J. Microbiol. Biotechnol. (2001), **11**(3), 389–393

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Characterization of an α-Glucosidase Inhibitor Produced by *Streptomyces* sp. CK-4416

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Received: October 23, 2000
Accepted: May 14, 2001

Abstract An α-glucosidase inhibitor, CK-4416, was identified from the culture broth of *Streptomyes* sp. CK-4416. CK-4416, which had some specificity against intestinal disaccharidases, especially sucrase and maltase activities, was purified by adsorption and cationic ion exchange chromatograpies. The molecular formula was determined to be $C_{37}H_{63}NO_{30}$ (MW 1001.31) by FAB-MS and NMR analyses. *In vitro* studies found CK-4416 to show a potent inhibitory activity against sucrase and maltase, but it had low inhibition against α-amylase.

Key words: CK-4416, α-glucosidase, inhibitor, *Streptomyces* sp.

Carbohydrates are the major constituents of human diet, and mono-, di-, and polysaccharides are major components of carbohydrates, which mainly supply energy. However, the relatively less frequent monosaccharides (glucose and fructose) can be absorbed only when they are readily taken up from the small intestine [3, 7, 8]. Therefore, the components of dietary carbohydrates should be broken down to monosaccharides by α-glucosidases such as sucrase, maltase, glucoamylase, dextrinase, and the pancreatic α-amylase before they can be absorbed. This enzymatic process is usually taking place rapidly in the upper part of the small intestine.

Based on this concept, Puls *et al.* [12] proposed a new pharmacological approach for improving the metabolic control of diabetics by inhibition of α-glucosidases, because postprandial hyperglycemia was not properly treated by standard pharmacotherapy such as sulfonylureas or insulin. A few inhibitors of intestinal brush-border α-glucosidases have been available for clinical use.

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Acarbose (BAY g 5421) [3, 14], an amino-oligosaccharide (pseudo-tetrasaccharide) which was isolated from the fermentation broth of *Actinoplanes* strain SE 50 strongly inhibited the brush-border enzymes of human subjects *in vitro* and *in vivo*. Inhibition of carbohydrate-digestive enzymes by acarbose resulted in a significant decrease in the postprandial rise of blood glucose level after a mixed carbohydrate diet. However, the major adverse effects of acarbose are abdominal distension, flatulence, meteorism, and possibly diarrhea. It has been suggested that such adverse effects might be caused by excessive inhibition of pancreatic α-amylase, which results in the bacterial abnormal fermentation of undigested carbohydrates in the colon [4, 9].

In our continuing search for bioactive microbial metabolites, we found a new α-glucosidase inhibitor, CK-4416, produced by a strain of *Streptomyces*, which was isolated from the mountain soil of Jeju Island in Korea (*Streptomyces* sp. CK-4416).

This paper describes the fermentation, isolation, physicochemical properties, and structure elucidation of CK-4416 in addition to the inhibition profile for α-glucosidases.

Materials and Method

Preparation of CK-4416

Spore suspension (0.1%) of the producing strain was inoculated into four 500-ml Erlenmeyer flasks containing 100 ml of sterile seed medium which was composed of 1.0% starch, 1.0% glucose, 0.25% peptone, 0.5% yeast extract, and 0.1% $CaCO_3$, and incubated on a rotary shaker at 28°C for 36 h at 240 rpm. The resulting 360 ml of seed culture was transferred to a 30-l jar fermentor (Korea Fermentor Co., Korea) containing 15-l of production medium which was composed of 3% corn starch, 1% glucose, 1.5% soybean flour, 1.5% corn steep liquor, 0.1% KH_2PO_4, 0.5% $CaCO_3$, and 0.05% antifoamer. The fermentation was carried

out at 28°C for 6 days while stirring at 200 rpm. After filtration, the filtrate was applied to activated carbon SK-3794 (Shinki Chem., Korea) and Dowex 50W-2X (Sigma, U.S.A.) ion-exchange chromatography which was eluted with a pyridine-formic acid buffer solution (0.1 N, pH 6.8). Active fractions were collected and concentrated *in vacuo*, followed by CM-Sephadex (Sigma, U.S.A.) ion-exchange chromatography. Active fractions were collected and lyophilized to obtain white powder.

