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Bioconversion of Capsaicin by *Aspergillus oryzae*

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ABSTRACT: This study identified metabolites of capsaicin bioconverted by *Aspergillus oryzae*, which is generally used for mass production of gochujang prepared by fermenting red pepper powder in Korea. *A. oryzae* was incubated with capsaicin in potato dextrose broth. Capsaicin decreased depending on the incubation period, but new metabolites increased. Five capsaicin metabolites purified from the ethyl acetate fraction of the capsaicin culture were identified as *N*-vanillylcarbamoylbutyric acid, *N*-vanillyl-9-hydroxy-8-methyloctanamide, ω-hydroxycapsaicin, 8-methyl-*N*-vanillylcarbamoyl-6(*E*)-octenoic acid, and 2-methyl-*N*-vanillylcarbamoyl-6(*Z*)-octenoic acid by nuclear magnetic resonance (NMR) and mass spectrometry (MS). The capsaicin metabolites in gochujang were confirmed and quantitated by selective multiple reaction monitoring detection after liquid chromatography electrospray ionization MS using the isolated compounds as external standards. On the basis of the structures of the capsaicin metabolites, it is proposed that capsaicin metabolites were converted by *A. oryzae* by ω-hydroxylation, alcohol oxidation, hydrogenation, isomerization, and α- and/or β-oxidation.

KEYWORDS: *capsaicin, Aspergillus oryzae, gochujang, hot pepper, secondary metabolites*

INTRODUCTION

Fruits of the hot pepper (*Capsicum* family) have been widely used as vegetable foodstuffs, spices, and external medicine. Capsaicinoids, such as capsaicin, dihydrocapsaicin, nordihydrocapsaicin, and homocapsaicin, are the pungent compounds in the hot pepper fruits. Capsaicin, *trans*-methyl-*N*-vainillyl-6-nonenamide, **1** (Figure 2), is the most abundant of the capsaicinoids contained in hot pepper and has a strong pungent taste.[1] Capsaicin has received clinical attention due to various biological effects, such as antioxidant,[2] antiobesity,[3] anticancer,[4] and antidiabetic[5] activities. Additionally, capsaicin has been widely consumed as the main ingredient of processed foods in many countries including China, Hungary, Japan, Korea, and Mexico.

Gochujang, a Korean fermented food product, is often used as a sauce in Korean cooking and spicy seasonings.[6] Gochujang is prepared by fermenting a mixture of, primarily, red hot pepper powder, waxy rice flour, *meju* (fermented soybean), and *koji*. Characteristically sweet, savory, and pungent flavors depend on the primary ingredients and different microorganism activities. Various natural microflora, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus pasteuri*, *Aspergillus oryzae*, *Saccharomyces rouxii*, and *Zygosacchromyces pseudorouxii* have been identified in gochujang.[7,8] Particularly, *A. oryzae* is often used to make gochujang in quality-controlled environment and mass production.[9,10]

The pungent taste of gochujang after fermentation is milder than that of the starting ingredient red hot pepper, which may be related to the bioconversion of capsaicin during gochujang fermentation. Previous studies reported the hydrolysis of capsaicin to vanillylamine and *trans*-8-methyl-6-nonenoic acid by microflora[10−14] and the degradation of the capsaicin side chain through hydroxylation and β-oxidation by fungi.[15−17] Also, the converted metabolites of the capsaicin side chain may have biological effects similar to those of capsaicin due to their similar structures.[17] However, no previous studies have investigated the metabolites of capsaicin derivatives during fermentation, comprehensively. Additionally, no study has assessed the effect of *A. oryzae* on the metabolism of capsaicin derivatives, although the starting ingredient has been widely used in gochujang factory mass production. Moreover, qualitative and quantitative information on gochujang capsaicin metabolites has not yet been reported.

In the course of investigations on capsaicin metabolites in gochujang, we isolated and identified five capsaicin metabolites from capsaicin culture incubated with *A. oryzae*. Three compounds, **2**, **4**, and **5**, isolated as capsaicin metabolites in this study have been previously reported,[15,16] but the nuclear magnetic resonance (NMR) assignments for **2** and **5** were not completely achieved. In this study, we report the complete NMR assignments of the two capsaicin metabolites, **2** and **5**, and the structures of two new capsaicin metabolites, **3** and **6**, were determined by NMR and MS. In addition, the five capsaicin metabolites identified were quantitated in gochujang prepared by using *A. oryzae* as the starter.

MATERIALS AND METHODS

Materials, Chemicals, and Strain. Gochujang fermented by *A. oryzae* as the starter was purchased from a local market in Korea in

