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Production of an Antihyperlipemial HMG-CoA Reductase Inhibitor from *Bacillus cereus* D-3

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For the purpose of production of a novel antihyperlipemial HMG-CoA reductase inhibitor from bacteria, a bacterium which showed the highest HMG-CoA reductase inhibitory activity was isolated from traditional *Doenjang*. This strain was identified as *Bacillus cereus* (D-3) based on its microbiological characteristics and 16S rRNA sequence analysis. The maximal HMG-CoA reductase inhibitor production from *Bacillus cereus* D-3 was obtained by cultivation in a Glucose-CSL broth containing 2% glucose, 0.6% corn steep liquor, 0.04% K_2HPO_4 and 0.05% KH_2PO_4 at 30°C for 36 h. The final HMG-CoA reductase inhibitory activity under the above conditions was 39.4%.

Key words: Antihyperlipemial substance, HMG-CoA reductase inhibitor, *Bacillus cereus* D-3

β-Hydroxy-β-methylglutaryl CoA reductase (E.C 1.1.1.34), a rate-limiting enzyme in endogenous cholesterol synthesis, is a 97 kDa glycoprotein. It catalyses the reductive deacylation of HMG-CoA to mevalonate in a two-step reaction [1, 5]. An increase in the blood cholesterol level by the HMG-CoA reductase can causes hyperlipemia or coronary heart diseases. Therefore, lowering the total cholesterol level through the HMG-CoA reductase inhibitor is very important as a remedy or prevention of hyperlipemia.

Some HMG-CoA reductase inhibitors from microbes and phytochemicals have been investigated and introduced into clinical use [5, 15]. Endo *et al.* [5] reported the isolation of mevastatin (formerly called mL236M or compactin) as a potent inhibitor of the β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase which was a key step toward attaining an effective means of lowering the plasma cholesterol level in humans. Subsequently, three drugs have been marketed in many countries: lovastatin (called Mevinolin) or Monacolin K, Simvastatin, and Pravastatin [6]. In addition to these, many other Mevastatin analogues have been synthesized, some of which are now under clinical development [18]. Mevastatin, a metabolite of *Penicillium citrinum,* was first isolated from *Penicillium brevicompactum* as an antibiotic. Following this, Lovastatin was isolated from *Monascus ruber* and *Aspergillus terreus.* Lovastatin is slightly more potent than Mevastatin for the inhibition of the HMG-CoA reductase. In addition to Mevastatin, Dihydrocompactin, mL-263A, and mL-236C have been isolated from *Penicillium citrinum.* Furthermore, Monacolin J, Monacolin L, Dihydromonacolin L acid are all minor metabolites of *Monasus ruber,* and Dihydromevinolin is a product of *Aspergillus terreus.* Extracts of medicinal plants such as *Typha augustifolia*, *Polygonum cuspidatum*, *Crataegus pinnatifida* and *Polygonum mulitflorum* [14] as well as Orengedokuto which consists of *Scutellariae radix, Coptidis rhizoma, Phellodendri cortex* and *Gardeniae fructus* and *Daio-Orengedokuto* [13] and *Pueraria thunbergiana* [11] show a high HMG-Co A reductase inhibitory activity. Methanol extracts of prosomillet and sorghum also show high HMG-CoA reductase inhibitory activities of 72.7% and 44.7%, respectively [10]. Recently, isoflavone compounds were found in Korean soybean paste as HMG-Co A reductase inhibitor [19].

However, commercial antihyperlipemial drugs, including Mevastatin, have some disadvantages, such as high cost, low yield, ineffectiveness *in vivo,* and some side effects. For these reasons, this study was carried out in order to isolate a potent HMG-CoA reductase inhibitor-producing

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bacterium and to optimize the production condition of the HMG-CoA reductase inhibitor for the purpose of developing of a new antihyperlipemial agent.

Material and Methods

Materials and chemicals

Escherichia coli BL21(DE3) was used as a host for a recombinant plasmid pKFT7-21 containing the HMG-CoA reductase gene, which was obtained from Professor Victor W. Rodwell of Purdue University [11].

