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Production of α-Glucosidase Inhibitor by β-Glucosidase Inhibitor-Producing *Bacillus lentimorbus* B-6

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Abstract A soil microorganism producing α- and β-glucosidase inhibitors was identified as *Bacillus lentimorbus*, based on the fatty acid and morphological analyses, along with biochemical and physiological tests. The α-glucosidase inhibitor was highly produced by this strain in a culture medium containing 0.25% of sodium glutamate and 0.5% of glucose, pH 8.0 at 30°C for 2 days. The α-glucosidase inhibitor from culture filtrate of this strain was identified as water soluble, organic solvent nonextractable, and heat stable. In addition to α-glucosidase inhibitor, this strain also produced β-glucosidase inhibitor in the same culture medium and this inhibitor showed an antifugal activity against *Botrytis cinerea*. While the production of α-glucosidase inhibitor was decreased by a glucose concentration higher than 1%, the production of β-glucosidase inhibitor was not influenced by a glucose concentration higher than 20%. The α-glucosidase inhibitor from culture filtrate of this strain was separated from the β-glucosidase inhibitor through Sephadex G-100 column chromatography.

Key words: *Bacillus lentimorbus*, α-glucosidase inhibitor, β-glucosidase inhibitor, *Botrytis cinerea*, antifugal activity

In the course of our intensive search for antifugal substances [4, 18, 21] against plant pathogen *Botrytis cinerea*, we have found a soil microorganism, *Bacillus lentimorbus*, which produces β-glucosidase inhibitor in the fermentation broth. The filamentous fungus *Botrytis cinerea* is a parasite of many fruits and vegetables [22]. During the host-parasite interaction, *Botrytis cinerea* synthesizes many enzymes [8, 10, 25], such as cellulolytic and pectinolytic ones, for the degradation of the plant cell wall [13]. β-glucosidase [20] of *Botrytis cinerea* is the key enzyme in the enzymatic hydrolysis of cell wall of fruits and vegetables [6]. To isolate antifungal substances from *Bacillus lentimorbus* against *Botrytis cinerea*, the β-glucosidase inhibitor was partially purified from the supernatant of the culture medium. The β-glucosidase inhibitor of the supernatant also showed inhibitory activity against α-glucosidase. But the inhibitor fraction purified by Sephadex G-100 column did not show any inhibitory activity against α-glucosidase. From this result, we concluded that both inhibitors could be separated. Therefore, we have separated the α-glucosidase inhibitor and β-glucosidase inhibitor by Sephadex G-100 column chromatography. α-Glucosidase is among the most important carbohydrate-splitting enzymes that also catalyze the hydrolysis of α-glucose linkage in the final step of the digestive process of carbohydrates [3, 16]. α-Glucosidase inhibitors [5] could retard the use of dietary carbohydrates to suppress postprandial hyperglycemia [15, 23, 24]. In this study, we describe the production of α-glucosidase inhibitor and β-glucosidase inhibitor from *Bacillus lentimorbus* in various culture conditions, and the antifungal activity [10] of β-glucosidase inhibitor. To the best of our knowledge, this is the first report on the production of α- and β-glucosidase inhibitors from *Bacillus lentimorbus*.

Materials and Methods

Microorganism, Media, and Culture Conditions

Botrytis cinerea was provided by KCTC (Korean Collection for Type Cultures). The organism was grown at 30°C on Potato Dextrose Agar (PDA). *Bacillus lentimorbus* was isolated from soil in Chungnam province Korea. Half g of soil was suspended in 50 ml of distilled water and the supernatant was spread on the following PGI agar medium (PGI medium containing 1.5 % agar). The colonies grown on PGI agar were cultured in PGI medium at 30°C for 5 days and the supernatants were used to assay for β-glucosidase inhibitor. The colony showing the highest

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inhibitory activity was selected for further investigation. The growth medium of *Bacillus lentimorbus* was a chemically defined one (PGI medium) with the following compositions (in grams per liter): glucose (5), sodium glutamate (2.5), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.01), and $MnSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CuSO_4 \cdot 7H_2O$ (all 0.002). The fermentation was carried out at 30°C for 2 days.