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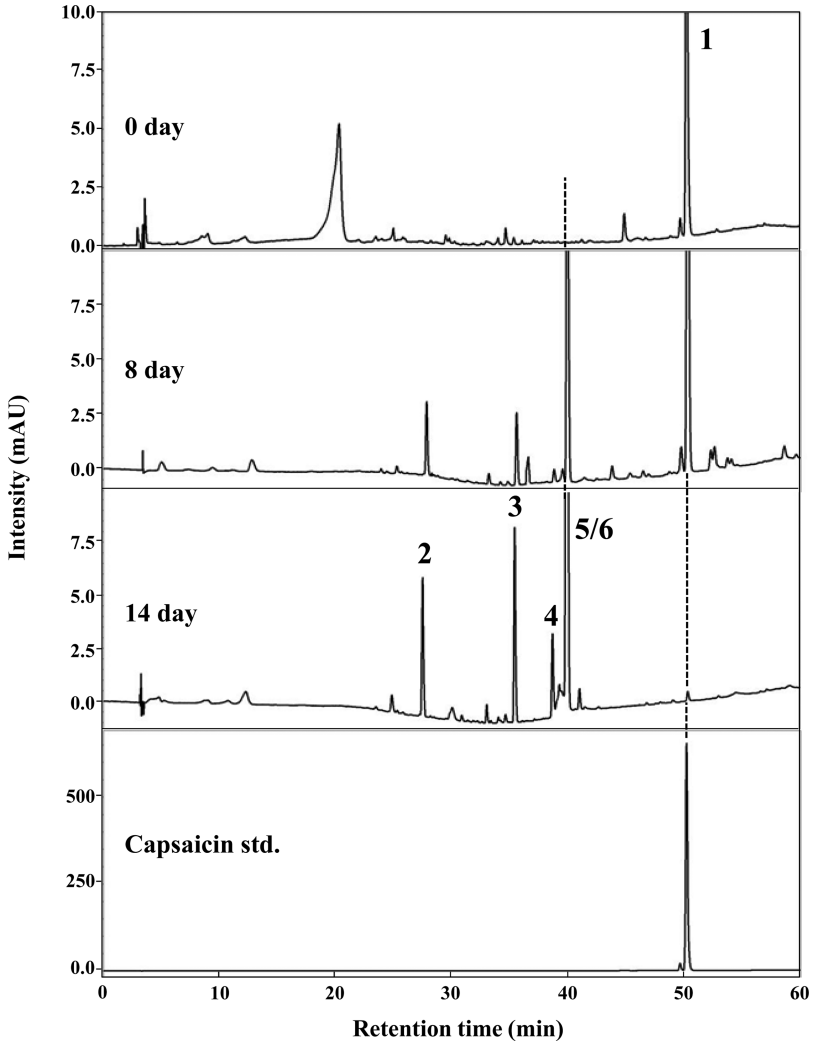

Figure 1. High-performance liquid chromatography chromatograms of capsaicin culture during incubation.

September 2014. The main ingredients were listed as red pepper powder, starch syrup, wheat flour, glutinous rice, *meju* made with soybean, and calcium. Capsaicin (purity, 50%) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and was purified as follows: Commercial capsaicin (purity, 50%) was dissolved in MeOH (0.3 mL). An aliquot of the capsaicin solution was subjected to preparative HPLC equipped with a Shim pack prep-ODS(H)KIT column (20 i.d. × 250 mm, 10 μm) (Shimadzu, Kyoto, Japan). Capsaicin was detected at 280 nm at a flow rate of 9.9 mL/min. Elution was achieved using a solvent mixture of MeOH/H_2O (7:3, v/v) as the mobile phase. Capsaicin (t_R 18.7 min, 240.6 mg, white powder) was collected, subjected to NMR and MS analyses, and used as a standard. The purity of capsaicin was >98.0%. *A. oryzae* KCCM 11372 was obtained from the Korean Culture Center for Microorganism (Seoul, Korea). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Difco Co. (Detroit, MI, USA). All solvents used for analyses were of HPLC grade. All other chemicals used were of reagent grade.

Incubation of Capsaicin with *A. oryzae*. Purified capsaicin (10 mg) was added to PDB (100 mL), inoculated with a 5% (v/w) spore suspension of *A. oryzae* and incubated for 14 days at 30 °C and 120 rpm in an incubator. The samples were collected at intervals of 0, 2, 4, 6, 8, 10, 12, and 14 days during the incubation and were stored immediately at −70 °C until analysis. *A. oryzae* was grown on PDA and maintained at 30 °C. The spores were inoculated to PDB and maintained in a shaker incubator at 30 °C and 120 rpm for 48 h. The cells were collected by centrifugation (890*g*, 15 min, at 4 °C), and starter culture was obtained.

HPLC Profile of Capsaicin Metabolites. The cultured capsaicin broth (1 mL) was centrifuged for 15 min, at 4 °C and 890*g*. The supernatant was extracted with EtOAc (6 mL, twice). The EtOAc fractions were combined and concentrated under vacuum. The EtOAc fraction was dissolved in 0.5 mL of 90% MeOH and subjected to reversed-phase-HPLC containing a photodiode array system operating at 200−800 nm. The capsaicin metabolites were separated on a Capcell Pak C18 column (UG-120, 4.6 mm i.d. × 250 mm, 5 μm) (Shisheido, Tokyo, Japan) at 40 °C using a gradient consisting of 100% H_2O (solvent A) to 100% MeOH (solvent B). The gradient was started with 50% B for 30 min, increased to 80% B at 50 min, and held at 100% B until 60 min. The flow rate was 1.0 mL/min.