All chemicals used in this study were of a special-pure grade.

Screening and identification of HMG-CoA reductase inhibitor-producing bacteria

Suspensions of several traditional *Doenjangs* and *Chunggukjangs* were inoculated into a nutrient agar plate and cultured at 37°C for 72 h. Bacteria which grew well on the nutrient agar plates were cultivated in a nutrient broth at 37°C for 72 h. After centrifugation at 15000×g for 15 min, HMG-CoA reductase inhibitory activity of the supernatants was determined. Finally, the bacterium that showed the highest HMG-CoA reductase inhibitory activity was selected.

Morphological and biochemical characteristics of the selected strain were investigated according to the Manual of Methods for General Bacteriology [9] and a phylogenetic analysis based on the 16S rRNA sequence was finally used to identify the D-3 strain. A 16S rRNA was amplified by the colony PCR. The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and its nucleotide sequence was analyzed. Its homology was investigated by using the BLAST program of the NCBI. The identification was performed using Bergey's Manual of Determinative Bacteriology [3].

Preparation of Syrian hamster HMG-CoA reductase

The catalytic domain of Syrian hamster HMG-CoA reductase with the C-terminal extension of Glu-Glu-Phe (RcatEEF) was overexpressed in *E. coli* BL21(DE3) under the control of a T7 promoter. The construction of pKFT7-21 was described by Frimpong [8]. *E. coli* BL21(DE3) freshly transformed with pKFT7-21 was inoculated in 100 mL of LBamp broth. The culture was shaken at 37°C. When the cell density (A_{600}) reached 0.5-0.7, isopropyl-β-D-thiogalactoside was added to the final concentration of 0.5 mM, and growth was allowed to continue further for exactly 5 h. Cells were harvested by centrifugation (10000 ×g, 15 min, 4°C), suspended in 15 mL of buffer A (pH 7.3, 20 mM Na_2PO_4, 50 mM NaCl, 10% glycerol, 100 mM sucrose, and 10 mM dithiothreitol), and used directly for the preparation of cell extract.

Cells suspended in buffer A were broken at a duty cycle of 40% and output control level 4 for 20 min by a sonicator (Branson 450 Sonifier) in an ice bath. The cell lysate was ultracentrifuged (100,000×g, 60 min, 4°C). The pelleted cell debris was discarded, and the supernatant was frozen in a -70°C deep-freezer before use for as assay of the HMG-CoA reductase inhibitory activity. Its protein concentration was determined according to the method of Bradford [2] using bovine serum albumin as a standard.

Assay of HMG-CoA reductase inhibitory activity

The HMG-CoA reductase inhibitory activity was assayed spectrophotometrically, following the method of Kleinsek *et al.* [11], whereby the rate of decrease in absorbance at 340 nm due to the oxidation of NADPH was measured. The spectrophotometer was equipped with a cell holder and maintained at 37°C. The standard assay mixture contained 300 μM HMG-CoA, 500 μM NADPH, 100 mM NaCl, 1.0 mM EDTA, 2 mM dithiothreitol, and 0.5 mM potassium phosphate buffer (pH 7.0) with a final volume of 150 μL. The reaction mixture containing the enzyme (100 μg/150 μL) and all components except HMG-CoA were first monitored to detect any HMG-CoA-independent oxidation of NADHP. The reaction was then initiated by adding HMG-CoA at 37°C. The HMG-CoA reductase inhibitory activity was calculated by applying the following equation [12];

$$\text{HMG-CoA reductase inhibitory activity (\%)} = \left(1-\frac{A_{340}\text{ of the reactants}-A_{340}\text{ of the sample blank}}{A_{340}\text{ of the control}-A_{340}\text{ of the control blank}}\right)\times 100,$$

where the control is the standard assay mixture and the control blank is an addition of water instead of HMG-CoA into the standard assay mixture. The sample control is the addition of the supernatant(sample) into the control blank. One unit of HMG-CoA reductase is defined as the amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH per minute. A statistical analysis was performed by a Sigma plot of SPSS Inc. using mean values of

triplicate measurements ($p \leq 0.05$).

