VOL. 55 NO. 5, MAY 2002 THE JOURNAL OF ANTIBIOTICS pp. 457 — 461

# Novel α-Glucosidase Inhibitors, CKD-711 and CKD-711a Produced by *Streptomyces* sp. CK-4416

## I. Taxonomy, Fermentation and Isolation

JONG-GWAN KIM, HUNG-BAE CHANG, YOUNG-IN KWON, SEUNG-KEE MOON, HYOUNG-SIK CHUN*, SOON KIL AHN and CHUNG IL HONG

CKD Research Institute,
Chonan P.O. Box 74, Chonan, 330-600, Korea

(Received for publication September 10, 2001)

New α-glucosidase inhibitors, CKD-711 and CKD-711a were produced from the fermentation broth of *Streptomyces* sp. CK-4416 which was isolated from a forest soil of Jeju Island, South Korea. CKD-711 and CKD-711a were purified by Dowex 50W-2X and Sephadex G-10 column chromatography. In *in vitro* studies, CKD-711 showed a potent inhibitory activity against α-glucosidase from mammalian, but less inhibition against α-amylase from microorganism and mammalian. CKD-711a showed a lower inhibitory activity than CKD-711.

Several α-glucosidase inhibitors such as acarbose,[1] trestatin,[2] amylostatin[3] and valiolamine[4] were isolated from microorganisms. Among these inhibitors acarbose[5,6] isolated from *Actinoplanes* sp. has been developed a clinically useful drug for the treatment of type II insulin-independent diabetes. And valiolamine isolated from *Streptomyces hygroscopicus* var. *limoneus* is an important component to obtain voglibose, another α-glucosidase inhibitor in clinical use[7,8].

In the course of screening for new bioactive metabolites from microorganisms, CKD-711 and CKD-711a, α-glucosidase inhibitors, were discovered in the fermentation broth of *Streptomyces* sp. CK-4416. Taxonomy of the producing organism, fermentation and isolation of CKD-711 and CKD-711a are discussed in this paper. Biological properties and structure elucidation are described in the following papers[9,10].

### Materials and Methods

#### Microorganism and Carbohydrate Hydrolases

*Streptomyces* sp. CK-4416 was isolated from a forest soil sample in Jeju Island, South Korea. Crude rat intestinal α-glucosidase was prepared from rat intestinal mucosa and other enzymes were purchased from Sigma chemical company.

#### Taxonomic Studies

For taxonomic studies, cultures were grown in accordance with methods adopted by the International *Streptomyces* Project. For experiments on cultural properties, all cultures were incubated at 28°C and observed for 15~20 days. The colors recorded for mature cultures were described scoring to the "Methuen Handbook of Colour"[11]. Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB[12,13]. Diaminiopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[14].

#### Analytical Method

The production and purity of CKD-711 and CKD-711a were monitored by high performance liquid chromatography (HPLC). Whole broth (10 ml) was adjusted to pH 3.0 with 2 N HCl. After being stirred at room temperature for 10 minutes and centrifuged at 4000 rpm for

* Corresponding author: hschun@ckdpharm.com

10 minutes, the supernatant (2 ml) was passed through a column (1 ml) of Dowex 50W-2X (pyridinium form). The column was washed with water and eluted with 1 N $NH_4OH$. The active fractions were concentrated to dryness under reduced pressure and the residue was dissolved with water. The amount of CKD-711 and CKD-711a was determined by HPLC: The HPLC (HP-1100, Hewlett-Packard, Germany) was performed using a column of Daisopak ODS-BP (4.6×250 mm, DAISO Co., Japan) and the column was eluted with deionized water at 1 ml per minute of flow rate. The elute was monitored on an evaporative light scattering detector (ELSD-Sedex 55, SEDERE Co., France) at 41°C.

Inhibitory Effect of Carbohydrate Hydrolase

The inhibitory effects of CKD-711 and CKD-711a were compared in *in vitro* experiments using carbohydrate hydrolases from microorganism, plant and mammalian sources. Enzyme activity was assayed according to the method of DAHLQVIST[15]. The inhibitory activity was determined by incubating a solution of an enzyme (50 $\mu$l), phosphate buffer (pH 7.0, 500 $\mu$l) containing 0.4 mg/ml sucrose or maltose, or 1% soluble starch, and a solution (50 $\mu$l) with various concentrations of CKD-711 and CKD-711a at 37°C for 30 minutes. The reaction mixture was heated in a boiling water bath to stop the reaction for 2 minutes, and then the amount of liberated glucose was measured by the glucose oxidase method[16]. The inhibitory activity was calculated from the formula as follows. Inhibition (%)=(C−T)/C×100, where C is the enzyme activity without inhibitor and T is the enzyme activity with inhibitor.