Purification and Isolation of Capsaicin Metabolites. Purified capsaicin (200 mg) was added to PDB (2.0 L) and incubated for 14 days under the same conditions described above. Samples were collected at intervals of 0, 2, 4, 6, 8, 10, 12, and 14 days during the incubation. The cultured capsaicin broth was centrifuged for 15 min, at 4 °C and 890*g*. The supernatant was extracted with EtOAc (4 L, twice), and then the EtOAc fractions were combined and concentrated under vacuum. The EtOAc fraction was fractionated by octadecylsilane (ODS, 70−230 mesh) (YMC, Kyoto, Japan) column (3.0 cm × 80 cm i.d.) chromatography eluted with H_2O/MeOH (7:3−0:10, v/v, stepwise elution, each 300 mL). The fractions were subjected to TLC and grouped into 13 fractions (A−M). Each fraction was analyzed by HPLC under the conditions described above. The HPLC

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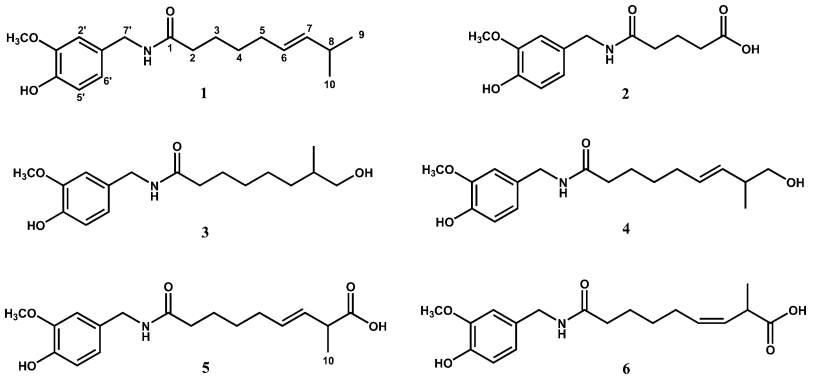

Figure 2. Structures of capsaicin and its metabolites isolated from capsaicin culture.

Table 1. ^{1}H Nuclear Magnetic Resonance Spectroscopic Data of the Capsaicin Metabolites

	δ_H (int, mult, J in Hz)				
position	2	3	4	5	6
1					
2	2.28 (2H, m)	2.22 (2H, t, 7.5)	2.23 (2H, m)	2.21 (2H, t, 7.5)	2.22 (2H, t, 7.5)
3	1.91 (2H, m)	1.63 (2H, m)	1.63 (2H, m)	1.63 (2H, m)	1.62 (2H, m)
4	2.32 (2H, m)	1.30 (2H, m)	1.38 (2H, m)	1.39 (2H, m)	1.40 (2H, m)
5		1.33 (2H, m)	2.02 (2H, m)	2.03 (2H, m)	2.04 (2H, m)
6a		1.39 (1H, m)	5.45 (1H, m)	5.53 (1H, dt, 15.5, 6.0)	5.54 (1H, dt, 12.5, 6.0)
6b		1.06 (1H, m)			
7		1.56 (1H, m)	5.33 (1H, m)	5.48 (1H, dt, 15.5, 7.5)	5.49 (1H, dt, 12.5, 7.0)
8		3.34 (2H, m)	3.40 (1H, m)	3.11 (1H, m)	3.02 (1H, m)
9		0.89 (3H, d, 7.0)	3.32 (2H, m)[a]		
10			0.89 (3H, d, 7.0)	1.20 (3H, d, 7.0)	1.19 (3H, d, 7.0)
2′	6.87 (1H, br s)	6.86 (1H, d, 1.0)	6.86 (1H, d, 1.5)	6.86 (1H, d, 1.5)	6.86 (1H, d, 1.5)
5′	6.73 (1H, d, 8.0)[a]	6.74 (1H, d, 8.0)	6.74 (1H, d, 8.0)	6.74 (1H, d, 8.0)	6.74 (1H, d, 8.0)
6′	6.73 (1H, br d, 8.0)[a]	6.71 (1H, br d, 8.0)	6.71 (1H, dd, 8.0, 1.5)	6.71 (1H, dd, 8.0, 1.5)	6.71 (1H, dd, 8.0, 1.5)
7′	4.26 (2H, s)	4.26 (2H, s)	4.26 (2H, s)	4.26 (2H, s)	4.52 (2H, s)
$-OCH_3$	3.84 (3H, s)	3.83 (3H, s)	3.83 (3H, s)	3.83 (3H, s)	3.82 (3H, s)

[a]The chemical shifts of H-5′ and H-6′ overlapped.

chromatograms showed high purity of fractions J (**3**, t_R 24.5 min, 4.5 mg), K (**4**, t_R 25.5 min, 93.6 mg), and L (a mixture of **5** and **6**, t_R 28.7 min, 12.8 mg). Fraction H (10.6 mg) was purified by HPLC equipped with a μBondapak C18 column (10 μm, 7.8 i.d. × 300 mm) (Waters, Milford, MA, USA) (flow rate, 5.0 mL/min; wavelength, 210 nm; elution, from 50 to 80% MeOH for 40 min) to give **2** (t_R 17.7 min, 1.0 mg). Thin-layer chromatography (TLC) was carried out by using silica gel TLC plates (0.25 mm thickness) (Merck, Rahway, NJ, USA) and developed using a mixture of EtOAc/HOAc/$CHCl_3$ (5:1:2, v/v/v). The spots were detected by UV light (254 nm) and 1% cerium(IV) sulfate ethanol solution spray.

Structural Analysis. NMR spectra were obtained with a ^{unit}INOVA 500 spectrometer (Varian, Walnut Creek, CA, USA). Methanol-d_4 (CD_3OD) (Acros Organics, Morris Plains, NJ, USA) containing tetramethylsilane was used as an analytical solvent. The mass spectra were acquired on a SYNAPT G2 hybrid (Waters, Cambridge, UK) and LCMS-8030 (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization source (ESI-MS).