Determination of protease activity and some physiological functionality

Acidic, neutral and alkaline protease activities were determined by the modified method of Lee [17]. An 0.6% skim milk solution (2.5 mL) which were dissolved in 0.5 M sodium dicarbonate buffer (pH 10.0), 0.5 M sodium phosphate buffer (pH 6.0) and 0.2 M citric acid buffer (pH 3.0), respectively was added into the concentrated supernatants (0.5 mL) and then reacted for 10 min at 30°C, placed 20 min at room temperature after the addition of 0.4 M TCA (3 mL). After centrifugation at 5000×g for 15 min, 0.4 M Na_2CO_3 (5 mL) and 1 N folin(1 mL) reagent were added to the supernatants (1 mL) and reacted for 30 min at 30°C. The absorbance was then determined at 660 nm.

The ACE inhibition was assayed by a modification of the method of Cushman and Cheung [4]. A mixture containing a 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 3 units of ACE, and an appropriate amount of supernatants was preincubated for 10 min at 37°C. The reaction was initiated by adding 50 μl of Hip-His-Leu with a final concentration of 5 mM, and terminated after 30 min of incubation by adding 250 μl of 1.0 N HCl. The hippuric acid liberated was extracted with 1 mL of ethyl acetate, and 0.8 mL of the extract was evaporated to dry by a Speed Vac Concentrator (Eyela Co., Tokyo). The residue was then dissolved in 1 mL of sodium borate buffer. The absorbance at 228 nm was measured to estimate the ACE inhibitory activity.

The fibrinolytic activity was assayed by the method of Fayek *et al.* [7]. 0.5 mL of supernatants was added to a 3 mL of substrate solution (0.6% fibrin in 0.1 M McIlvaine buffer, pH 7.0) and incubated at 40°C for 10 min. The reaction was stopped with the addition of 3 mL of 0.4 M TCA for 30 min and then filtered by Whatman filter paper No. 2. A reaction mixture consisting 1 mL of the filtrates, 5 mL of 0.4 M Na_2CO_3, and 1 mL of 1 N Folin reagent was placed at room temperature for 30 min and the amount of tyrosine released from the fibrin as substrate was determined from a tyrosine standard curve by measuring the absorbance at 660 nm. One unit of activity was defined as the production of 1 μg of the tyrosine per minute by 1 mL of crude enzyme.

Results and Discussion

Screening and identification of the extracellular HMG-CoA reductase inhibitor-producing bacteria

To select a potent extracellular HMG-CoA reductase inhibitor-producing bacteria, culture broth of 150 colonies from the cultivation of a nutrient agar plate was tested for HMG-CoA reductase inhibitory activities. Firstly, 11 strains were screened that were showed more than 15% of HMG-CoA reductase inhibitory activity. Among these, the strain D-3 which was isolated from the deteriorated traditional *Daenjang* showed the highest HMG-CoA reductase inhibitory activity of 27.5% (Table 1). Therefore, the strain D-3 was selected finally as a bacterium for the production of the extracellular HMG-CoA reductase inhibitor.

Morphological and biochemical characteristics of the D-3 strain are summarized in Table 2. The selected strain, D-

Table 1. Growth and HMG-CoA reductase inhibitory activity of secondary screened bacteria.

Strain No.[1]	Growth (A_{660})	HMG-CoA reductase inhibitory activity (%)
D-4	0.76	24.5
D-3	0.73	27.5
D6-1	0.54	13.3
D10-1	0.66	20.7
C2-1	0.77	18.4
C2-4	0.86	22.1
C2-5	0.92	18.9
C4-2	0.58	16.2
C5-8	0.71	15.8
C8-2	0.69	21.3
C11-3	0.88	19.9

[1]Eleven strains which were showed more than 15% of HMG-CoA reductase inhibitory activities were selected in secondary screening.

Table 2. Microbiological characteristics of the selected strain, D-3.

