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ARTICLE

Biodegradation of Capsaicin by *Bacillus licheniformis* SK1230

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Abstract Capsaicin is a major representative component in pepper and it has gained interest by its health beneficial effects. An enzymatic hydrolysate of capsaicin has also been of great interest, because it can be applied to the reduction pungency of pepper or production of natural flavor. We first developed a simple screening method to isolate capsaicin-degrading bacteria showing a clear zone. A cloudy solid medium was prepared by the addition of capsaicin as sole carbon and nitrogen sources to 3-(Nmorpholino) propanesulfonic acid (MOPS) minimal medium, and the bacteria showing clear zone around the colony was isolated from Korean traditional pickled pepper. The isolated strain was identified as *Bacillus licheniformis* using 16S rRNA gene sequence analysis. *B. licheniformis* SK1230 was able to utilize capsaicin for both of carbon and nitrogen sources for its growth. The hydrolysis of capsaicin by *B. licheniformis* was verified using high-performance liquid chromatography, and capsaicin in medium was depleted depending on culture time. Capsaicin hydrolysate was composed of vanillylamine and capsiate (8-methyl-6-*trans*-nonenoic acid).

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Introduction

Hot pepper is fruit of *Capsicum* and has been used as a food, spice, and medicine. The pungency of *Capsicum* gives rise to wide range of application, and its strong taste is caused by capsaicinoid, a lipophillic alkaloid capsaicin or its analogues. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is a major component of pepper and has shown various biological activities such as thermogenesis, improved fat utilization, interaction with sensory neuron, and thermal pain-mimicking (Castillo et al., 2007; Lee et al., 2010; De Petrocellis et al., 2011). It is also reported that capsaicin can be a good precursor compound for the production of natural flavor, particularly vanillin (Van den Heuvel et al., 2001). For the application of capsaicin, structural change should be advanced, because the strong pungency of capsaicin can restrict its use. For a bioactive compound, vanillin is the product of enzymatic conversion of capsaicin (Romano et al., 2011). Vanillin can be produced by the action of two enzymes, acylase that hydrolyzes capsaicin or dihydrocapsaicin into vanillylamine and amino oxidase that produces vanillin from vanillylamine. Capsaicin analogues with long alkyl side chains have no pungency, but they can stimulate adrenaline secretion (Wang et al., 2009). Capsiate, a capsaicin analogue included in non-pungent *Capsicum*, is reported to have similar bioactivities to those of capsaicin (Inoue et al., 2007). Therefore, biological or biochemical conversion including substitution or breakdown of capsaicin was employed to develop it as a commercially bioactive compound.

For biological conversion of capsaicin, microorganisms have been isolated and their enzymes were studied. As bacteria for hydrolyzing capsaicin, *Variovorax paradoxus* from pepper and

tomato leaf was isolated, and the activity of capsaicin hydrolysis using acylase was reported (Flagan and Leadbetter, 2006). *Bacillus subtilis* isolated from Korean pickled pepper was reported to hydrolyze capsaicin, although the related enzyme has not yet been revealed (Lee et al., 2010). For yeast, *Candida antarctica* was reported to secrete lipase B, which could degrade capsaicin (Torres et al., 2009). For fungi, *Actinoplanes utahensis* producing acylase and *Streptomycin mobaraensis* producing penicillin V acylase were reported to hydrolyze capsaicin (Zhang et al., 2007; Romano et al., 2011). In enzymatic production of vanillin from capsaicin, the flavoprotein vanilly alcohol oxidase (VAO; EC 1.1.3.38) was reported to effectively produce vanillin from vanillyamine, hydrolysate of capsaicin by the action of acylase (Van den Heuvel et al., 2001).

In the present study, we aimed to isolate bacteria degrading capsaicin from Korean traditional pickled pepper and verify the hydrolysis of capsaicin using high-performance liquid chromatography (HPLC).