Identification of Glucosidase Inhibitor-Producing Bacteria

The bacterial strain with the highest β-glucosidase inhibitor production was then identified by using the Gram staining as well as morphological, biochemical, and physiological tests. Morphological characteristics of this strain were observed with a scanning microscope and a light microscope Thereafter, the strain was identified according to *Bergey's Manual of Determinative Bacteriology* [7] based on its morphological, biochemical, and physiological characteristics. The morphology of the isolated bacterium was observed by using a scanning electron microscope (SEM). In order to study the biochemical and physiological properties of the isolated strain, an API kit (BioMerieux, France) was used.

Fatty Acid Analysis of the Cell Wall

Fatty acids analyses of the cell wall were performed by following the Microbial Identification System (MIDI Inc., U.S.A.) with Sherlock software. Fatty acids were separated and esterified to determine the composition according to the method of Miller and Berger [14]. Methylesters were analyzed by gas chromatographic separation on a 25 m × 0.2 mm ultra 2.5% phenylmethylsiloxane capillary column [26].

Assay for α- and β-Glucosidase Inhibitors

para-Nitrophenyl (PNP) α-, β-glucopyranoside and commericial α- and β-glucosidases (from Baker's Yeast and Almond, respectively) were purchased from Sigma. The inhibitory activities of α- and β-glucosidases were determined colorimetrically by monitoring the release of ρ-nitrophenol from an appropriate PNP-glycoside substrate [1, 9]. The reaction mixture contained 20 mU α- or β-glucosidase in 50 mM acetate buffer (pH 6.0 or 5.0, respectively) and culture filtrate in a final volume of 200 μl, and the mixture was preincubated at 37°C for 10 min. Half ml of 2 mM PNP-glycoside was added to the mixture and it was incubated for 10 min. The reactions were terminated by adding 0.9 ml of 0.2 M sodium carbonate. The release of ρ-nitrophenol during the reaction was measured at 405 nm. Values for percentage of inhibition were calculated relative to a control sample.

$$\text{Inhibition } (\%) = \frac{A_{con} - A_{samp}}{A_{con}} \times 100$$

A_{con} : Absorbance of control at 405 nm
A_{samp}: Absorbance of sample at 405 nm

Isolation of α- and β-Glucosidase Inhibitors

For the isolation of α- and β-glucosidase inhibitors from culture supernatant, culture supernatant was concentrated by lyophilization. The concentrated sample was suspended in distilled water and passed through a Sephadex G-100 column (2×40 cm), and the column was eluted with distilled water at a flow rate of 15 ml/h. Fractions of 3 ml were collected, and the inhibitory activities against α- and β-glucosidases were measured.

Properties of α- and β-Glucosidase Inhibitors

To study the degree of stability at different pHs, the α- and β-glucosidase inhibitors were kept in different pH buffers at 4°C for 24 h, dialyzed again against 50 mM sodium acetate buffer (pH 6.0 and pH 5.0), and then subjected to the reaction. The thermal stability of the inhibitors was studied by measuring the activity after incubation at 100°C for 15 min. Solubility of the inhibitors was compared at the concentration of 1 mg/ml.

Inhibition of Fungal Growth by α- and β-Glucosidase Inhibitors

A spore suspension of *Botrytis cinerea* was uniformly spread on plates of PDA (potato dextrose agar). Discs soaked with Sephadex G-100 fractions containing α- or β-glucosidase inhibitor were laid on the seeded plates; discs soaked with distilled water served as control. Fungal growth was observed during 2 days of incubation at 30°C.