HPLC ESI MS/MS Analysis of the Capsaicin Metabolites Identified in Gochujang. Freeze-dried gochujang (1 g) was homogenized in MeOH (20 mL, twice). The mixture was filtered under vacuum through no. 2 filter paper (Whatman, Maidenstone, UK). The MeOH solutions were combined and concentrated under vacuum at 38 °C. The extracts were suspended in distilled water (10 mL) and partitioned with EtOAc (10 mL, three times). The EtOAc fraction was evaporated under vacuum at 38 °C and dissolved in MeOH/$CHCl_3$ (1:1, v/v, 4 mL), and then the fraction was analyzed using a high-performance liquid chromatography/electrospray ionization tandem mass spectrometer (HPLC-ESI/MS) (Shimadzu). Capsaicin and its metabolites (**1**−**6**) were separated under the chosen HPLC conditions [column, MG III (C18, 3 μm, 3.0 i.d. × 100 mm) (Shiseido); column temperature, 35 °C; flow rate, 0.3 mL/min (Shimadzu)]. The sample was eluted using a gradient system of H_2O (solvent A) to MeCN (solvent B) (both containing 0.3% formic acid), starting with 100% B, increasing to 20% A for 8 min, holding to 20% A for 16 min, increasing to 50% A for 30 min, increasing to 80% A for 35 min, increasing to 100% A for 40 min, and holding at 100% A for 45 min. The mass spectrometer was set up respectively for multiple reaction monitoring (MRM) with a dwell time of 0.1 s per transition to monitor the capsaicin metabolites: m/z 304.1 $[M - H]^-$ → 168.2 for **1**; m/z 268.3 $[M + H]^+$ → 137.1 for **2**, m/z 310.1$[M + H]^+$ → 174.3 for **3**, m/z 320.1$[M - H]^-$ → 184.1 for **4**, m/z 334.0 $[M - H]^-$→ 154.2 for **5** and **6**. The optimal MS conditions for **1**−**6** were employed: ESI source voltage, 3.5 kV; detector voltage, 45 V; heat block temperature, 400 °C; desolvation line temperature, 250 °C. Nebulizing gas and drying gas flows were 3.0 and 15.0 L/min, respectively. Argon was used as the collision gas at a pressure of 230 kPa. The optimized collision energies for **1**−**4** and **5** and/or **6** were 13.0, −16.0, −11.0, 13.0, and 19.0 V, respectively.

The contents of **1**−**6** in gochujang were quantitatively analyzed by HPLC ESI MS/MS analysis. Sample and standard solutions were prepared just before analysis. The calibration curves (n = 6) were constructed using **1**−**6** (0.1−50 ng) that were isolated from capsaicin broth incubated with *A. oryzae*. Accuracy and reproducibility were evaluated using the standard spike method. External standards of capsaicin and **1**−**6** were added to aliquots of gochujang at three

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concentrations to determine their precision. The quantification and quantitation of capsaicin and its metabolites **1**−**6** in gochujang were performed in triplicate.

RESULTS AND DISCUSSION

Changes in Capsaicin and Its Metabolites in the Medium during *A. oryzae* Incubation. *A. oryzae* was incubated with capsaicin in PDB at 30 °C for 14 days. The supernatants (1 mL) obtained with the passage of time were extracted with EtOAc, and the H_2O and EtOAc fractions were analyzed by reversed-phase-HPLC. In the EtOAc fraction, the capsaicin peak decreased gradually depending on the incubation period (Figure 1). New metabolites were detected from 6 days of incubation, and then capsaicin was detected at detection limit concentration (100 pmol) at 14 days of incubation. No chemical transition was observed from H_2O fraction (data not shown), suggesting that capsaicin metabolites converted by *A. oryzae* were extracted in the EtOAc fraction. However, no chemical change of capsaicin was observed in PDB without *A. oryzae* at 30 °C for 14 days (data not shown).

Isolation and Identification of the Capsaicin Metabolites Converted by *A. oryzae*. Capsaicin was incubated with *A. oryzae* for 14 days, and the culture was solvent-fractionated to obtain the EtOAc fraction. Five converted metabolites **2**−**6** were purified and isolated from the EtOAc fraction of the capsaicin culture by ODS open-column chromatography and ODS-HPLC. The structures of the converted compounds were determined by NMR and ESI-MS analyses.

The molecular weight of metabolite **2** was determined to be 267, as established by a pseudomolecular ion peak at m/z 268.3 $[M + H]^+$ in the ESI-MS (positive) spectrum. The ^1H NMR spectrum of **2** was partially similar to that of **1**.[16] The ^1H NMR spectrum exhibited three trisubstituted benzene ring proton signals at δ 6.87 (1H, br s, H-2′), 6.73 (1H, d, J = 8.0 Hz, H-5′), and 6.73 (1H, d, J = 8.0 Hz, H-6′), one methoxyl proton signal at δ 3.84 (3H, s, $-OCH_3$), and one nitrogen-bearing methylene proton signal at δ 4.26 (2H, s, H-7′) (Table 1), indicating a vanillylamine moiety as a partial capsaicin structure. In addition, three sp^3 methylene carbon protons at δ 2.28 (2H, m, H-2), 1.91 (2H, m, H-3), and 2.32 (2H, m, H-4) were observed in the ^1H NMR spectrum. This result was also supported by the ^{13}C NMR spectrum, which contained 13 carbon signals including 7 sp^2 carbons with 5 quaternary carbons at δ 177.0−112.5 and 4 sp^3 carbons at δ 56.5−12.8 (Table 2). Considering the molecular weight of **2**, the chemical shifts in five sp^2 quaternary carbons indicated that one was a nitrogen-bearing carbonyl carbon at δ 175.3 (C-1) with evidence of a carbonyl carbon at δ 177.0 (C-4). Metabolite **2** was suggested to be *N*-vanillylcarbamoylbutanoic acid based on the ESI-MS and 1D NMR spectra. The complete NMR assignment and connectivity of **2** were further studied by $^1H-^1H$ correlation spectroscopy ($^1H-^1H$ COSY), heteronuclear single-quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC) experiments. Vanillyl and carbamoylbutanoic acid moieties were confirmed by the 2D NMR spectra. In particular, H-7′ was correlated with C-1 in the HMBC spectrum (Figure 3), indicating a vanillyl group was connected to the C-1 position of carbamoylbutanoic acid. Consequently, the structure of metabolite **2** was unambiguously determined to be *N*-vanillylcarbamoylbutanoic acid (Figure 2).

