JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 2001−2004 **2001**

Precursors of 2-Acetyl-1-pyrroline, a Potent Flavor Compound of an Aromatic Rice Variety

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The biological formation of a potent flavor compound, 2-acetyl-1-pyrroline, in the aromatic rice variety (Khao Dawk Mali 105) was studied in seedlings and callus of the rice. Concentrations of 2-acetyl-1-pyrroline were determined by GC−MS−SIM using an isotope dilution method. Increases in concentration occurred when proline, ornithine, and glutamate were present in solution, with proline increasing the concentration by more than 3-fold compared to that of the control. Results of tracer experiments using ^{15}N-proline, ^{15}N-glycine, and proline-1-^{13}C indicated that the nitrogen source of 2-acetyl-1-pyrroline was proline, whereas the carbon source of the acetyl group was not the carboxyl group of proline. 2-acetyl-1-pyrroline was formed in the aromatic rice at temperatures below that of thermal generation in bread baking, and formed in the aerial part of aromatic rice from proline as the nitrogen precursor.

KEYWORDS: 2-Acetyl-1-pyrroline; proline; aromatic rice; Khao Dawk Mali 105; *Oryza sativa* L.; Thailand

INTRODUCTION

Aromatic rice varieties are very popular in Southeast Asia and have recently gained wider acceptance in Europe and the U.S. (*1*, *2*). Because of their characteristic aroma and flavor, they are highly favored and command higher prices in rice markets.

A "popcorn"-like flavor compound, 2-acetyl-1-pyrroline, has been reported as a potent flavor component of an aromatic rice (*3*). It had a lower odor threshold than other volatile compounds in rice. Other sources of this compound, such as pandan (*Pandunus amaryllifolius* Roxb.) leaves, have been identified (*4*−*6*). It was also isolated from the "roasted aroma" of cooked beef and crusts of both wheat and rye breads (*7*).

Many studies on genetic control of the aroma of aromatic rice have been reported. For example, a gene responsible for aroma was located on chromosome 8, and at least 2 chromosomal regions regulated concentrations of 2-acetyl-1-pyrroline in rice (*8*). Previous investigations concluded that this trait of rice underwent monogenic inheritance (*9*, *10*), whereas others stated that two or three recessive or dominant genes controlled the construction of the trait (*11*).

In fact, there have been no reports on the formation of 2-acetyl-1-pyrroline in aromatic rice varieties. However, the formation was reported in cocoa fermentation (*12*) and baked products, such as breads (*13*, *14*). This compound formed in bread from ornithine and triose phosphates during baking through Strecker degradation of ornithine (*13*). On the other hand, this compound in cocoa fermentation was produced from both L-proline and L-ornithine by *Bacillus cereus*. It was indicated that microorganisms, especially *B. cereus*, may have a possible role in formation of this compound in rice (*12*). Our previous investigations reported that 2-acetyl-1-pyrroline did not form during cooking or postharvest processing of aromatic rice, but instead it was formed in aerial parts of plants during growing in paddy fields (*15*). However there have been no data on precursors of 2-acetyl-1-pyrroline in aromatic rice varieties. Therefore, this study was carried out to reveal precursors of this compound in the aromatic rice variety, Khao Dawk Mali 105.

MATERIALS AND METHODS

Rice Samples and Preparation. Rough rice samples of the Khao Dawk Mali 105 rice, grown in 1999, were obtained from the International Training Center for Agricultural Development, Khon Kaen, Thailand. They were hulled using a rice huller (TR-200, Kett, Tokyo, Japan). Broken kernels were removed by an automated rice analyzer (RN-500, Kett, Tokyo, Japan).

Rice seedlings were grown under an artificial light (16 h light/8 h dark) at 27 °C. The apical 5-cm segments excised from 14-day-old seedlings were used. Callus of rice was initiated from mature embryos on Linsmaier and Skoog (LS) basal agar medium (*16*) containing 58.4 μM sucrose and 9 μM 2,4-dichlorophenoxyacetic acid. Callus cultures were incubated at 27 °C by routinely transferring at 30 day intervals.