Materials and Methods

Medium and culture conditions. For the isolation of bacteria degrading capsaicin, we used 3-(Nmorpholino) propanesulfonic acid (MOPS)-capsaicin solid medium consisted of MOPS powder 8.37, tricine 0.07, NaCl 0.3, NH_4Cl 0.05, KOH 0.16, K_2SO_4 0.28 mM, $MgCl_2$ 0.53 mM, K_2HPO_4 2 mM, Bacto agar 1.6% (w/v) and 0.05 mM (*z*) capsaicin ((*Z*)-N-[(4-hydroxy-3-methoxyphenyl) methyl]-8-methyl-6-nonenamide, Tocris Bioscience, USA) (Neidhardt et al., 1974). For the validation of capsaicin degradation using HPLC, bacillus minimal medium (BMM) consisting of $Na_2HPO_4 \cdot 7H_2O$ 33.5 mM, KH_2PO_4 22 mM, NaCl 8.6 mM, NH_4Cl 18.7 mM, $MgSO_4$ 1 mM, $CaCl_2$ 0.1 mM, glucose 10 mM (Brans et al., 2004), and (*z*) capsaicin 0.05 mM were used. The growth of isolate in BMM supplemented with capsaicin (0.05 mM) and glucose (10 mM) was determined using spectrophotometer (Shimazu, Japan) at 600 nm, and the remaining glucose in culture supernatant during growth was analysed by glucose assay kit (GAGO20, Sigma Aldrich, USA) according to manufacturer's recommendation. Maintenance and enrichment of isolate was performed using LB broth or solid medium (Difco™, BD, USA). Culture conditions for screening, isolation, enrichment, and maintenance were 30°C with 150 rpm agitation for 20 h incubation.

Screening of bacteria degrading capsaicin. Bacteria degrading capsaicin was screened and isolated on modified MOPS minimal medium that contained (*z*) capsaicin as sole carbon and nitrogen sources. Korean pickled pepper was used for bacterial isolation and diluted with sterilized 0.8% (w/v) NaCl solution. Diluted samples were spread on the screening medium and then incubated until colonies appeared. The colonies showing clear zone were selected as candidates of bacteria-degrading capsaicin and isolated.

Taxonomic identification. To identify the isolated strain, 16S rRNA gene sequence was analysed by polymerase chain reaction using universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG- 3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Weisburg et al., 1991), and the sequence of PCR product was determined by ABI PRISM 3730XL DNA analyser (Applied Biosystem, USA). Multiple alignment was performed using CLUSTRAL W program (Thompson et al., 1994) with 16S rRNA gene sequences of type strains involved in the isolate as a result of previous sequence comparison in GenBank. The evolutional distance for determination and phylogenetic tree building were conducted using Maximum Composite Likelihood method in MEGA 4 program (Tamura et al., 2004; Tamura et al., 2007). For biochemical test relevant to carbohydrate utilization profile, API 50 CH kit (bioMérieux, France) was used.

Analysis of capsaicin degradation. For the validation of capsaicin degradation, the remaining capsaicin concentration was determined using HPLC in the culture of BMM supplemented with glucose (10 mM) and capsaicin (0.05 mM). The culture supernatants taken according to incubation time intervals (4, 8, and 25 h) were prepared by centrifugation (10,000 rpm, 10 min) and used for analysis. HPLC (Waters delta prep 4400, USA) equipped Capselpak C18 column and absorbance detector (model 486; Waters) was employed, and capsaicin separation was performed according to ASTA method with slight modification (Choi and Ko, 1990). For analysis condition, mobile phase used are 0.1% acetic acid in 70% methanol in isocratic, and the flow rate was 0.7 mL/min. The wavelength for detection was 280 nm. For the investigation of the cleavage site in capsaicin molecule, MS (mass spectrophotometer, Thermo Quest Instrument, USA) combined with HPLC was used. MS was equipped with an electrospray ionization source, and selected-reaction monitoring mode was operated for the detection of hydrolysed derivatives from capsaicin according to Reilly et al. (2002).