The molecular weight of metabolite **3** was 309 based on the pseudomolecular ion peaks detected at m/z 332.1 $[M + Na]^+$ and 310.1 $[M + H]^+$ in the ESI-MS (positive) spectrum, and the molecular formula ($C_{17}H_{27}NO_4$) was established by the HRESI-MS data (m/z 310.2021 $[M + H]^+$, calculated for $C_{17}H_{28}NO_4$, m/z 310.2018, +0.3 mDa). The ^1H NMR spectrum of **3** was related to that of **1**.[18] However, the ^1H NMR spectrum of **3** exhibited two upfield-shifted sp^3 methylene proton signals at δ 1.30 (2H, m, H-6) and 1.56 (1H, m, H-7) and one downfield-shifted sp^3 oxygenated methylene proton signal at δ 3.34 (2H, m, H-9′) (Table 1), compared to the proton signals of the olefinic double bond and the methyl group in capsaicin. The ^{13}C NMR spectrum revealed the presence of 17 carbon signals, including 7 sp^2 carbons with 4 quaternary carbons at δ 176.2−112.6 and 10 sp^3 carbons at δ 68.5−17.2 (Table 2). The side chain of **3** was suggested to be hydroxymethyloctanamide on the basis of the ESI-MS and 1D NMR spectra. The complete NMR assignment and connectivity of **3** were further determined by 2D NMR. The important correlations observed from the HMBC experiment are shown in Figure 3. In particular, correlations between H-9 and C-7 and C-8 were observed in the HMBC spectrum, indicating that the side chain of **3** was 8-hydroxy-7-methyloctanamide (Figure 3). Therefore, the structure of metabolite **3** was *N*-vanillyl-9-hydroxy-8-methyloctanamide (Figure 2), which is a new compound.

The molecular weight of metabolite **4** was 321 on the basis of the pseudomolecular ion peak detected at m/z 320.1 $[M - H]^-$ in the ESI-MS (negative) spectrum. The ^1H and ^{13}C NMR spectra of **4** were similar to those of **1**, except for an oxygenated methylene group. An oxygenated methylene proton and carbon signals were detected at δ 3.34 (2H, m, H-9) in the ^1H NMR spectrum (Table 1) and at δ 68.5 (C-9) in the ^{13}C NMR spectrum (Table 2), suggesting that **4** is a hydroxylated capsaicin (ω-hydroxycapsaicin). The ^1H and ^{13}C NMR spectra of **4** were consistent with those of ω-hydroxycapsaicin, which was isolated from *Capsicum annuum* (Banshou) previously.[19] Therefore, **4** was identified as ω-hydroxycapsaicin (Figure 2).

The molecular weight of fraction L was 335 on the basis of the pseudomolecular ion peak detected at m/z 334.2 $[M - H]^-$ in the ESI-MS (negative) spectrum, and the molecular formula ($C_{18}H_{25}NO_5$) was established by the HRESI-MS data (m/z

Table 2. ^{13}C NMR Nuclear Magnetic Resonance Spectroscopic Data of the Capsaicin Metabolites

	δ_C				
position	**2**	**3**	**4**	**5**	**6**
1	175.3	176.2	176.1	176.0	176.0
2	36.3	37.2	37.1	37.0	37.0
3	12.8	27.2	26.7	26.7	26.6
4	22.5	30.7	30.2	29.9	29.9
5	177.0	27.9	33.5	33.3	33.2
6		34.4	131.2	131.0	130.6
7		36.9	131.7	132.7	133.0
8		68.5	40.8	44.0	44.0
9		17.2	68.4	179.0	177.3
10			17.4	18.1	18.0
1′	142.1	131.7	134.5	131.6	131.6
2′	112.5	112.6	112.6	112.5	112.6
3′	149.2	149.1	149.1	149.1	149.1
4′	147.0	146.9	146.9	146.9	146.9
5′	116.2	116.2	116.2	116.2	116.2
6′	121.6	121.5	121.5	121.5	121.5
7′	44.1	44.1	44.1	44.1	44.2
$-OCH_3$	56.5	56.5	56.5	56.5	56.5

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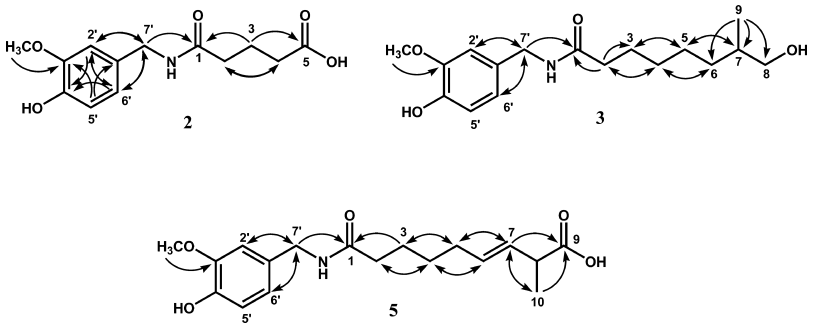

Figure 3. Heteronuclear multiple-bond correlation (HMBC) correlations (arrows) for **2**, **3**, and **5**.