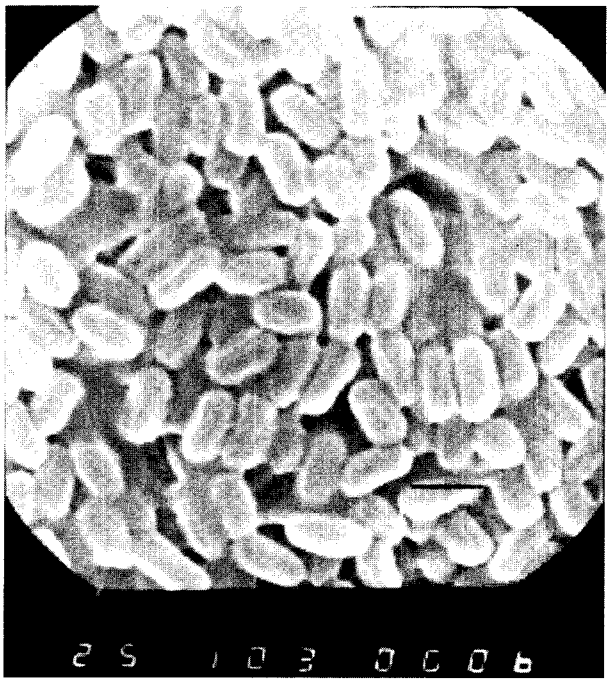

Fig. 1. Scanning electron microscopic observation of *Bacillus lentimorbus* B-6.

able 1. Taxonomical properties of the strain B-6.

Characteristics	Strain B-6
Gram stain	positive
Shape	rod
Cell size	0.5~0.6×1.2~1.4 μm
Motility	nonmotile
Optimum temperature	30°C
Oxidase	+
Catalase	+
Urease	-
Methyl red test	+
Voges-Proskauer reaction	-
Indole test	+
Casein hydrolysis	+
Starch hydrolysis	-
H_2S production	-
Gelatin liquefaction	+
Nitrate reduction	+
Denitrification	-

: positive, - : negative.

ESULTS AND DISCUSSION

solation and Identification of Glucosidase Inhibitor-roducing *Bacillus lentimorbus*

-Glucosidase inhibitor-producing bacterial strains were olated from soil by using PGI media. β-Glucosidase hibitory activities of supernatants of soil microorganisms ere measured as the method described in Materials and ethods. Five strains showed β-glucosidase inhibitory ctivity. The bacterial strain B-6 showing the highest β-lucosidase inhibitory activity was selected. Morphological haracteristics were observed by an electron microscope Fig. 1). The results obtained for the morphological, iochemical, and physiological characteristics of strain B-6 are summarized in Table 1. As shown in Table 2, the cellular fatty acids of strain B-6 were composed of five major fatty acids, iso-$C_{15:0}$ (18.1%), anteiso-$C_{15:0}$ (39.08%), n-$C_{16:0}$ (8.85%), iso-$C_{17:0}$ (10.04%), anteiso-$C_{17:0}$ (9.63%), showing a pattern similar to that of *Bacillus lentimorbus*. On the basis of its morphological, biochemical, and physiological characteristics, the strain B-6 was identified as a species of *Bacillus lentimorbus*.

able 2. Fatty acid composition of total membrane lipid extracts om *Bacillus lentimorbus* B-6.

Fatty acid (s)[a]	Fatty acid (%) in total membrane lipid extracts
Iso-$C_{14:0}$	1.11
n-$C_{14:0}$	1.54
Iso-$C_{15:}$	18.1
Anteiso-$C_{15:0}$	39.08
Iso-$C_{16:0}$	2.22
n-$C_{16:0}$	8.85
n-$C_{16:1}$ ω11c	3.85
Iso-$C_{17:1}$ ω10c	2.36
Iso-$C_{17:0}$	10.04
Anteiso-$C_{17:0}$	9.63
n-$C_{18:0}$	0.98

atty acids are abbreviated according to the number of carbon atoms recedes the colons and the number of double bonds follows the colons. he prefixes anteiso and iso represents the type of branched chain structure.

