and 2-oxopropanal will be published elsewhere (Schieberle, P, unpublished results).

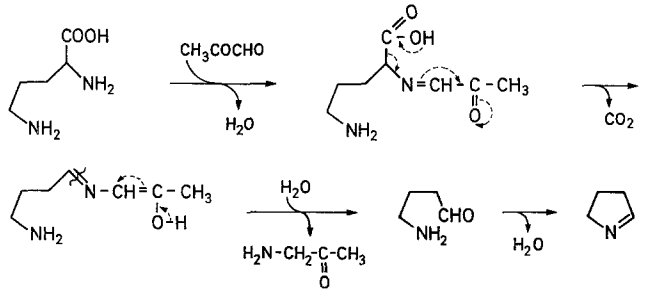

Fig. 2. Hypothetical pathway leading to the formation of 1-pyrroline from ornithine

The reaction between the ornithine homologue lysine with 2-oxopropanal did not lead to formation of the corresponding 2-acetyl-1-piperideine (2-acetyltetrahydropyridine). This was probably due to the fact that the possible intermediate 1-piperideine was not stable in the monomeric form and rapidly trimerized [16]. The amount of free ornithine present in yeast was more than three times the amount of free proline. In the model reactions both amino acids released the same molar amounts of ACPY. In addition, a bread crust from an ornithine-enriched dough contained more ACPY than a crust from a dough enriched in free proline. These data allowed the conclusion that ornithine was the most important precursor for the roast-smelling odorant ACPY in wheat dough. In the proline/2-oxopropanal reaction ACPY and ACTPY were formed, while in the presence of fructose ACTPY exclusively was liberated from proline. Hodge et al. [15] proposed a mechanism for ACTPY formation from proline and 2-oxopropanal. Our data suggest that an additional way of forming ACTPY from proline and fructose exists without the intermediate formation of 2-oxopropanal. The reaction pathway, which is governed by phosphate ions, is yet unclear.

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