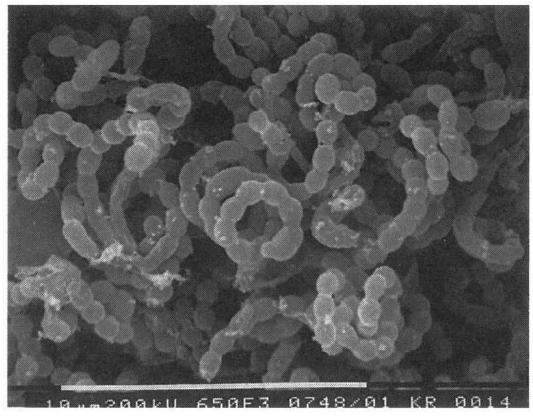
## Results

### Taxonomy of the Producing Strain

A scanning electron micrograph of spores of the strain CK-4416 is shown in Fig. 1. The spores were oval in shape, 0.5~0.6×0.8~1.0 $\mu$m in size with a smooth surface. The strain produced aerial mycelium with spiral, which comprised of more than 20 spores per chain. Sclerotic granules, sporangia and motile spores were not observed. Substrate mycelium of the strain was developed well and branched irregularly.

The cultural and physiological characteristics are shown in Table 1 and 2, respectively. Aerial mass color was grayish white on yeast extract-malt extract agar and reverse color of growth was yellowish. Melanoid pigments were not produced in trypton-yeast extract broth and on peptone-yeast extract-iron agar. Soluble pigments were not produced. L,L-Diaminopimelic acid was detected in the whole cell hydrolysates of the strain.

Fig. 1. Scanning electron micrograph of strain CK-4416.



Based on the morphological and physiological characteristics described above, the strain CK-4416 is considered to belong to the genus *Streptomyces* sp. The strain has been deposited in the Korean Collection Type Culture (KCTC), Korea, as *Streptomyces* sp. CK-4416 with the accession No. KCTC 0131 BP.

### Fermentation of CKD-711 and CKD-711a

A mature slant culture of *Streptomyces* sp. CK-4416 was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of medium consisting of corn starch 1%, glucose 1%, peptone 0.25%, yeast extract 0.5% and $CaCO_3$ 0.1%. The pH was adjusted to 7.0 before sterilization and the culture was incubated for 22 hours at 28°C on a rotary shaker (Infors Shaker, Switzerland) at 240 rpm. Fifty milliliters of the seed culture was transferred into 5-liter Erlenmeyer flasks containing 1000 ml of the same medium. After shaken for 36 hours, 360 ml of the second seed culture was inoculated to a 30-liter tank fermentor containing 15-liter of the production medium consisting of corn starch 5%, glucose 3%, soybean flour 1.5%, corn steep liquor 1.5%, $KH_2PO_4$ 0.1%, $CaCO_3$ 0.5% and chemical antifoamer 0.05%. The fermentation was carried out at 28°C for 7 days. The dissolved oxygen level was maintained around 5 ppm by adjusting the agitation rate. As shown in Fig. 2, the production of CKD-711 and CKD-711a started at the third day after maximum levels of growth were achieved. During growth in the production

Table 1. Cultural characteristics of *Streptomyces* sp. CK-4416.

| Medium | Growth | Aerial mycelium | Soluble pigment |
|---|---|---|---|
| Yeast-malt ext. agar (ISP No.2) | Moderate | Good, greyish white (1B1) | None |
| Oat meal agar (ISP No.3) | Good | Good, greyish yellow (2B2) | None |
| Inorganic salt-starch agar (ISP No.4) | Poor | None | None |
| Glycerin-asparagine agar (ISP No.5) | Moderate | Moderate, yellowish white (2A2) | None |
| Peptone-yeast ext.-iron agar (ISP No.6) | Poor | None | None |
| Tyrosine-agar (ISP No.7) | Good | Good, grey (3B1) | None |
| Sucrose-nitrate agar (Waksman No.1) | Poor | None | None |
| Glucose asparagine agar (Waksman No.2) | Good | Thin, greyish white (1B1) | None |
| Nutrient agar (Waksman No.14) | Poor | None | None |
| Emmerson's agar (Waksman No.28) | Poor | Thin, yellowish white (2A2) | None |

Table 2. Physiological properties of *Streptomyces* sp. CK-4416.

| | | | |
|---|---|---|---|
| Spore chain | Spiral | Utilization of | |
| Spore surface | Smooth | D-Glucose | + |
| Aerial mass color | Yellowish white | L-Arabinose | + |
| Melanin formation | Negative | D-Xylose | + |
| Soluble pigment | Negative | D-Fructose | + |
| Hydrolysis of starch | Positive | Sorbitol | - |
| Temperature range for growth | 15 ~ 45°C | D-Raffinose | + |
| | | Inositol | - |
| | | D-Mannose | + |
| | | L-Sorbose | - |
| | | Sucrose | + |
| | | L-Rhamnose | - |