Instrumental Analysis

The ultraviolet spectrum was monitored by a UV-160A UV-spectrometer (Shimadzu, Tokyo, Japan). The infrared spectrum was recorded on the Shimadzu IR 435 spectrophotometer (Shimadzu, Tokyo, Japan), nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR, and DEPT) spectra on a Brucker DPX-600 spectrophotometer (Brucker, Frankfurt, Germany) using tetramethylsilane as the internal standard, and FAB-MS on a JMS-DX 300 FAB-mass spectrometer (JEOL, Tokyo, Japan).

Enzymological Determination of CK-4416

Enzyme activity was assayed according to the method of Dahlqvist [6]. Fifty microliters of the enzyme solution (100 mU of α-amylase from porcine pancreas, 10 mU each of sucrase, maltase, and glucoamylase from porcine intestine in 0.25 M of phosphate buffer at pH 7.0) was incubated at 37°C for 5 min, along with 50 μl of 0.25 M phosphate buffer (pH 7.0) as a control or with the inhibitor in 50 μl of the same buffer solution. The reaction was started by adding 500 μl of each of carbohydrate solutions (400 μg/ml) as the substrate. After 30 min, the reaction

Table 1. Physicochemical properties of CK-4416.

Appearance	White powder
Solubility	Soluble in water Insoluble in $CHCl_3$, *n*-hexane
UV $\lambda_{max}^{H_2O}$ nm (ε)	End absorption
Molecular formula	$C_{37}H_{63}NO_{30}$
FAB-MS (m/z)	1002.31 $(M+H)^+$
IR ν_{max}^{KBr} cm^{-1}	3380, 2920, 1640, 1400, 1360, 1250, 1150, 1025
TLC (R_f)[a)]	0.2
Color reaction:	
$AgNO_3$-NaOH	Positive
$KMnO_4$	Positive
Sakaguchi	Negative

[a)]EtOAc:MeOH:H_2O=5:3:2 (silicagel TLC plates).