Results and Discussion

Isolation and identification of bacteria utilizing capsaicin. For the isolation of bacteria that were able to degrade capsaicin, the colony showing a clear zone was selected on MOPS-capsaicin solid agar medium. Capsaicin is water insoluble but is soluble in dimethyl sulfonic acid (DMSO) up to 50 mM. The turbidity of medium is gradually increased according to the addition rate of capsaicin to medium. However, capsaicin has an antibacterial activity that restricts the rate of addition to medium. To reconfirm that the appearance of clear zone was caused by the function of extracellular enzyme produced from the isolate, 100 μL of the culture supernatant grown in LB broth overnight was loaded on BMM-capsaicin solid medium and incubated overnight. Finally, culture supernatant of the isolate showed clear zone with shape of moon halo (Fig. 1). Capsaicin concentration of medium in the present study was optimized to 50 μM. The concentration showed a clear zone around colony without the effect of antibacterial activity. It was reported that above 150 μM of capsaicin completely

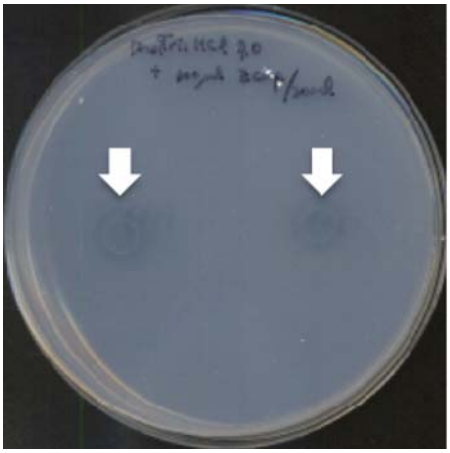

Fig. 1 Appearance of clear zones by culture supernatant. Culture supernatant of *Bacillus licheniformis* SK1230 was dropped on bacillus minimal medium.

inhibited the growth of *Helicobacter pyrori* (Yildiz Zeyrek and Oguz, 2005). The 16S rRNA of the isolate was sequenced and a phylogenetic tree was built based on gene sequences of seven different type strains in *Bacillus* genus (Fig. 2). The phylogenetic analysis showed the isolate was closely related to *Bacillus licheniformis* NBRC12200T [AB680255] with 99.5% (1312/1318 bp) identity and was designated as *Bacillus licheniformis* SK1230 [JQ864313]. Carbohydrate utilization profiles of *B. licheniformis* SK1230 were tested by API50 CH kit (Table 1). *B. licheniformis* is a widespread saprophytic bacterium involved in GRAS status, and it has been employed in various industrial fields for the production of natural compounds such as foods, enzymes, bacteriocin among other compounds (He et al., 2006).

Growth of *B. licheniformis* SK1230 by caspaicin utilization as carbon and nitrogen sources. We further investigated in detail how *B. licheniformis* SK1230 can utilize (*z*)-capsaicin as a substrate for its growth. Four different kinds of solid media were prepared as described in Table 2. *B. licheniformis* SK1230 showed growth in the tested medium after 24 h incubation at 37°C, suggesting the utilization of either carbon (medium B) or nitrogen (medium C) sources. As shown in medium D, in the absence of glucose and NH_4Cl, *B. licheniformis* SK1230 also showed the growth with utilizing capsaicin only as carbon and nitrogen sources.

Table 1 Biochemical characteristics of isolate SK1230

Substrate	Utilization[1]	Substrate	Utilization
Control	-	Galactose	-
Glycerol	+	Glucose	+
Erythritol	-	Fructose	+
D-Arabinose	-	Mannose	+
L-Arabinose	+	SorBosE	-
Ribose	+	Rhamnose	-
D-Xylose	+	Dulcitol	-
L-Xylose	-	Inositol	-
Adonitol	-	Mannitol	+
β-Methyl-D-xyloside	-	Sorbitol	-
α-Methyl-D-mannoside	-	Melibiose	-
α-Methyl-D-glucoside	+	Sucrose	+
N-Acetyl-glucosaine	+	Trehalose	+
Amygdalin	+	Inulin	+
Arbutin	+	Melezitose	-
Esculin	+	Raffinose	+
Salicin	+	Starch	+
Cellobiose	+	Glycogen	+
Maltose	+	Xylitol	-
Lactose	-	Gentiobiose	+
D-Turanose	+	D-Arabitol	+
D-Lyxose	+	L-Arabitol	-
D-Tagatose	+	Gluconate	-
D-Fucose	-	2-Keto-gluconate	-
L-Fucose	-	5-Keto-gluconate	-

[1].+ and – mean utilize and non-utilize tested carbohydrate, respectively.