+ : utilization, - : no utilization

medium, the broth pH was maintained neutral through day 7 after an alkaline shift was observed. The increase in pH on day 7 was associated with a decrease in titers of CKD-711 and CKD-711a. The production of CKD-711 and CKD-711a was measured by the method described in the above. CKD-711 and CKD-711a were eluted with retention times of 9.7 and 11.3, and 21.4 and 23.2 minutes, respectively, indicating the presence of $\alpha,\beta$-isomers in aqueous solution. The analytical HPLC elution profile of CKD-711 and CKD-711a is shown in Fig. 3. The

production of CKD-711 and CKD-711a in the fermentation broth reached about 800 and 1200 $\mu$g/ml on the sixth day, respectively.

Isolation of CKD-711 and CKD-711a

CKD-711 and CKD-711a were isolated according to the scheme as shown in Fig. 4. The 7-day old whole broth (15 liters) of *Streptomyces* sp. CK-4416 filtered with the aid of diatomaceous earth. The filtered broth (10 liters) was applied to an active carbon column (8×50 cm) and the column was eluted with 50% acetone. The active fractions were concentrated under reduced pressure, passed through a column of Dowex 50W-2X ($H^+$, 5.5×50 cm) and washed with water (2 liters) to remove neutral oligosaccharides. CKD-711 and CKD-711a were eluted from a column with 0.1 N $NH_4OH$ (1.2 liters). The active fractions were combined for concentration under reduced pressure and applied to a Sephadex G-10 column (2×100 cm), and the column was eluted with deionized water. The active fractions were concentrated carefully under reduced pressure below 35°C, followed by lyophilization to yield a powder (pale-yellowish brown). Fig. 4 shows the over-all process and amount of isolated CKD-711 and CKD-711a. Each compound could be further purified with Sephadex G-10 to give colorless amorphous powder.

Fig. 2. Typical HPLC chromatogram of CKD-711 and CKD-711a.

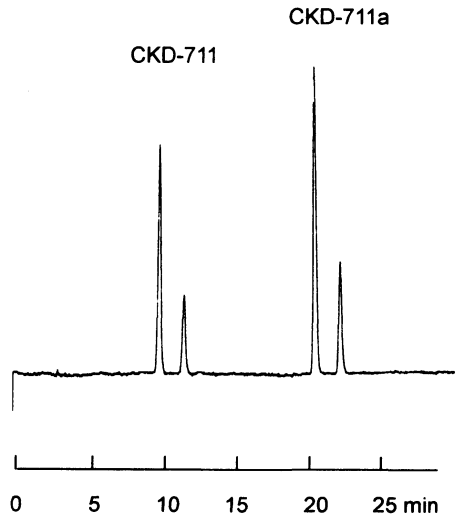



Fig. 3. Isolation procedure for CKD-711 and CKD-711a.

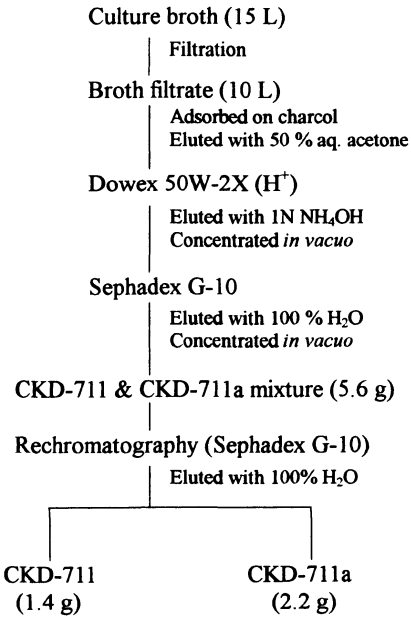



Table 3. Carbohydrate hydrolase inhibitory activity of CKD-711 and CKD-711a.

| Enzymes | Origin | CKD-711 | CKD-711a |
|---|---|---|---|
| | | $IC_{50}$ ($\mu$g/ml) | |
| α-amylase | Porcine pancreas | 78 | 104 |
| | *Bacillus* sp. | > 200 | > 200 |
| | Barley Malt | > 200 | > 200 |
| | *Aspergillus oryzae* | > 200 | > 200 |
| α-glucosidase | Rat intestine | 2.5 | 6.5 |
| | Baker's yeast | > 200 | > 200 |

### Enzyme Inhibitory Activities

CKD 711 and CKD-711a were tested *in vitro* against $\alpha$-amylase and $\alpha$-glucosidase. Of particular interest was that the inhibitory activity was more effective to the mammalian origin than the microorganism and plant origin. CKD-711 and CKD-711a showed a strong inhibitory activity against some selected $\alpha$-glucosidases as shown in Table 3. Interestingly, CKD-711 ($IC_{50}$, 78 $\mu$g/ml) was shown similar effect to CKD-711a ($IC_{50}$, 104 $\mu$g/ml) on porcine pancreatic $\alpha$-amylase, whereas it had about 3-fold higher inhibitory activity ($IC_{50}$, 2.5 $\mu$g/ml) on rat intestinal $\alpha$-glucosidase than CKD-711a ($IC_{50}$, 6.5 $\mu$g/ml).

