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Free Amino Acid and Reducing Sugar Composition of Pandan *(Pandanus amaryllifolius)* Leaves

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ABSTRACT

Pandan leaves are commonly used to flavor desserts in Southeast Asia. The key aroma compound in pandan, 2-acetyl-1-pyrroline (ACPY), probably forms through Strecker degradation during cooking. This study investigated the composition of free amino acids and reducing sugars that could be precursors of ACPY in pandan leaves. Fresh pandan leaves contained 2.38 mg/g fructose and 1.77 mg/g glucose. Major free amino acid in pandan was glutamic acid (0.41 mg/g). Proline, a precursor of ACPY in fragrant rice, was at 0.12 mg/g. Pandan samples were heated at 100 °C for 10 min at pH 7.0. Aroma impact compounds in heated pandan were ACPY and 3-methyl-2(5*H*)-furanone. In this experiment, glutamic acid, proline, ornithine, fructose and glucose were added to pandan samples separately. Addition of glutamic acid, proline, and ornithine increased ACPY and generated 4-vinylphenol and 3-ethyl-4-methyl-1*H*-pyrrole-2,5-dione that were not normally detected in heated pandan samples. Addition of fructose and glucose did not affect ACPY concentrations but decreased in 2-ethyl-5-methyl furanone.

Key words: pandan, 2-acetyl-1-pyrroline, proline, glutamic acid

INTRODUCTION

Pandan leaves *(Pandanus amaryllifolius Roxb.)* are used as a flavoring ingredient in many of Southeast Asian dishes. The key aroma compound in pandan is 2-acetyl-1-pyrroline (ACPY) (Laksanalamai and Ilangantilek, 1993) which is described as "pandan" by Asian and "popcorn and roasty" by Westerners (Paule and Power, 1989). ACPY is also a key odorant in Jasmine and Basmati rice. In pandan, the ACPY concentration has been reported as 1 ppm; which is 10 times higher than fragrant rice (Buttery *et al.*, 1982, 1986; Laksanalamai and Ilangantilek, 1993). The detected concentration of ACPY in pandan, however, could depend on the methods used to isolate the volatile component. Using supercritical carbon dioxide instead of solvent extraction or simultaneous distillation extraction (SDE), could extract up to 7.16 ppm ACPY in pandan leaves (Bhattacharjee *et al.*, 2005). Amino acids believed to be precursors of ACPY are proline and ornithine (Weenen *et al.*, 1997). Most ACPY in foods, such as bread, crackers, and sesame, is formed by Strecker degradation during the heating process (Schieberle, 1998). Pathways for the formation of ACPY through heating have been studied extensively by Hofmann and Schieberle (1998a, 1998b).

Besides heating, ACPY can also be

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generated through biosynthesis in plants or by as microbial metabolism. Biosynthesis of ACPY in plants has proline and ornithine as its precursors (Delauney and Verma, 1993). For example, fragrant rice (Khao Dwak Mali 105) has proline as the precursor of ACPY (Yoshihashi *et al.*, 2002). Its aroma can be observed during growth in the paddy field (Yoshihashi, 2002). By microbial metabolism, ACPY can be formed from ornithine during fermentation of cocoa by *Bacillus cereus (*Romanczyk *et al.*, 1995) or in wine by lactic acid bacteria such as *Lactobacillus hilgardii, L. brevis, L. plantarum,* and *Pediococcus* sp. (Costello *et al.*, 2001).

Formation of aroma compounds in pandan could be different from rice. The aroma of pandan is generated only during heating. Fresh pandan leaves have green aroma characteristic. Volatiles of fresh pandan leaves without heating did not contain ACPY but have 73.07% 3-methyl-2(5*H*)-furanone, 7.09% 3-hexanol, 6.13% 4-methyl-2-pentanol, 2.97% 3-hexanone, 2.65% 2-hexanone, and a few miscellaneous compounds (Jiang, 1999).