Degradation of capsaicin by *B. licheniformis* SK1230. For the verification of capsaicin degradation, *B. licheniformis* SK1230 was cultured in BMM supplemented with 0.05 mM of (*z*) capsaicin and 10 mM of glucose for 48 h. The growth of *B. licheniformis* SK1230 and its glucose utilization are shown in Fig. 3. Glucose was added in BMM to stimulate initial growth of the strain. Optical density of cells gradually increased until 9 h of incubation and then the growth was temporary ceased from 9 to 12 h of incubation. During this period, glucose was depleted and

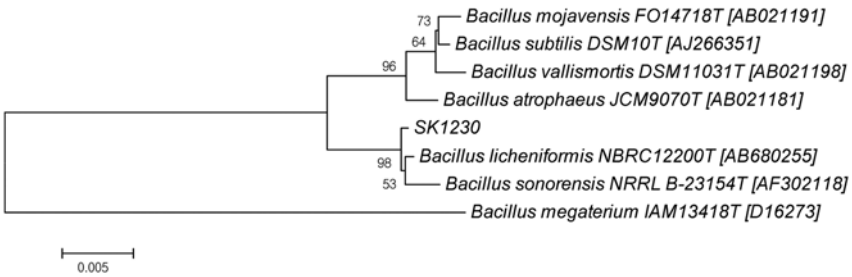

Fig. 2 Phylogenetic tree based on the 16S rRNA gene sequence of isolate SK1230 and other type strains of bacilli. Bootstrap values (based on 1,000 trials and only values >50%) are shown at the nodes. Bar denotes 0.01 substitutions per nucleotide position.

Table 2 Capsaicin utilization as carbon and nitrogen sources on the growth of *B. licheniformis* SK1230

Medium	Glucose, g/l	NH_4Cl, g/l	(*z*) Capsaicin, μM	Growth
A	0.2	0.1	-	+
B	-	0.1	50	+
C	0.02	-	50	+
D	-	-	50	+

Basal medium was composed of 33.5 mM $Na_2HPO_47H_2O$, 22 mM KH_2PO4, 8.6 mM NaCl, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$.

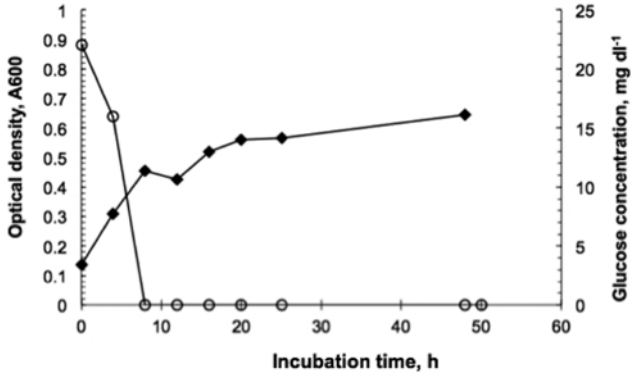

Fig. 3 Growth and glucose utilization of *Bacillus licheniformis* SK1230. Cultivation was performed in bacillus minimal medium containing (*z*) capsaicin and 10 mM glucose. Empty circle and filled diamond indicate glucose concentration and optical density, respectively.

B. licheniformis SK1230 appeared to change substrate from glucose to (*z*) capsaicin for subsequent growth. The concentration

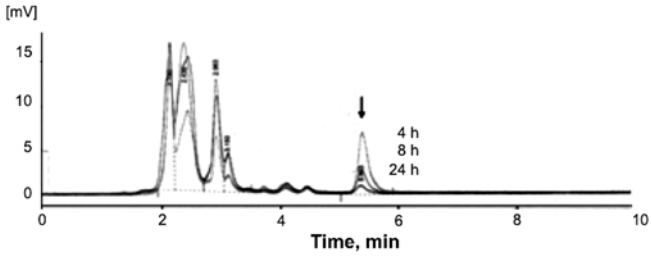

Fig. 4 Chromatography of capsaicin content in culture supernatant of *Bacillus licheniformis* SK1230 in minimal medium containing (*z*) capsaicin.

of remaining capsaicin was determined (Fig. 4). The peak (retention time 5.4 min) representing capsaicin decreased depending on incubation time.