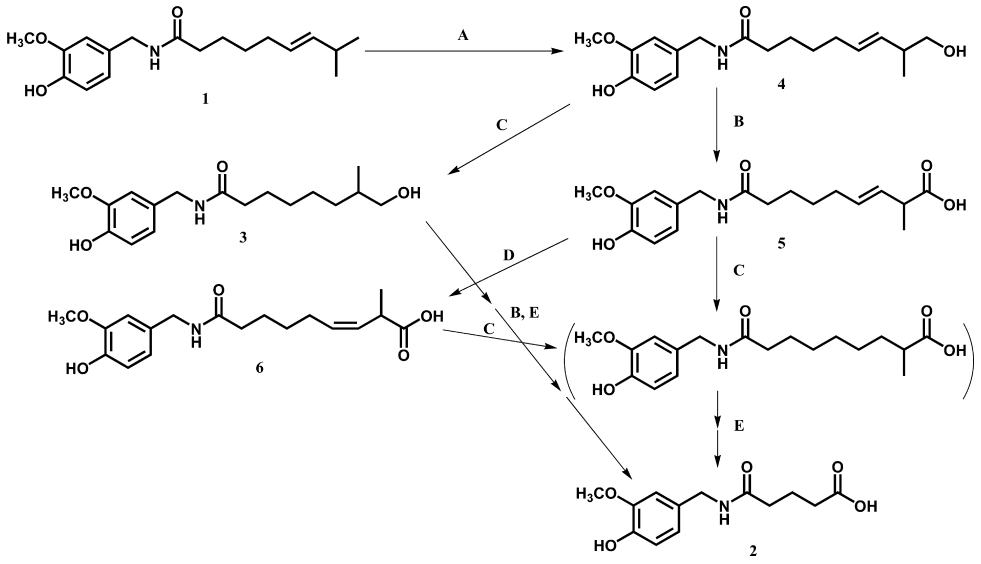

Figure 4. Proposed bioconversion mechanisms of the metabolite conversion from capsaicin by *A. oryzae*: (A) ω-hydroxylation; (B) alcohol oxidation; (C) hydrogenation; (D) isomerization; (E) α- and β-oxidations.

334.1655 $[M - H]^-$, calculated for $C_{18}H_{24}NO_5$, m/z 334.1654, +0.1 mDa). The ^1H and ^{13}C NMR spectra of fraction L showed evidence of a mixture of two compounds. These data suggest that the skeletal structure was similar, but the olefinic double bond differed. The ^1H NMR spectrum of fraction L had unequal methine proton signal intensity at a ratio of 2.5:1 (the main metabolite, **5**; the minor metabolite, **6**). The ^1H and ^{13}C NMR spectra of **5** were related to those of **1** and **4**, except for a carbonyl group. A carbonyl carbon signal at δ 179.0 (C-9) was observed in the ^{13}C NMR spectrum (Table 2), indicating that **5** contained a carboxylic group rather than a methyl alcohol group contained in **4**. The ESI-MS and 1D NMR results suggested that **5** was methyl-*N*-vanillylcarbamoyloctenoic acid. The complete NMR assignment and connectivity of **5** were further determined by 2D NMR. The olefinic double-bond position was side chain C-6 and C-7, based on the proton−proton correlations in the $^1H-^1H$ COSY experiment and the proton−carbon correlations in the HMBC experiment. In particular, correlations (Figure 3) of H-10 and H-7 to C-9 and of H-10 to C-7 were observed in the HMBC spectrum, indicating that the side chain of **5** was determined to be 8-methylcarbamoyl-6-octenoic acid (Figure 2). The olefinic double-bond signals of **5** overlapped with those of **6**. The olefinic double-bond signals of **5** were confirmed by the NOE experiment. The proton signals of the methyl group at δ 1.20 (H-10) and the olefinic double bond at δ 5.48−5.54 (H-6 and H-7) were enhanced when the proton signal of H-8 at δ 3.11was irradiated. The geometry of the olefinic double bond at C-6 and C-7 was the *trans* form, on the basis of their coupling constant value (15.5 Hz). Therefore, the structure of metabolite **5** was 8-methyl-*N*-vanillylcarbamoyl-6(*E*)-octenoic acid (Figure 2).