The objective of this study was to investigate the free amino acid and reducing sugar compositions in pandan leaves as possible precursors for ACPY formation during the heating process.

MATERIALS AND METHODS

Chemicals

D-(-)-ribose, D-(-)-fructose, D-(+)-glucose, maltitol, nor-leucine, *n*-alkane series (C5-C18) and 2,4,6-trimetylpyridine were from Aldrich Chemical (Milwaukee, WI, USA). Dichloromethane was from Mallinckrodt Baker (Phillipsburg, NJ, USA). Sodium sulfate was from Fisher Scientific UK (Leicestershire, UK). Amino acid hydrolysate standard, L-proline, L-ornithine, and glutamic acid were from Pierce Biotechnology (Rockford, IL, USA).

Isolation of amino acids and sugars

Isolation of free amino acids and sugars was adapted from the methods described by Kuo *et al.* (2003) and Yang *et al.* (1999). Freeze-dried pandan leaves (1.00 g) were mixed with 25 ml of 75% ethanol. Fifty microliters of nor-leucine (6 mg/ml) was added as an internal standard to the sample for amino acid analysis. In case of sugar analysis, 250 mg maltitol was added as an internal standard. Each sample was stirred at room temperature for 12 h and then centrifuged at 12,000 × g for 20 min. Precipitate was washed with 25 ml ethanol and centrifuged. The extract (75 ml) was concentrated under vacuum until the volume was reduced to 2.5 ml. The concentrated extract (100 µl) was diluted with deionized water to 1,000 µl. Samples were stored at –40 °C before analysis with high performance liquid chromatography (HPLC).

Free amino acid analysis

Free amino acids were analyzed by the method adapted from Cohen and Denis (1993). Fifty microliters of the sample was subjected to derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, Waters, Milford, MA, USA).

Analysis was performed using a Waters HPLC system composed of Model 600 controller and pump, and photodiode array detector (Waters, Milford, MA, USA). Five microliters of the derivatized extract was injected into an AccQTag^{TV} column (3.9 mm × 150 mm) at 37 °C. Mobile phase (mobile phase A : 140 mM sodium acetate + 17 mM triethylamine [TEA] titrated with 85% *o*-phosphoric acid, mobile phase B: acetonitrile 60% in water) was flown at 1.0 ml/min using the gradient elution program shown in Table 1. Amino acids were detected by photodiode array detector at 254 nm. Amino acids were identified and quantified using an amino acid hydrolysate standard.

Table 1 Gradient condition for free amino acid analysis.

Time (min)	% Mobile phase A[1/]	% Mobile phase B[2/]
0.0	100	0
0.5	98	2
15	93	7
19	87	13
33	68	32
43	0	100
53	0	100
54	100	0

1/ mobile phase A : sodium acetate 140 mM + triethylamine 17 mM.

2/ mobile phase B : 60% acetonitrile in water.

Sugar analysis

Sugars were analyzed using the HPLC system composed of a Waters Model 600 controller, Waters Model 600 pump and Waters 410 differential refractive detector. Samples (20 µl) were injected into a normal phase silica column (Waters, Milford, MA, USA). The separation was at 37 °C. The mobile phase, 75% acetonitrile in water was flown at 1.0 ml/min. Quantification and identification used D-(-)-fructose, D-(+)-glucose, and used D-(-)-ribose as an internal standard.

Addition of amino acids and reducing sugars in pandan leaves

Fresh pandan leaves were washed with water and then blanched in boiling water for 10 s to inactivate enzymes. Thirty grams of pandan leaves was added and blended thoroughly with 30 g sodium chloride, and 30 ml of 0.05% (w/v) concentration of standard amino acids and reducing sugars. The standards were L-proline, L-ornithine, glutamic acid, D-(-)-fructose, and D-(+)-glucose. Ground sample mixtures were transferred into 500 ml glass jars and phosphate buffer was used to adjust pH to 7.0. The samples were heated in a water bath to 100 °C for 10 min before isolation and evaluation of their volatile compounds.