Bacterial Growth and Production of α- and β-Glucosidase Inhibitors

To determine the relationship of cell growth and glucosidase inhibitory activity, *Bacillus lentimorbus* was cultured in a PGI broth at 30°C for 6 days with shaking (180 rpm). Figure 2 shows the bacterial growth curve and α- and β-glucosidase inhibitory activities of *Bacillus lentimorbus*. Maximum α- and β-glucosidase inhibitory activities were observed at the beginning of the stationary phase, suggesting that both inhibitors were produced during the active growth phase. After 2 days, the β-glucosidase inhibitory activity remained almost the same, but α-glucosidase inhibitory activity was decreased. It was reported that the inhibitory effect of α-glucosidase inhibitors varies greatly depending on the origin of the enzyme [17, 23]. α-Glucosidase inhibitor from *Bacillus lentimorbus* showed inhibitory activity against α-glucosidase from Baker's yeast. Further research on the α-glucosidase origin is needed to evaluate its *in vitro* inhibitory effect.

Effect of pH, Glucose Concentration, and Sodium Glutamate Concentration on the Production of Glucosidase Inhibitors of *Bacillus lentimorbus*

Under the various culture conditions, the production of α- and β-glucosidase inhibitors from *Bacillus lentimorbus* was investigated. Growth of *Bacillus lentimorbus* was tolerant

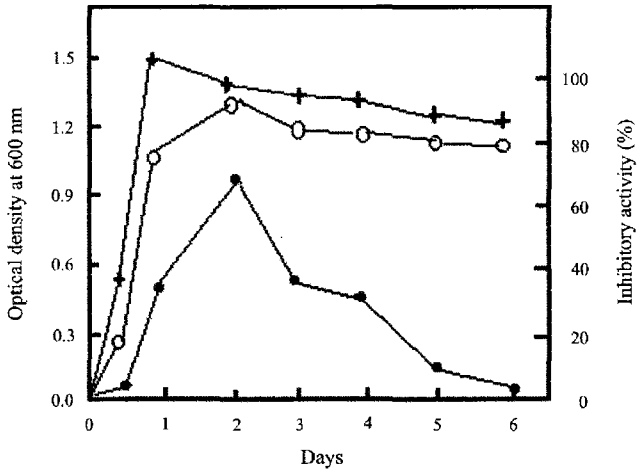

Fig. 2. Time course of microbial growth (+) and α- (●) and β-glucosidase (○) inhibitory activities of *Bacillus lentimorbus*.

Table 3. Effect of pH on production of α- and β- glucosidase inhibitors.

pH	α-Glucosidase inhibition (%)	β-Glucosidase inhibition (%)
4.0	ND	ND
5.0	0	49
6.0	49	92
7.0	63	93
8.0	72	93
9.0	67	93
10.0	ND	ND

One hundred μl of supernatant of *Bacillus lentimorbus* at 2 days was assayed as described in Materials and Methods.
ND: not determined.

to high concentration of glucose (20%), but was not tolerant to extreme pHs. No growth of *Bacillus lentimorbus* was observed at pH 4 and 10. Optimal pH for the production of α- and β-glucosidase inhibitors were pH 8.0 and pHs 6–9, respectively (Table 3). While the production of α-glucosidase inhibitor was decreased by a concentration of glucose higher than 1% [2, 19], the production of β-glucosidase inhibitor was not influenced by glucose concentration between 0.5% and 20%. Optimal concentration of glucose for the production of α-glucosidase inhibitor was 0.5% (Table 4). Optimal concentration of sodium glutamate for the production of α- and β-glucosidase inhibitors was 0.25%, however, the effect of sodium glutamate concentration on the production of glucosidase inhibitor was negligible (data not shown).

Isolation of α- and β-Glucosidase Inhibitors

α- and β-glucosidase inhibitors from the culture filtrate were isolated by lyophilization, followed by Sephadex G-100 column chromatography (Fig. 3). Elution profile showed that the molecular weight of the α-glucosidase inhibitor was higher than that of the β-glucosidase inhibitor.

Properties of α- and β-Glucosidase Inhibitors

The pH stability of the α-glucosidase inhibitor was different from that of β-glucosidase: the former was stable at pH 6

Table 4. Effect of glucose concentration on production of α- and β-glucosidase inhibitors.