The ^1H and ^{13}C NMR spectra of metabolite **6** were closely related to those of metabolite **5**, except for different chemical shifts in the methine proton and olefinic double-bond carbon signals (Table 1). In particular, methine proton signals at δ 3.02 (H-8) were observed at 0.09 ppm upfield, the C-7 signal at δ 133.0 was shifted by 0.3 ppm, and the signals of C-6 at δ 130.6 and of C-9 at δ 177.3 were shifted by above 0.4 ppm compared to the ^1H and ^{13}C NMR spectra of **5**. The complete NMR assignment and connectivity of **6** were further confirmed by the $^1H-^1H$ COSY, HSQC, and HMBC experiments. The geometry of the olefinic double bond at C-6 and C-7 was the *cis* form, based on the NOE data and their coupling constant value (12.5 Hz). Consequently, the structure of metabolite **6** was 2-methyl-*N*-vanillylcarbamoyl-6(*Z*)-octenoic acid (Figure 2).

The structures of five metabolites converted from capsaicin by *A. oryzae* were determined by NMR and MS analyses. Metabolites **2**, **4**, and **5** have already been reported as capsaicin metabolites converted by *A. niger*.[15,16] However, the NMR study for the capsaicin metabolites **2** and **5** converted by *A. niger* was insufficient. Therefore, we performed complete assignments of them using NMR analysis to provide data as models for establishing the structures of the capsaicin

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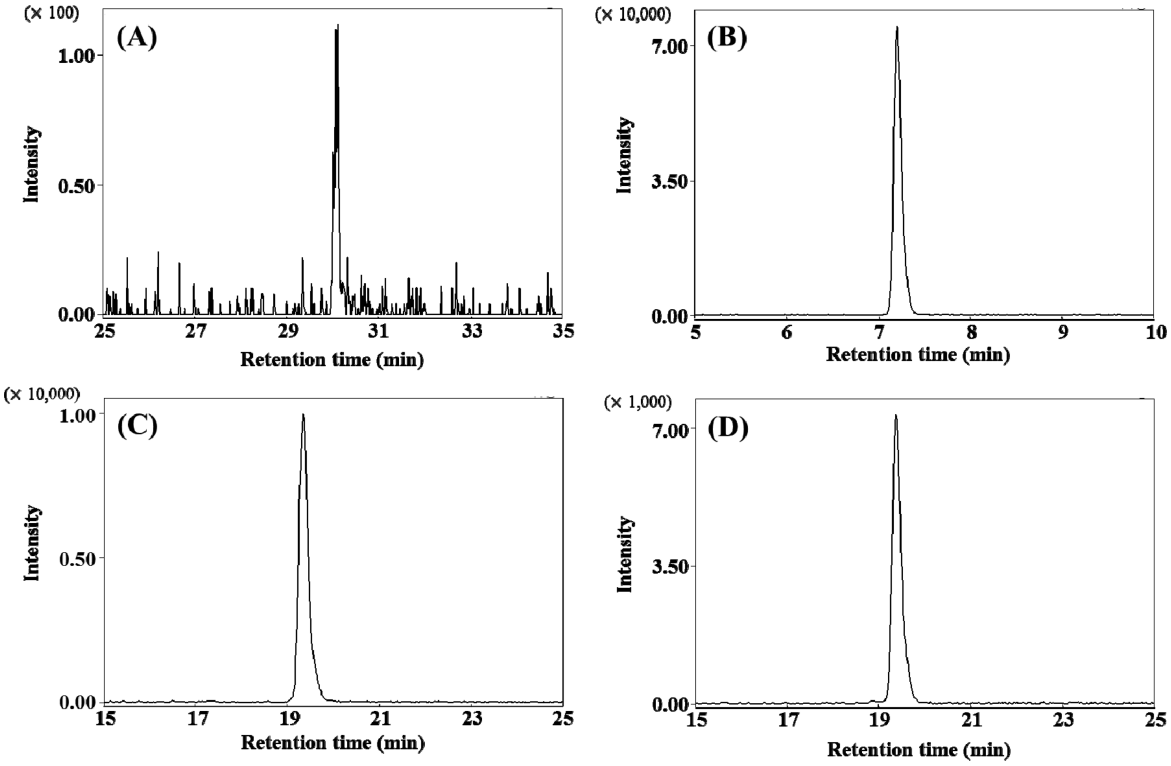

Figure 5. Multiple reaction monitoring chromatograms of (A) **1**, (B) **2**, (C) **4**, and (D) **5** and/or **6** in gochujang. Capsaicin and its metabolites were analyzed by LC-ESI-MS/MS. Capsaicin metabolites were detected by selective multiple reaction monitoring (MRM): m/z 304.1 $[M - H]^-$ → 168.2 for **1**; m/z 268.3 $[M + H]^+$ → 137.1 for **2**; m/z 320.1 $[M - H]^-$ → 184.1 for **4**; m/z 334.0 $[M - H]^-$→ 154.2 for **5** and **6**. Detailed procedures for LC-ESI-MS/MS analysis have been described under Materials and Methods.

metabolites in future research. Among the five metabolites, **3** and **6** are new capsaicin metabolites first reported in this study. *Streptomyces mobaraensis* and *Actinoplanes utahensis* hydrolyze capsaicin to vanillylamine and *trans*-8-methyl-6-nonenoic acid.[10−14] However, capsaicin hydrolysates (vanillylamine and *trans*-8-methyl-6-nonenoic acid) were not identified in this study. These observations indicate that capsaicin hydrolysis may not be a predominant pathway in *A. oryzae*. The conversion mechanisms by *A. oryzae* of capsaicin metabolites are proposed in Figure 4. Metabolite **4** was produced by the hydroxylation of capsaicin and was converted to **3** and **5** by β-oxidation and alcoholic oxidation, respectively. In addition, metabolite **6** was probably degraded from **5** by α- and β-oxidations via isomerization and/or hydrogenation, and **2** was likely produced from **3** by α- and β-oxidations.