Chemicals. Iodomethane-d_3 and iodomethane-^{13}C were purchased from CDN isotopes (Quebec, Canada). ^{15}N-L-glycine and ^{15}N-L-proline were obtained from Euriso-top (Cedex, France). l-Proline-1-^{13}C was from Cambridge Isotope Laboratories, Inc. (Andover, MA). Ethanol (residual pesticide analysis grade), acetonitrile (environmental analysis

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10.1021/jf011268s CCC: $22.00
Published on Web 02/26/2002

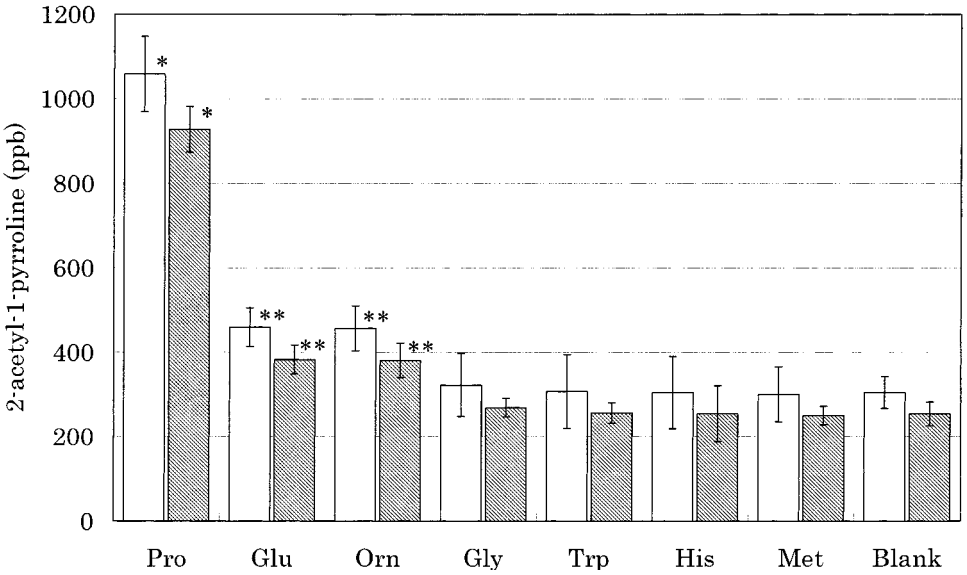

Figure 1. Effect of amino acid addition to rice seedlings and callus on 2-acetyl-1-pyrroline formation. Nonshaded bar shows 2-acetyl-1-pyrroline recovery from treated rice seedlings, and shaded bar shows recovery from treated rice callus. Asterisks indicate statistical significance ($P > 0.05$).

Table 1. Tracer Experiments for 2-Acetyl-1-pyrroline Formation Using Rice Seedlings and Callus

	^{15}N-proline		^{15}N-glycine		proline-1-^{13}C
	seedlings[a]	callus[b]	seedlings[a]	callus[b]	callus[b]
2-acetyl-1-pyrroline (*m/z* 111; ppb)	745.7 ± 34.5	589.5 ± 25.4	315.2 ± 20.8	248.5 ± 22.5	932.5 ± 55.1
labeled derivatives (*m/z* 112; ppb as ^{13}C-labeled analog)	286.8 ± 15.8	243.4 ± 18.6	2.8 ± 1.5	2.1 ± 0.2	1.5 ± 1.0
ratio of nonlabeled to labeled derivatives	2.6	2.4	112.6	118.3	621.7

[a] Excised rice seedlings were immersed in test solutions (200 ppm) and then incubated (27 °C, 8 h). Triplicate samples were analyzed; for detailed procedure, see Materials and Methods. [b] Rice callus samples were immersed in test solutions (based on LS medium, final concentration 200 ppm) followed by incubation (27 °C, 8 h). Triplicate samples were analyzed; for detailed procedure, see Materials and Methods.

grade), and all nonlabeled amino acids were bought from Wako Chemicals (Osaka, Japan). Other reagents used were of analytical grade.