Isolation of volatile compounds from pandan leaves

Heated samples were transferred into 500 ml glass jars and along with 50 µL of the internal standard (1,500 mg/ml of 2,4,6-trimetylpyridine). Volatile compounds were extracted with dichloromethane (3 × 60 ml) by shaking in a water bath at 40 °C for 1 h. The pooled extract was evaporated under mild nitrogen stream until the volume was reduced to 50 ml. The concentrated extract was distilled under high vacuum distillation (vacuum pressure at 10^{-5} Torr) for 1 h. The distillate was added with 10 g anhydrous sodium sulfate to eliminate trace water in the sample and then filtered through a pasture pipette contained glass wool and anhydrous sodium sulfate. The solvent was evaporated under mild nitrogen stream until the volume reached 0.5 ml. Samples were then kept in brown glass vials at –40 °C and analyzed within 24 h after extraction.

Analysis of volatile compounds by gas chromatography-mass spectrometry (GC-MS)

Volatile compounds from pandan sample extracts were analyzed using an HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP 5973 mass selective detector (MSD, Agilent Technologies, Palo Alto, CA, USA). Two microliters of concentrated extract was injected into a splitless injection port at 155 °C. Capillary column HP-5 (60 m × 0.25 mm × 0.32 µm, Agilent Technologies, Palo Alto, CA, USA) and HP-Innowax (60 m × 0.25 mm × 0.32 µm, Agilent Technologies, Palo Alto, CA, USA). High purity helium (99.999%) was used as a carrier gas at the flow rate of 2.0 ml/min. Temperature program was holding at 35 °C for 3 min, 9 °C/min to 120 °C, 25 °C/min to 200 °C and maintained at 200 °C for 3 min. Ion source was by Electron-Impact Ionization. Ionization

energy was 70 eV, scan range was 30-300 amu, and scan rate was 2.74 scan/s.

Identification of volatile compounds was done tentatively by comparison of mass spectrum data of volatile compounds with Wiley 275 mass spectrum library and comparison of retention index (RI) with the literatures. Retention indices were calculated by using a series of *n*-alkanes (C5-C18). Informations on odor description and threshold of aroma compounds were obtained from Flavor-Base 2004 (Leffingwell & Associates, Canton, GA, USA).

Analysis of volatile compounds by gas chromatography-olfactometry (GCO)

Two experienced panelists conducted GCO for identification purpose. The GCO system consisted of a HP5890 GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID), a sniffing port (SGE, Australia), and a splitless injector. Each extract (2 µl) was injected into a capillary column (HP-5, 15 m × 0.32 mm × 0.25 µm film thickness, Agilent Technologies, Palo Alto, CA, USA). GC oven temperature was programmed from 35 °C to 200 °C at the rate 10 °C/min with initial and final hold times of 5 and 30 min, respectively. The carrier gas was helium at a constant flow of 2.2 ml/min.

Statistical analysis

Experiments were performed using a Complete Randomized Design. All experiments were done in duplicate. Mean separation was analyzed by Duncan's multiple range test using SPSS version 12.0.

RESULTS AND DISCUSSION

Sugar and amino acid composition in fresh pandan leaves

Fresh pandan leaves contained 2.38 mg/g fructose and 1.77 mg/g glucose (Table 2). Glucose, although was at lower concentration than fructose, might play significant role in ACPY formation. Previous study by Blank *et al.* (2003) showed that glucose/proline model system produced more ACPY than fructosyl-proline system when heated at pH 7.0.

Free amino acids are listed in Table 2. In this study glutamic acid was the major free amino acid (0.41 mg/g), followed by aspartic acid, threonine, serine, histidine, alanine, and proline (0.12 mg/g). Amino acids previously reported to be possible precursors for ACPY, are ornithine, proline, and glutamic acid (Yoshihashi *et al.*, 2002). In pandan, it is possible that glutamic acid and proline may play an important role in pandan aroma compound formation. Ornithine, although is interconvertible with glutamic acid and proline, was not detected in the sample.