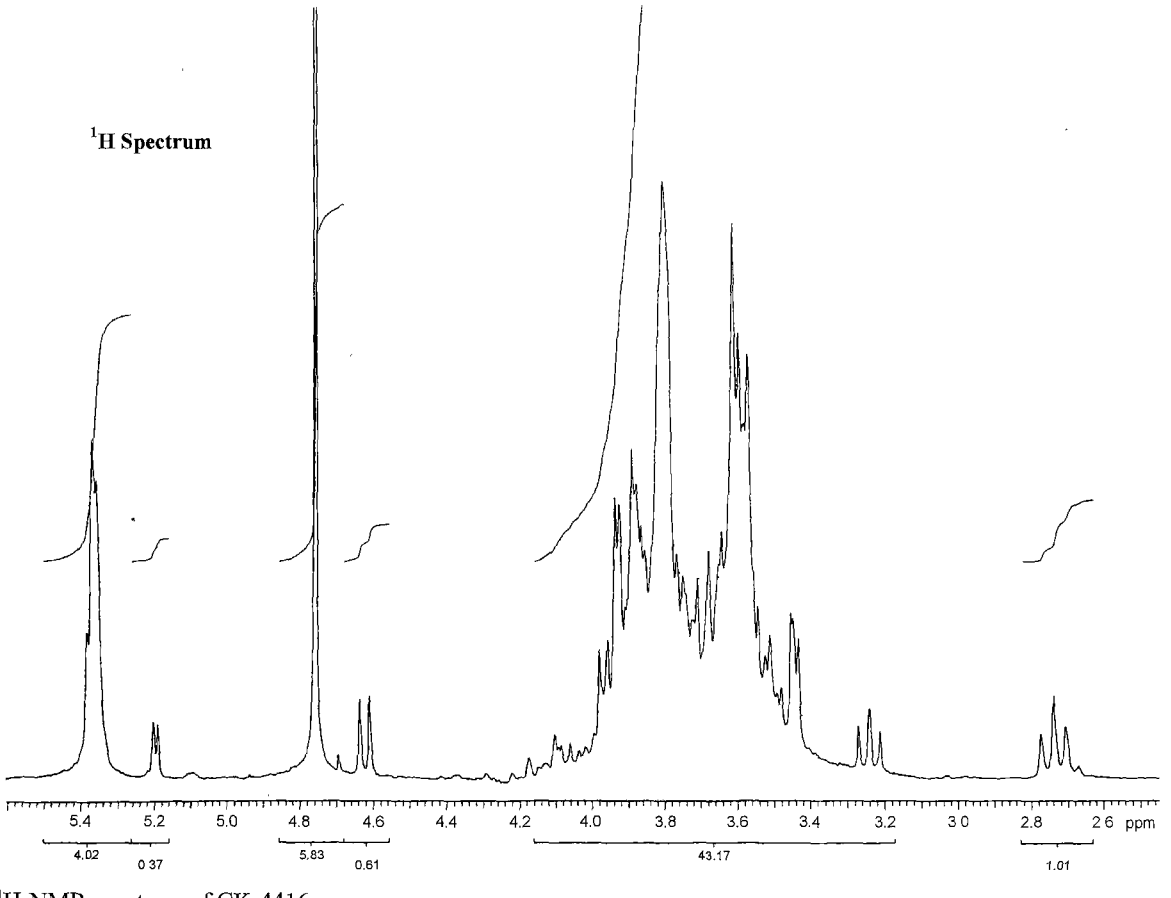

Fig. 1. ^{1}H-NMR spectrum of CK-4416.

mixture was heated in a boiling water bath for 2 min to stop the reaction, and the amount of liberated glucose was determined by the glucose oxidase method [10] by measuring absorbance at 660 nm. The inhibitory activity was calculated from the formula: Inhibition (%)=100×(C–T)/C, where C is the enzyme activity without the inhibitor and T is the enzyme activity with the inhibitor.

RESULTS AND DISCUSSION

Fermentation and Isolation of CK-4416

The production of CK-4416 by *Streptomyces* sp. CK-4416 was carried out in a 30-l jar fermentor. The production of CK-4416 started on the second day, and the maximal yield of 150 μg/ml was observed on the sixth day. The culture broth was filtered through a diatomaceous earth. Broth filtrate (10-l) was adsorbed onto 700 ml of activated carbon column and eluted with 50% MeOH. The active fractions (1.0 l) were collected and concentrated under reduced pressure. It was chromatographed on a Dowex 50W-2X (H^+ form, 0.5-l) column and the column was washed with distilled water (2-l) in order to remove neutral oligosaccharides: Neutral oligosaccharides such as maltotriose or pentaose passed through the column without binding. The active fraction (55 ml) was then eluted with pyridine-formic acid buffer (0.1 N, pH 6.8) and concentrated under reduced pressure. It was then applied to CM-sephadex (NH_4^+ form) ion-exchange chromatography and the column was eluted with 0.1 N NH_4OH. The active fraction (15 ml) was carefully concentrated under reduced pressure below 35°C, followed by lyophilization to yield white powder (92 mg).

Physicochemical Properties and Structure Elucidation of CK-4416

Physicochemical properties of CK-4416 are summarized in Table 1. CK-4416 was soluble in acetone, methanol, and water, and insoluble in ethylacetate, chloroform, and *n*-hexane. The molecular weight of CK-4416 was found to be 1000.31 according to the FAB-MS analysis, and the molecular formula was $C_{37}H_{63}NO_{30}$ based on the FAB-MS and NMR analysis. CK-4416 gave positive color reactions with $AgNO_3$ and $KMnO_4$ but negative to ninhydrin and Sakaguchi reagent. ^{1}H-NMR spectra of CK-4416 are shown in Fig. 1 and ^{13}C-NMR spectrum in Fig. 2. ^{1}H-NMR and ^{13}C-NMR spectra and color reactions strongly indicated that CK-4416 was of the oligosaccharide nature. From the ^{1}H-NMR analysis, 2 doublet peaks at 4.6 and 5.2 ppm were observed and these anomeric protons were assigned for the α and β isomers of the terminal hexose moiety [1]. Tandem mass spectrum and NMR spectra showed that CK-4416 contained four more hexoses in addition to the anomeric hexose. The physicochemical properties of CK-4416 were quite similar to that of NS-520 [11]. However, the ^{13}C-NMR and DEPT (Fig. 3) showed that there was no CH_3 in CK-4416, which is often present in other α-glucosidase inhibitors including NS-520 or acarbose [14]. The molecular formula showed seven unsaturation equivalents consisting of seven rings including five hexoses. The ^{13}C-NMR and IR (1,250 cm^{-1}) indicated the presence of epoxide.