Synthesis of 2-Acetyl-1-pyrroline and Stable Isotope-Labeled Analogues. 2-Acetyl-1-pyrroline and 2-acetyl-d_3-pyrroline were synthesized as described by De Kimpe et al. (*17*). 2-Acetyl-(^{13}C-methyl)-1-pyrroline was also synthesized by the procedure above using ^{13}C-methyl-magnesium iodide from ^{13}C-iodomethane. Purity of 2-acetyl-1-pyrroline and its labeled analogues was confirmed by GC−MS and ^{1}H- and ^{13}C-NMR (GSX-500, JEOL, Tokyo, Japan); yields were 35, 36, and 34%, respectively. The yield of the last reaction for labeling the acetyl group was 99%. The actual quantity was confirmed by ^{1}H-NMR using ethanol as an internal standard. Stock solutions (2%) were prepared with dichloromethane and stored at −80 °C until used.

Effect of Amino Acid Addition on 2-Acetyl-1-pyrroline Formation in Rice Seedlings. A group of 20 segments of excised rice seedlings was floated in a Petri dish containing 20 mL of distilled water (control) or amino acid solutions (Pro, Orn, Glu, Gly, Met, Trp, or His; 500 ppm, as a final concentration) previously adjusted to pH 5.5. Incubation of seedlings was carried out at 27 °C in darkness for 8 h, after which segments were removed and rinsed with distilled water, and 2-acetyl-1-pyrroline was then extracted. Quantitative analysis was carried out using ^{13}C-labeled analogue as an internal standard with ethanol extraction. GC−MS−SIM was performed by monitoring *m/z* 111 and 112.

Precursors of 2-Acetyl-1-pyrroline in Rice Seedlings. Solutions containing 200 ppm of ^{15}N-L-glycine or ^{15}N-L-proline were adjusted to pH 5.5. Excised rice seedlings were immersed in these solutions, and then incubated at 27 °C in darkness for 8 h. Triplicate samples were analyzed using deuterium-labeled analogue with acetonitrile extraction. GC−MS−SIM was performed by monitoring *m/z* 111 and 114.

Precursors of 2-Acetyl-1-pyrroline in Rice Callus. Filter sterilized solutions of ^{15}N-L-glycine, ^{15}N-L-proline, or L-proline-1-^{13}C were individually added to LS medium (without agar) to make a final concentration of 200 ppm. A 1-g portion of callus was floated in a test tube containing 5 mL of LS medium containing amino acids, then incubated at 27 °C in darkness for 8 h; medium without added amino acids was used as a control. After incubation, callus tissues were rinsed with distilled water and then removed for 2-acetyl-1-pyrroline extraction. Triplicate samples were analyzed using deuterium-labeled analogue as an internal standard and extracted with acetonitrile. GC−MS−SIM was performed by monitoring *m/z* 111 and 114.

Extraction of 2-Acetyl-1-pyrroline from Rice Seedlings and Callus. Extraction vials used were 12 × 32 mm with a PTFE septa and a screw cap. Portions of samples (0.2 g) were homogenized (Ika Ultra-Turrax T8 homogenizer, Staufen, Germany) with 0.75 mL of acetonitrile or ethanol solution containing 200 ppb internal standard of isotope-labeled analogue. They were then extracted at room temperature for 2 h. After the extracted samples were centrifuged, the supernatant was subjected to GC−MS−SIM analysis.

Gas Chromatography−Mass Spectrometry−Selected Ion Monitoring (GC−MS−SIM). A 2-μL portion of the extract was injected into a DB-Wax (60 m × 0.25 mm i.d. × 0.25 μm film thickness) fused silica capillary column (J&W Scientific, Folsom, CA) installed in a Hewlett-Packard (HP) 5980 Series 2 gas chromatograph (Palo Alto, CA). Helium gas (purity 99.9999%, passed through a molecular sieve and an oxygen trap) with carrier velocity of 41.2 cm/sec was used as the carrier gas. The injector and the GC−MS interface temperatures were set at 150 °C and 250 °C, respectively. The following oven temperature program was used: column temperature was isothermally maintained at 40 °C for 2 min, programmed at a rate of 10 °C/min to