Table 2 Concentrations of sugars and free amino acids (dry basis) in fresh pandan leaves.

Compound	Concentration (mg/g)
Sugars	
fructose	2.38 ± 0.88
glucose	1.77 ± 0.65
Amino acids	
aspartic acid	0.21 ± 0.19
serine	0.17 ± 0.09
glutamic acid	0.41 ± 0.04
glycine	0.05 ± 0.73
histidine	0.16 ± 0.04
arginine	0.02 ± 0.11
threonine	0.21 ± 0.05
alanine	0.16 ± 0.07
proline	0.12 ± 0.04
tyrosine	0.03 ± 0.06
valine	0.03 ± 0.02
lysine	0.03 ± 0.04
isoleucine	0.02 ± 0.36
leucine	0.02 ± 0.69
phenylalanine	0.03 ± 0.08

Volatile compounds of heated pandan leaves

Heated pandan leaves at 100 °C for 10 min at pH 7.0 produced a clean pandan aroma. Heated samples had 10 volatile compounds as listed in Table 3. The major volatile compound in pandan was 3-methyl-2(5*H*)-furanone (with a harsh, sweet, medicinal odor) followed by ACPY (pandan odor). 3-Methyl-2(5*H*)-furanone was also the major volatile compounds in unheated pandan (Jiang, 1999). Considering OAV (odor activity value), which is volatile concentration divided by odor threshold in water, ACPY was the most potent odorant. The other compounds that had threshold values previously reported in the literatures had OAV less than one. This agreed with GCO data as indicated in the odor description (Table 3) that the aroma active compounds in pandan were ACPY and 3-methyl-2(5*H*)-furanone. The panelists could not detect other aroma active compounds from GCO. Total volatile compound concentration in pandan was low in comparison to other studies. This is due to the use of limited heating in this study. The heating condition in this experiment was based on the typical cooking conditions for pandan. Extraction with dichloromethane followed by high vacuum distillation was chosen to isolate volatile compounds to avoid excessive heating.

ACPY is unstable even when kept at -20 °C under vacuum conditions but has a very low odor threshold of 0.1 ppb (Buttery *et al.*, 1983). In our early experiment, we experienced significant lost of ACPY in the concentrated extracts that were kept at -40 °C longer than one week. The aroma was still strong because of its low odor threshold but the compound could not be detected by MSD. Therefore, analysis for volatile component should be conducted immediately after isolation or not later than 24 h.

Addition of reducing sugars and amino acids

Addition of fructose and glucose did not affect concentrations of most volatile components

Table 3 Volatile compounds of heated pandan leaves.

Compounds	Odor description	RI		Threshold in water (ppb) [1/]	Concn. (ng/g)	OAV
		HP-5	HP-Innowax			
cyclohexanol	fermented, yeasty odor [1/]	890	-	90-3,500	0.89	<1
cyclohexanone	sweet ketonic solvent-like, minty-camphoraceous odor [1/]	900	-	280-14,400	0.88	<1
2-acetyl-1-pyrroline	pandan [2/]	926	1323	0.1	19.86	198
3-methyl-2(5*H*)-furanone	harsh, sweet, medicinal [2/]	1083	1795	*na* [3/]	102.23	*na*
N-octanal	fatty-fruity [1/]	1077	-	0.7	0.14	<1
nonanal	green, waxy [1/]	1107	1078	1.0	0.11	<1
2-ethyl-5-methylfuran	fresh gassy, burnt note [1/]	1453	-	*na*	0.94	*na*
1-isocyanato-2-methoxy benzene	slightly musty odor [1/]	1470	1874	*na*	2.01	*na*

[1/] Odor description or odor threshold from Flavor-Base.

[2/] Odor description from GCO of 30 g heated pandan leaves.

[3/] *na* = not available.

including ACPY (Table 4). This could be because pandan leaves contained excess amount of glucose and fructose when compared to the concentrations of individual amino acids. The only effect of addition of reducing sugars was the reduction of 2-ethyl-5-methylfuran that had fresh gassy and burnt aroma characteristic.