The culture supernatant at the end of incubation (48 h) was analysed using HPLC-MS to investigate the molecular weight of capsaicin hydrolysates. Two compounds showing the molecular weights 154 and 170 were detected (Fig. 5). It is expected that *B. licheniformis* SK1230 can cleave both sides of nitrogen atom on capsaicin structure. One of the hydrolysates could be assumed to be vanillylamine or vanillin, and these were reported as products from enzymatic hydrolysis by the action of acylase, amino oxidase, or vanillylamine amidase (Flagan and Leadbetter, 2006; Romano et al., 2011). Penicillin acylase has been supposed to be related to breakdown of capsaicin and production of vanillin (Van den Heuvel et al., 2001). Penicillin amidase (EC. 3.5.1.11) produced by *B. licheniformis* 749 strain was reported to be able to hydrolyze carbon and nitrogen bonds in peptide bond and producing 6 aminopenicillanic acid and phenylacetic acid (PA)

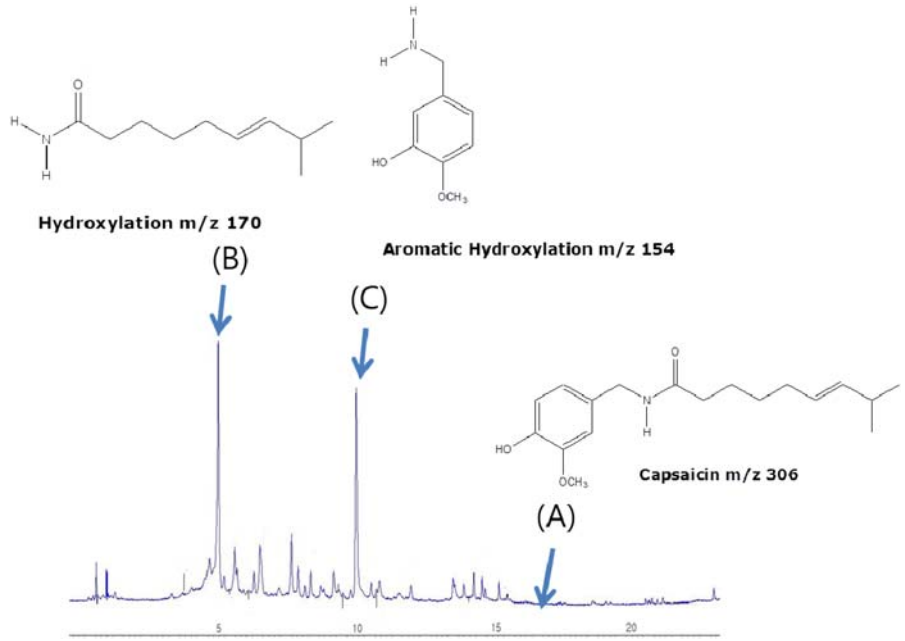

Fig. 5 Mass spectrum showing the products derived from the dissociation of capsaicin by *Bacillus licheniformis* SK1230 in minimal medium.

from penicillin, and one of its other names commonly used is penicillin acylase (Priest, 1977; Ferreira et al., 2004). Penicillin acylases are produced by numerous microorganisms such as *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus spaericus*, *Bacillus subtilis*, *Cryptococcus* sp., *Erwinia aroideae*, *Micrococcus ureae*, *Pseudomonas acidovorans*, *Rhodotorula glutins*, *Penicillium* sp., *Fusarium* sp., *Streptomyces lavendulae*, *Streptomyces mobaraensis, Streptoveticillium* sp., and *Pleurotus ostreatus* via intracellular production or extracellular secretion (Van den Heuvel et al., 2001; Zhang et al., 2007; Kumar et al., 2008). Most of the enzymes are intracellularly retained, and few of them can be excreted extracellularly. To date, there has been no direct report about extracellular secretion of capsaicin degrading enzyme in *Bacillus licheniformis*. In conclusion, it was confirmed that *B. licheniformis* SK1230 produces an enzyme related to capsaicin breakdown extracellularly and may degrade capsaicin into vanillylamine and capsiate (8-methyl-6-*trans*-nonenoic acid). *B. licheniformis* SK1230 can be used for the production of functional capsaicin derivatives or the decrease of strong pungency in pepper-containing food. Molecular and biochemical studies are needed to understand the mechanism of capsaicin degradation in the strain.

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