## Discussion

In the course of screening for new bioactive metabolites from soil actinomycetes, we first targeted active components to Gram-negative bacteria. From 600 selected actinomycetes in the first screening, we discovered CKD-711 and 711a having an $\alpha$-glucosidase inhibitory activity. The producing strain was identified *Streptomyces* sp. CK-4416. Furthermore, CKD-711 and 711a showed more $\alpha$-glucosidase inhibitory activity to the mammalian origin than the microorganism and plant origin. More detail investigations to characterize the pecise inhibitory activity of $\alpha$-amylase and $\alpha$-glucosidase are in the accompanying paper.[10]

### Acknowledgments

The authors are grateful to Ms. YUN SUN LEE for skillful technical assistance, and Dr. SUNGSOOK LEE for helpful discussions.

## References

1) SCHMIDT, D.; W. FORMER, B. JÜNGE, L. MULLER, W. WINGENDER & E. TRUSCHEIT: $\alpha$-Glucosidase inhibitor: New complex oligosaccharides of microbial origin. Naturwissenschaften 64: 535~536, 1977
2) WATANABE, K.; T. FURUMAI T., M. SUDOH, K. YOKOSE & H. B. MARUYAMA: New alpha-amylase inhibitor, trestatins. IV. Taxonomy of the producing strains and fermentation of trestatin A. J. Antibiotics 37: 479~486, 1984
3) MURAO, S.; K. OHYAMA & S. OGURA: Isolation of amylase inhibitor-produng microorganism. Agric. Biol. Chem. 41: 919~924, 1977
4) HORII, S.; H. FUKASSE, T. MATSUO, Y. KAMEDA, N. ASANO & K. MASUI: Synthesis and $\alpha$-D-glucosidase inhibitory activity of *N*-substituted valiolamine derivatives as potent oral antidiabetic agents. J. Med. Chem. 29: 1038~1046, 1987
5) BISCHOFF, H.: Pharmacology of glucosidase inhibitor. Eur. J. Clin. Invest. 24 (Suppl.): 3~10, 1994
6) PULS, W. & U. KEUP: Recent advances in obesity research volume 1. ED., A. Haward, p. 391, 1975
7) ODAKA, H.; N. MIKI, H. IKEDA & T. MATSUO: Effect of disaccharidase inhibitor, AO-128, on postprandial hyperglycemia in rats. J. Jpn. Soc. Nutr. Food Sci. 45: 27~31, 1992
8) MATSUO, T.; H. ODAKA & H. IKEDA: Effect of an intestinal disaccharidase inhibitor (AO-128) on obesity and diabetes. Am. J. Clin. Nutr. 55: 314~317, 1992
9) CHANG, H. B.; S. H. KIM, Y. I. KWON, D. H. CHOUNG, K. H. HAN, W. K. CHOI, T. W. KANG, S. LEE, J. G. KIM, H. S. CHUN, S. K. AHN & C. I. HONG: Novel $\alpha$-glucosidase inhibitors, CKD-711 and CKD-711a produced by *Streptomyces* sp. CK-4416. III. Physico-chemical properties and structure elucidation. J. Antibiotics 55: 467~471, 2002
10) KWON, Y. I.; H. J. SON, K. S. MOON, J. K. KIM, J. G. KIM, H. S. CHUN, S. K. AHN & C. I. HONG: Novel $\alpha$-glucosidase inhibitors, CKD-711 and CKD-711a produced by *Streptomyces* sp. CK-4416. II. Biological properties. J. Antibiotics 55: 462~466, 2002
11) KORNERUP, A. & J. H. WANSCHER: Methuen Handbook of Colour. 3rd Ed. Fletcher and Son Ltd., Norwich, Great Britain, 1983
12) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
13) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some actinomycetales as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
14) BECKER, B.; M. P. LECHEVALIER & H. LECHEVALIER: Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~248, 1965
15) DAHLQVIST, A.: Method for assay of intestinal disaccharidases. Anal. Biochem. 7: 18~25, 1964
16) OVE, N.; H. SJOSTROM, G. M. COWELL, J. TRANUM-JENSER, O. C. HANSEN & K. G. WELINDER: Pig intestinal microvillar maltase-glucoamylase. J. Biol. Chem. 261: 12306~12309, 1986