Addition of proline, ornithine and glutamic acid to pandan samples increased ACPY formation when compared to the samples without addition of amino acids (Table 4). Free glutamic acid (0.41 mg/g) and proline (0.12 mg/g) could play an important role in pandan aroma as previously mentioned. Similar work has been conducted in fragrant rice; Yoshihashi *et al.* (2002) added proline, ornithine, and glutamic acid to Thai jasmine rice, Khoa Dwak Mali 105. They found that proline was the most effective amino acid in increasing ACPY in jasmine rice whereas ornithine and glutamic acid increased ACPY to lesser levels.

Pandan samples with amino acids had additional volatile compounds, i.e., 4-vinylphenol and 3-ethyl-4-methyl-1*H*-pyrrole-2,5-dione, that were not found in the control sample and the samples added with reducing sugars added. The volatile 4-vinylphenol has medicinal and phenolic aroma characteristic with the odor threshold of 10 ppb. It is possible that high amounts of free amino acid present in the mixture could lead to an off-flavor. The concentrations of 4-vinylphenol in the samples with amino acids in this experiment, however, were below its threshold value.

Proline and ornithine are precursors in biosynthesis of ACPY in plants (Delauney and Verma, 1993). Enzyme that involves in proline synthesis is pyrroline-5-carboxylate synthetase (P5CS). Activity of P5CS depends on stress such as drought and high salt concentration in soil. In case of fragrant rice, growing in dry area or high salt area can result in irregular osmotic pressure in cells. Rice raised proline accumulation by increase P5CS activity to alleviate the stress. Formation of ACPY in pandan could be from different mechanism. It is interesting to study amino acid biosynthesis and the ACPY precursor in pandan because the plant is generally grown in wet areas but still has higher ACPY contentration than fragrant rice when heated.

Table 4 Volatile compounds of pandan leaves with added amino acids and reducing sugars after heating at 100 °C for 10 min at pH 7.

Compounds	Concentration (ng/g)					
	Control	Proline	Ornithine	Glutamic acid	Fructose	Glucose
cyclohexanol	0.89 a [1/]	0.81 a	0.90 a	0.85 a	1.02 a	0.96 a
cyclohexanone	0.88 a	0.77 a	0.56 b	1.00 a	0.86 a	0.65 ab
2-acetyl-1-pyrroline	19.86 b	27.36 a	26.50 a	24.39 a	20.10 b	19.89 b
3-methyl-2(5*H*)-furanone	102.23 a	103.35 a	111.10 a	107.14 a	99.94 a	112.88 a
N-octanal	0.14 a	0.18 a	0.15 a	0.11 a	0.16 a	0.19 a
nonanal	0.11 a	0.09 a	0.06 a	0.07 a	0.16 a	0.19 a
4-vinylphenol	-	1.20 a	0.96 a	0.95 b	-	-
3-ethyl-4-methyl-1 *H*-pyrrole-2,5-dione	-	0.25 a	0.17 a	0.22 a	-	-
2-ethyl-5-methylfuran	0.94 a	0.70 ab	0.46 c	0.79 ab	0.61 bc	0.65 bc
1-isocyanato-2-methoxy-benzene	2.01 b	2.98 ab	2.76 ab	3.18 ab	3.88 a	2.16 b

Note: [1/] Different alphabets (a-c) in the same row are significantly different ($p \leq 0.05$).

CONCLUSION

Pandan leaves contained glutamic acid, proline, glucose, and fructose as possible precursors to ACPY. Heating pandan leaves to 100 °C for 10 min at pH 7 generated pandan aroma without off-flavor. The result from GCO analysis indicated that ACPY was the only compound that possessed pandan aroma characteristic. Most compounds in pandan leaves had OAV less than one. Addition of reducing sugars reduced 2-ethyl-5-methylfuran concentration and only samples that added with amino acids added had their ACPY increased.

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