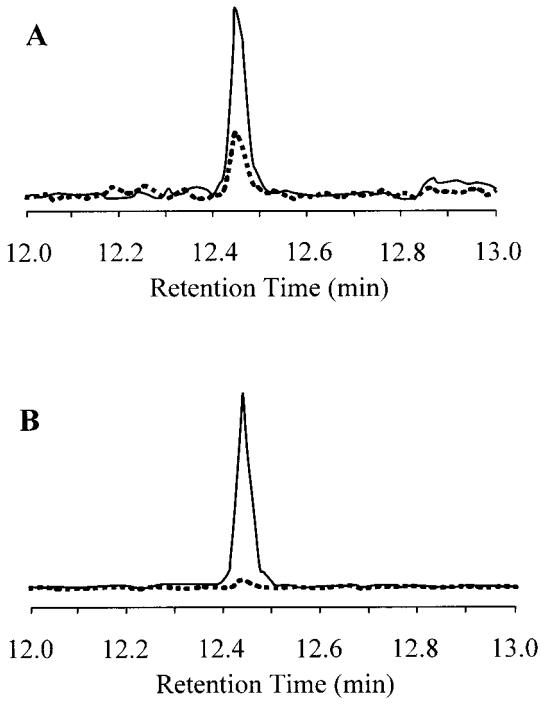

Figure 2. (A) Selected ion-monitoring chromatogram of rice callus treated with ^{15}N-proline. Solid line shows *m*/*z* 111 (2-acetyl-1-pyrroline), and broken line shows *m*/*z* 112 (labeled product; estimated as 2-acetyl-1-^{15}N-pyrroline). (B) Selected ion-monitoring chromatogram of rice callus treated with proline-1-^{13}C. Solid line shows *m*/*z* 111 (2-acetyl-1-pyrroline), and broken line shows *m*/*z* 112 (labeled product; estimated as 2-acetyl-(^{13}C-carbonyl)-1-pyrroline).

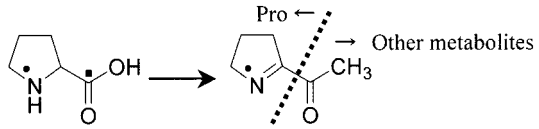

Figure 3. Possible pathway for 2-acetyl-1-pyrroline formation from proline. Dot shows labeled nitrogen-15 on proline, and square shows carbon-13 on proline.

100 °C, then at a rate of 5 °C/min to 140 °C, and then at a rate of 20 °C/min to 250 °C; the column temperature was then maintained isothermally at 250 °C for 10 min. An HP 5989A mass spectrometer was used in the electron ionization mode with the ion source temperature set at 250 °C, the analyzer temperature set at 100 °C, and ionization energy set at 70 eV. SIM was set up to monitor *m*/*z* 111 for 2-acetyl-1-pyrroline, *m*/*z* 112 for ^{13}C-labeled analogue, and *m*/*z* 114 for deuterium-labeled analogue. MS detection dwell time was 100 ms for each ion. Under these conditions, the retention times of 2-acetyl-1-pyrroline, ^{13}C-labeled and deuterium-labeled analogues were found to be 12.46, 12.46, and 12.44 min, respectively. Quantification was performed by measuring the area ratios between ions at *m*/*z* 111, 112 and 114, corresponding to 2-acetyl-1-pyrroline, ^{13}C-labeled and deuterium-labeled analogues, respectively. Each extract was analyzed three times by GC−MS−SIM to obtain an average peak area and ± SD. The amounts of 2-acetyl-1-pyrroline and its labeled derivatives were calculated from a calibration curve.

RESULTS AND DISCUSSION

Quantitative Analysis of 2-Acetyl-1-pyrroline and Labeled Derivatives by GC−MS−SIM. Because proton/deuterium exchange on the methyl group of 2-acetyl-1-pyrroline in protogenic solvents was reported during quantitative analysis using stable isotope dilution by addition of its deuterium-labeled analogue (*14*), the ^{13}C-labeled analogue was synthesized in our laboratory as an alternative. It showed stability in protogenic solvents such as water, methanol, and ethanol. The molecular weight of the ^{13}C-labeled analogue was 112. Similarly, that of ^{15}N-labeled 2-acetyl-1-pyrroline, the expected product of tracer experiments using ^{15}N-labeled amino acids, was also 112. Therefore, deuterium-labeled analogue whose molecular weight was 114, was used to replace ^{13}C-labeled analogue, using nonprotogenic extraction solvents.