Cell shape	Rod
Cell size	0.7× 2.0 - 2.2 μm
Gram-staining	Positive
Endospores formation	+ (ellipsoidal)
Motility	+ (Peritrichous flagella)
Voges-Proskauer test	+
Catalase	+
Acid production from glucose	+
Anaerobic growth	+

3 was rod-shaped bacterium, that formed an endospore. It was Gram positive, motile and facultative anaerobic. Furthermore, its 16S rRNA sequences revealed that it belongs to *Bacillus cereus* (similarity: 99.9%). Based on its microbial characteristics and 16S rRNA sequence, the D-3 strain was identified with *Bacillus cereus* D-3 using Bergey's manual.

Although many fungi including *Penicillum citrnum*, *Penicillium brevicompactum*, *Aspergillus terreus* and *Monascus ruber* are known to be produce HMG-CoA reductase inhibitors [5, 11] and also many microorganisms were isolated from the Korean traditional *Doenjang* [16, 20], *Bacillus cereus* D-3 from the Korean traditional *Doenjang* is the first bacterium discovered which produces HMG-CoA reductase inhibitor and may be useful in the development of a new antihyperlipemial drug.

Meanwhile, the culture supernatant of *Bacillus cereus* D-3 showed 37.0% of antihypertensive angiotensin I-converting enzyme inhibitory activity, whereas its protease activity against skim milk was minor (2.2 U/mL) and also did not showed any hydrolysis activity against fibrin (data not shown).

Optimization of HMG-CoA reductase inhibitor production

The effects of nitrogen sources on the production of the HMG-CoA reductase inhibitor was shown in Table 3. 28.3% of the HMG-CoA reductase inhibitory activity was shown by the addition of corn steep liquor into a basal medium containing 2.0% glucose, 0.04% K_2HPO_4 and 0.05% KH_2PO_4. However, the addition of other nitrogen sources such as urea, NH_4Cl, $(NH_4)_2SO_4$ and NH_4HCO_3 did not have an effect on the HMG-CoA reductase inhibitory activity.

Table 3. Effect of nitrogen sources on the production of HMG-CoA reductase inhibitor from *Bacillus cereus* D-3.

Nitrogen sources[1]	Growth (A_{660})	HMG-CoA reductase inhibitory activity (%)
Tryptone	2.10	2.7
Peptone	1.23	6.0
Yeast extract	1.97	20.3
Beef extract	1.93	12.3
Casamino acid	1.17	13.9
Corn steep liquor	0.68	28.3
Urea	0.85	N.D[2]
NH_4Cl	0.49	N.D
Ammonium sulfate	0.56	N.D
Ammonium citrate	0.31	N.D
Ammonium oxalate	0.58	3.6
NH_4NO_3	0.60	9.0
NH_4HCO_3	1.40	N.D
$NaNO_3$	0.73	7.0
KNO_3	0.77	14.2

[1]Organic nitrogen and inorganic nitrogen sources were added 0.6% and 0.1% into basal medium, respectively. Basal medium was composed with glucose (2%), K_2HPO_4 (0.04%), and KH_2PO_4 (0.05%)
[2]N.D. :not determined.

In addition, the effect of sugars on the production of the HMG-CoA reductase inhibitor was examined (Table 4). The highest HMG-CoA reductase inhibitory activity (32.1 %) was found to occur with the addition of glucose (2%) in CSL medium containing corn steep liquor 1.0%, K_2HPO_4 0.04% and KH_2PO_4 0.05%. However, fructose, sucrose, maltose and xylose did not have an influence on

Table 4. Effect of sugars on the production of HMG-CoA reductase inhibitor from *Bacillus cereus* D-3.

Sugars[1]	Growth (A_{660})	HMG-CoA reductase inhibitory activity (%)
Glucose	0.63	32.1
Galactose	0.72	3.2
Fructose	0.56	N.D[2]
Lactose	0.44	5.4
Sucrose	0.92	N.D
Maltose	0.01	N.D
Xylose	0.19	N.D
Glycerol	1.30	1.2
Arabinose	0.58	3.4
Raffinose	0.91	12.2

[1]Each sugars were added 2% into CSL medium which was composed with corn steep liquor 0.6%, K_2HPO_4 0.04%, and KH_2PO_4 0.05%
[2]N.D. : not determined.