Qualitative and Quantitative Analyses of the Capsaicin Metabolites in Gochujang. Capsaicin and its metabolites **1**−**6** in gochujang were quantitated by HPLC-ESI-MS using the isolated compounds as external standards. The EtOAc fraction obtained after solvent fractionation from gochujang MeOH extracts was analyzed by selective MRM detection and MS/MS. The capsaicin metabolites **1**, **2**, **4**, and **5** and/or **6** were detected at t_R 30.8, 7.2, 19.3, and 19.4 min on the MRM chromatogram of gochujang (Figure 5). The metabolites were identified using the retention times of authentic compounds **1**, **2**, **4**, and **5** and/or **6**. In this study, the identity of **5** and **6** on the EtOAc fraction MRM chromatogram could not be determined because **5** and **6** were a mixture when they were purified, suggesting that **5** and **6** are present in gochujang. However, metabolite **3** (t_R 16.0 min; limit of detection, 0.5 ng) was not detected on the MRM chromatogram of gochujang, suggesting no production of it or rapid conversion to other capsaicin metabolites, although this compound is produced during fermentation of gochujang. These results indicate that capsaicin metabolites **2**, **4**, and **5** and/or **6** are unambiguously present in gochujang.

Capsaicin and its metabolites **1**, **2**, **4**, and **5** and/or **6** were also analyzed quantitatively by selective MRM detection and MS/MS. The external calibration curve of each compound at a concentration range of 0.1−50 ng produced a good linear correlation ($R^2 > 0.99$). The dilution ratio of gochujang extract in MeOH/$CHCl_3$ was 1:800 (w/v). The repeatability of each metabolite ranged from 1.1 to 2.6%: **1**, 1.1%; **2**, 1.9%; **4**, 2.6%; and **5** and/or **6**, 2.4%. The recoveries of **1** (99.0 ± 1.1%), **2** (99.5 ± 1.9%), **4** (100.3 ± 2.6%), and **5** and/or **6** (101.0 ± 2.4%) ranged from 99.0 to 101.1%. Of the capsaicin metabolites, **5** and **6** (5607.8 ± 53.0 μg/100 g dry wt) were most abundantly found in gochujang, yet the abundance was lower than that of capsaicin (7671.4 ± 278.6 μg/100 g dry wt) (Table 3). Metabolites **2** (74.8 ± 1.4 μg/100 g dry wt) and **4**

Table 3. Contents of Capsaicin and Its Metabolites in Gochujang

metabolite	content[a] (μg/100 g dry wt)
1	7671.4 ± 278.6
2	74.8 ± 1.4
3	ND
4	38.1 ± 0.9
5 and/or **6**	5607.8 ± 53.0

[a]Data are expressed mean ± standard deviation of triplicate experiments.

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(38.1 ± 0.9 μg/100 g dry wt) were present in small amounts in gochujang compared to **5** and/or **6**. In this study, most capsaicin was converted when *A. oryzae* was incubated with capsaicin in PDB at 30 °C for 14 days. However, capsaicin was found at greater content than capsaicin metabolites in gochujang product. It is considered because gochujang is a high viscous paste that is fermented from a primary mixture of red hot pepper powder, waxy rice flour, fermented soybean, and koji. Although capsaicin metabolite-related enzymes were secreted from *A. oryzae* during the fermentation of gochujang, the amounts and activities of the secreted enzymes may be relatively lower than those in PDB culture. Therefore, capsaicin may be converted slowly to capsaicin metabolites during the fermentation of gochujang. The varieties and contents of capsaicin metabolites in gochujang may be dependent upon the fermentation microorganisms and the extent of fermentation.

The contents of capsaicinoids in hot pepper were 46−77% for capsaicin, 21−40% for dihydrocapsaicin, 2−12% for nordihydrocapsaicin, and >2% for other capsaicin derivatives.[1,18] According to the results of a capsaicinoid sensory evaluation, the pungent intensity decreases in the order **1** (100) = dihydrocapsaicin (100) > nordihydrocapsaicin (57) > homodihydrocapsaicin (50) > homocapsaicin (43).[20] In addition, *N*-nonanoylvanillylamide, which contains a nine-carbon side chain, has the most pungent taste.[21] However, the pungency of capsaicin derivatives, which have more variable carbon side chains than that of *N*-nonanoylvanyllylamide, is lower.[22,23] Thus, the pungency of capsaicin derivatives may depend on the amide acylation and carbon side chain length. In addition, ω-hydroxycapsaicin has slightly weaker pungency than **1**.[19] All capsaicin metabolites identified in this study contain the carboxylic acid or hydroxyl groups, and **2** and **3** had fewer carbons in their side chain than **1**. Therefore, capsaicin metabolites are likely to be less pungent than capsaicin, although pungency was not evaluated in this study.

The five capsaicin metabolites converted by *A. oryzae* were elucidated by NMR and MS analyses. Also, the capsaicin metabolites in gochujang were all found except for **3**. Therefore, the capsaicin metabolites are likely produced from **1** during gochujang fermentation, and the product becomes less pungent than red hot pepper. These results will provide useful information to improve the quality and biological activity of gochujang to control pungency during fermentation. New alternatives for capsaicin products in food and pharmaceutical applications can be suggested for future study.

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