Concentration of glucose	α-Glucosidase inhibition (%)	β-Glucosidase inhibition (%)
0.1	45	86
0.5	75	92
1.0	52	90
2.0	28	92
5.0	10	86
10.0	5	90
20.0	5	90

One hundred μl of supernatant of *Bacillus lentimorbus* at 2 days was assayed as described in Material and Methods.

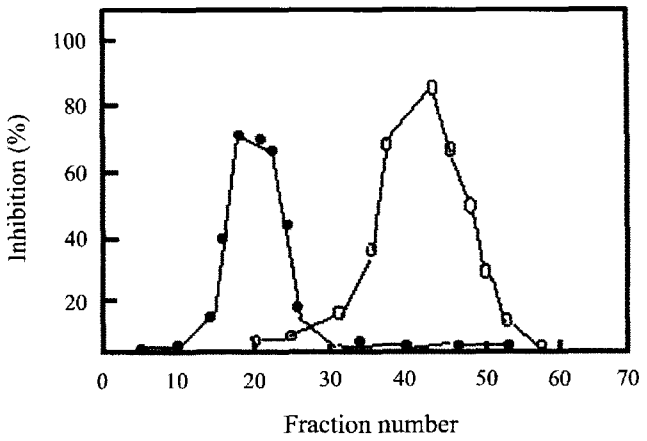

Fig. 3. Elution profile of α- (●) and β-glucosidase (○) inhibitors on Sephadex G-100 column.
Details are described in Materials and Methods.

to 7, but the latter was stable at pH 4 to 10 (data not shown). Thermal stability of the α-glucosidase inhibitor was the same as that of β-glucosidase inhibitor: Both inhibitors were stable at 100°C for 15 min (data not shown). Both inhibitors were soluble in water, ethanol, and methanol, but not soluble in butanol, chloroform, ether, hexane, and ethyl acetate. Physicochemical properties of the inhibitors are under investigation. Acarbose [16], oleanolic acid [2], and cyclo(dehydroala-L-Leu) [11] as α-glucosidase inhibitors, and 1-deoxynojirimycin [12] as a β-glucosidase inhibitor have been reported.

Inhibition of Fungal Growth by α- and β-Glucosidase Inhibitors

A spore suspension of *Botrytis cinerea* was uniformly spread on plates of PDA (potato dextrose agar). Discs

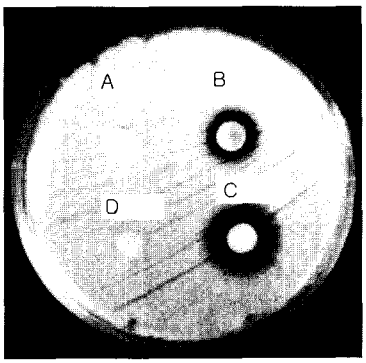

Fig. 4. Effect of α- and β-glucosidase inhibitors on the growth of *Botrytis cinerea* in agar diffusion assay.
A spore suspension of *Botrytis cinerea* was uniformly spread on plates of PDA (potato dextrose agar). Discs soaked with Sephadex G-100 column fractions containing α-(A: 80 μg) or β-glucosidase inhibitor (B: 40 μg, C: 80 μg) were laid on the seeded plates; control (D) was disc soaked with distilled water. Fungal growth was observed over 2 days of incubation at 30°C.

s)aked with Sephadex G-100 fractions containing α- or ∫ -glucosidase inhibitor were laid on the seeded plates; c)ntrols were discs soaked with distilled water. Fungal g ·owth was observed during 2 days of incubation at :)°C. β-Glucosidase inhibitor (40 μg and 80 μg) showed a ntifungal activity against *Botrytis* cinerea (Fig. 4). *In* ı *'tro* experiment confirms that *Bacillus lentimorbus* can t e used as a biological organism against *Botrytis cinerea*. ∫ -Glucosidase inhibitor from *Streptomyces* sp. is known t ı have inhibitory effect on hydrolytic enzyme action of ı *enicillium* sp. [12]. However, α-glucosidase inhibitor showed ı ɔ antifugal activity against *Botrytis cinerea* (Fig. 4).

ı cknowledgment

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