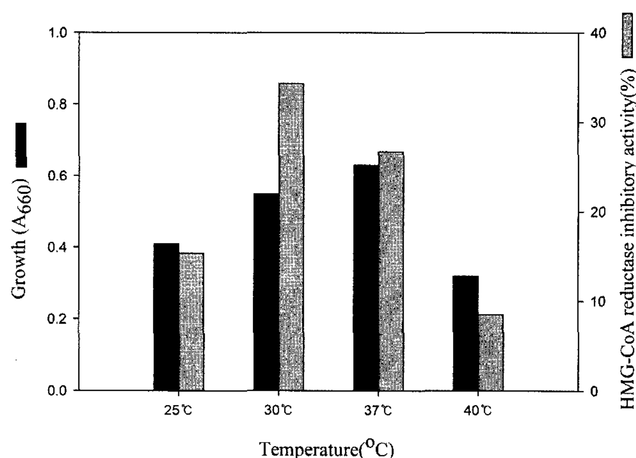

Fig. 1. Effect of temperature on the production of HMG-CoA reductase inhibitor from *Bacillus cereus* D-3.

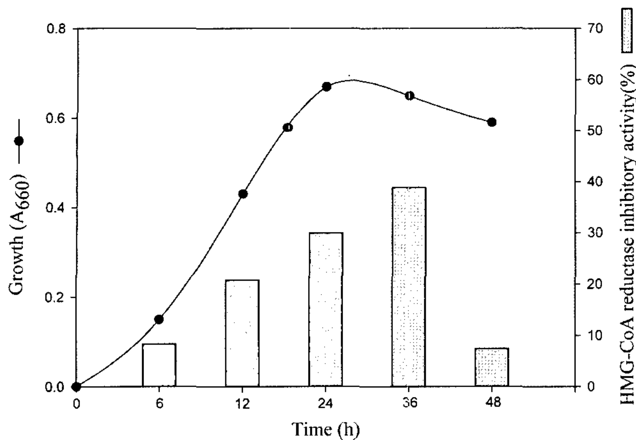

Fig. 2. Effect of cultural time on the production of HMG-CoA reductase inhibitor from *Bacillus cereus* D-3.

the HMG-CoA reductase inhibitory activity.

The effects of the cultural temperature and time course on the production of HMG-CoA reductase inhibitor were investigated at various temperatures (25~40°C) and at different time periods (6~48 h) (Fig. 1 and Fig. 2). The maximum production of HMG-CoA reductase inhibitor was obtained from a cultivation of 36 h at 30°C and the HMG-CoA reductase inhibitory activity was 39.4%.

Considering the above results, the optimum culture condition for the production of the HMG-CoA reductase inhibitor was a medium composed of 2.0% glucose, 0.6% corn steep liquor, 0.04% K_2HPO_4 and 0.05% KH_2PO_4 with culture temperature and time of 30°C and 36 h, respectively.

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Bacillus cereus D-3로부터 항고지혈증 HMG-CoA Reductase 저해제의 생산

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새로운 고지혈증 예방 제품 개발을 위한 기초 자료를 얻고자 우선 전통 된장으로부터 HMG-CoA reductase 저해물질을 강력하게 세포외로 생산하는 D-3 세균을 최종 분리 하였다. D-3 균의 미생물학적 특성을 조사한 결과 그람 양성의 간균으로 운동성이 있었고 통성 혐기성이었다. 이들 특성과 16S rRNA 염기서열의 분석 결과 등을 종합하여 Bergey's manual로 동정한 결과 *Bacillus cereus* D-3로 동정되었다. HMG-CoA reductase 저해물질 생산 최적 조건을 조사한 결과 *Bacillus cereus* D-3를 glucose 2%, corn steep liquor 0.6%, K_2HPO_4 0.04%, KH_2PO_4 0.05%를 함유한 Glucose-CSL 배지에 접종하여 30°C에서 36시간 배양하여 얻은 상등액의 HMG-CoA reductase 저해활성이 39.4%로 저해물질을 가장 많이 생성 하였다.

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