Based on these physicochemical properties, it was suggested that CK-4416 is a new hexose homologue. The detailed structural elucidation of CK-4416 is in progress using various 2D-NMR analyses including ^{1}H-^{1}H COSY,

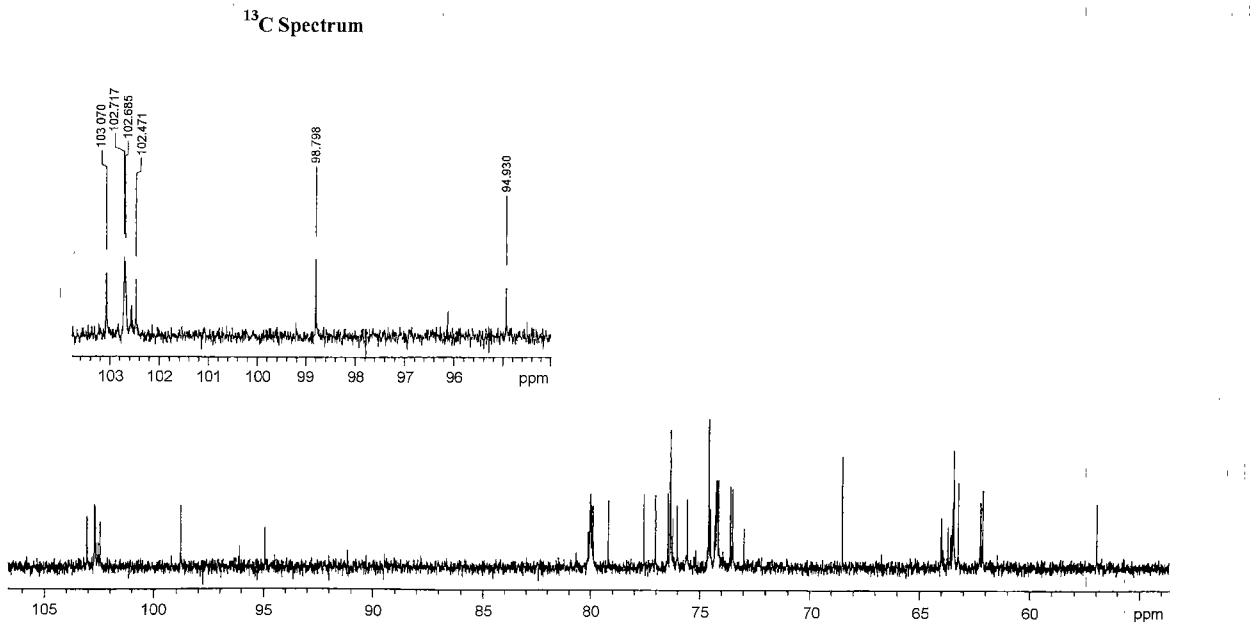

Fig. 2. ^{13}C-NMR spectrum of CK-4416.