Effect of Amino Acids Addition on 2-Acetyl-1-pyrroline Formation in the Aromatic Rice. Schieberle et al. (*13*) reported that ornithine found in baker's yeast was a principal precursor of 2-acetyl-1-pyrroline during baking, whereas L-proline led to formation of this compound by *B. cereus* (*12*). It was also reported that 2-acetyl-1-pyrroline was present in aerial parts of rice plants (*15*). On the basis of these reports, those amino acids related to proline and glutamate interconversion pathway (i.e., Pro, Orn, and Glu) were used as probable precursors of 2-acetyl-1-pyrroline. Gly, Met, Trp, and His were used as controls. The results obtained (**Figure 1**) show that increases in 2-acetyl-1-pyrroline concentration occurred when Pro, Orn, and Glu were present in solution. When Pro was added to the solution, it increased the concentration by more than 3-fold compared to that of the control. Addition of Gly, Met, Trp, and His did not increase its content in rice leaves. From the results, it is assumed that L-proline was related to 2-acetyl-1-pyrroline formation in rice leaves. Romanczyk et al. (*12*) indicated the possible role of contaminating microorganisms, especially *B. cereus*, in its formation in aromatic rice varieties. However, this compound was detected in aseptic rice callus, and addition of filter-sterilized amino acids to rice callus gave results similar to those of excised rice leaves (**Figure 1**). These results indicated that the formation of 2-acetyl-1-pyrroline was inherent in the aromatic rice variety tested.

Precursors to 2-Acetyl-1-pyrroline in Aromatic Rice. GC−MS−SIM analysis clearly showed that the formation of ^{15}N derivative occurred only from ^{15}N-proline (**Table 1** and **Figure 2A**). The ratios for nonlabeled to labeled 2-acetyl-1-pyrroline were 2.6 and 2.4 in rice leaves and callus, respectively.

To reveal the carbon source of the acetyl group, L-proline-1-^{13}C was used as precursor. If the carbonyl group was not originated from other carbon sources, carboxyl ^{13}C-labeled derivative (*m*/*z* 112) would be observed. However, only nonlabeled compound (*m*/*z* 111) was observed (**Table 1** and **Figure 2B**). These results indicated that the nitrogen source of 2-acetyl-1-pyrroline was L-proline, whereas the carbon source of acetyl group was not the carboxyl group of L-proline.

Collectively, these results were similar to those of Romanczyk et al (*12*) on *B. cereus*. However, findings in this study pointed to a biological origin and nitrogen source for 2-acetyl-1-pyrroline in aromatic rice. 2-Acetyl-1-pyrroline in aromatic rice was formed in the aromatic rice at the temperature below that of thermal generation in bread baking, and formed in the aerial part of aromatic rice from L-proline as nitrogen source. **Figure 3** shows a suggested formation pathway of 2-acetyl-1-pyrroline in aromatic rice. This result may shed light on genetic control on mechanisms of the formation in Khao Dawk Mali 105 rice.

It is a fact that L-proline accumulation is a common metabolic response of higher plants to water deficits and salinity stresses, and has been the subject of numerous reviews (*18*, *19*). In this regard, it has been stated that the quality of Khao Dawk Mali 105 is influenced by the area of production, especially the difference in water supply and salinity of soil (*20*). Hence, it was suspected that the amount of L-proline in rice plants was related to the concentration during maturation. Loss of this

compound by volatilization during postharvest processes of aromatic rice, such as drying and storage, could not be ruled out.

Further studies are needed to connect both genetic and chemical studies of aromatic rice varieties, especially the carbon source of acetyl group in 2-acetyl-1-pyrroline. This also could reveal key enzymes involved in its formation.

ACKNOWLEDGMENT

We thank Dr. Nobuyuki Kabaki (JIRCAS) for providing Khao Dawk Mali 105 rice samples. We also thank Dr. Sayuki Nikkuni and Dr. Kazuhiko Nakahara (JIRCAS) for helpful advice in tracer experiments.

ABBREVIATIONS USED

GC−MS−SIM, gas chromatography−mass spectrometry−selected ion monitoring; LS, Linsmaier and Skoog.

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Received for review September 4, 2001. Revised manuscript received December 28, 2001. Accepted December 28, 2001. This research was supported by "Comprehensive Research Project in Agricultural Development in Northeast Thailand" provided from Japan International Research Center for Agricultural Sciences (JIRCAS).

JF011268S