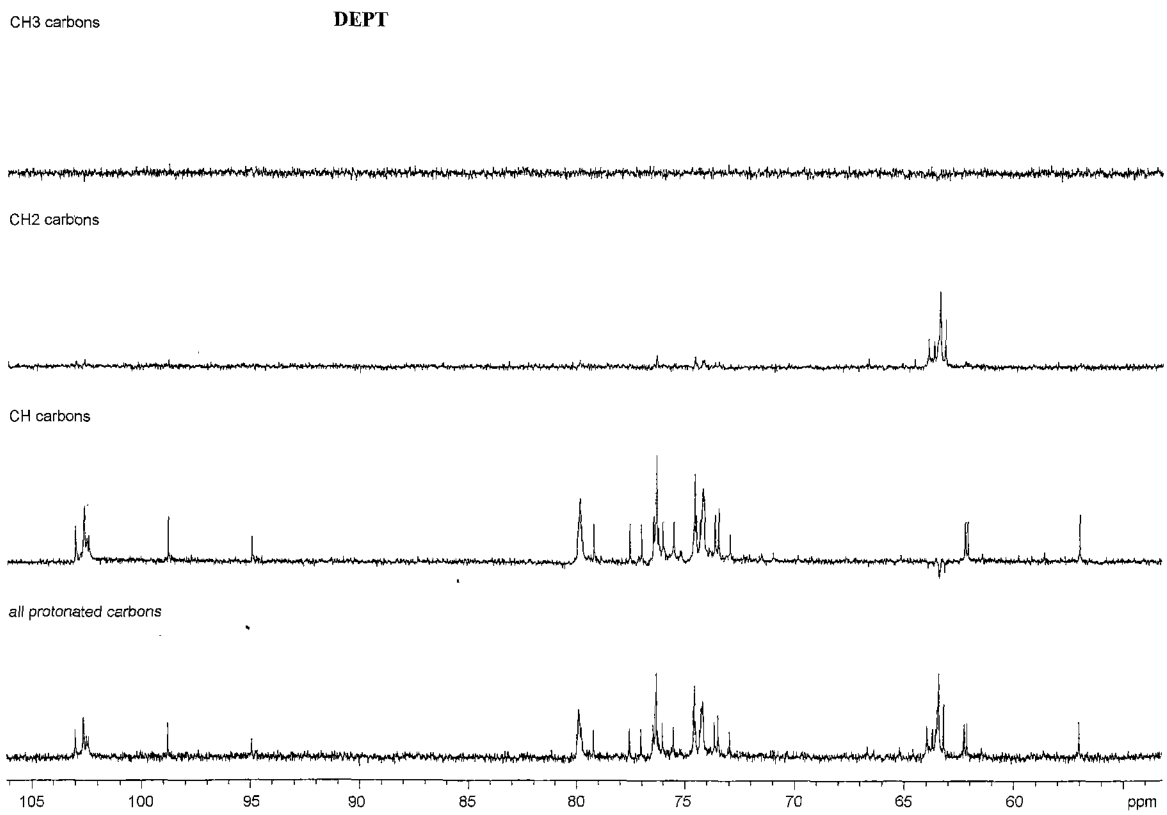

Fig. 3. DEPT spectrum of CK-4416.

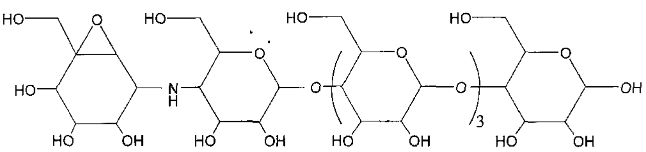

Fig. 4. The proposed structure of CK-4416.

^{1}H-^{13}C COSY, TOCSY, HMQC, and PFG-HMBC. The proposed structure of CK-4416 is shown in Fig. 4.

Characterizations of Enzyme Inhibitory Activities

CK-4416 was tested *in vitro* against intestinal α-glucosidases and pancreatic α-amylase of porcine origin, and found to have lower inhibitory activity than acarbose on porcine intestinal enzymes and α-amylase (Table 2). Consequently, although CK4416 showed lower inhibitory activity than acarbose, CK-4416 is expected to improve glycemic response in diabetes mellitus with less adverse effects such as flatulence, meteorism, abdominal distension, and diarrhea than acarbose for clinical use.

Table 2. Inhibitory activity of CK-4416 against porcine intestinal enzymes *in vitro* (IC_{50}: μg/ml).

Enzymes	CK-4416	Acarbose
α-amylase	104.0	36.0
maltase	6.5	2.5
sucrase	1.5	0.6

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