A Novel Chitinase Inhibitor from a Marine Bacterium, *Pseudomonas* sp.

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A new chitinase inhibitor, CI-4, was isolated from the culture broth of a marine bacterium *Pseudomonas* sp. IZ208. The structure of CI-4 was determined to be cyclo(L-Arg–D-Pro) by spectral studies and comparison with a synthetic sample. CI-4 showed inhibitory activity against chitinase.

Chitinase is widely distributed among plants, microorganisms, marine invertebrates, fish, insects[1] and marine bacteria[2]. It is known to be a key enzyme for the ecdysis of insects through the degradation of chitin. Inhibition of chitinase is expected to control insects. However, there are no known chitinase inhibitors except for allosamidin[3], its derivatives[4] and the styloguanidines[5]. We have developed a screening system for chitinase inhibitor from marine bacteria using a new agar plate method for the determination of chitinase inhibitory activity[6], and found that strain IZ208 produced the chitinase inhibiting substance.

In this paper we describe the identification of chitinase inhibitor producing bacterium, as well as the isolation, structural determination and biological properties of CI-4.

Materials and Methods

General Material

Chitinase from *Bacillus* sp. was purchased from Wako Pure Chemical Ind., Ltd. IR spectra were measured with a Jasco FT/IR-7000 FT-IR spectrometer. Optical rotation was taken with a Horiba SEPA-300 polarimeter with a 5 cm micro cell. ^{1}H NMR, ^{1}H-^{1}H COSY, ^{13}C NMR, HSQC, NOESY and HMBC spectra were recorded with a Varian Unity 500 spectrometer, using D_2O and DMSO-d_6 as solvents. The FAB-MS spectrum (FABMS) was measured with a Jeol JMS-SX102 mass spectrometer.

Microorganisms

Marine bacteria were isolated from seawater samples and sediment samples, which were collected at several points at Hamanako, Yaizu, Shimizu, Numazu and Izu areas in Shizuoka Prefecture, Japan.

Fermentation

A loopful of microorganisms grown on an agar slant was inoculated into a 500 ml Erlenmeyer flask containing 150 ml of E1 medium composed of seawater 750 ml, distilled water 250 ml, Bacto-peptone 5.0 g, Bacto-yeast extract 1.0 g, glucose 2.0 g and $FePO_4$ 0.01 g (pH 7.7 before sterilization) and incubated for 4 days at 20°C on a rotary shaker (120 rpm).

Taxonomic Characterization

The bacterial strain was identified according to BERGEY's Manual of Systematic Bacteriology[7]. Physiological and biochemical studies were mainly performed with the bacterium grown in E1 medium. The mol% guanine-cytosine (G+C) content of bacterial DNA was determined by HPLC with a catalytic reaction using nuclease P1 (Yamasa Shoyu Co.)[8]. The morphological study was carried out mainly by transmission electron microscopy. The isoprenoid quinone composition of each strain was analyzed by LC-MS[9].

Measurement of Chitinase Inhibitory Activity by Agar Plate Method

Chitinase inhibitory activity was measured by the agar plate method using copper sulfate as a positive standard as reported previously[6].

Chitin degrading bacterium EY410 was suspended in 1.0 ml of 75% seawater, the concentration of which was controlled to absorbancy 1.0 at 660 nm. The suspension (150 μl) was spread on the surface of an agar plate containing squid chitin. Paper disks were impregnated with 50 μl of a test sample, dried and placed on the agar plate. After incubating at 24°C for 3 days, the chitinase inhibitory activity was evaluated according to the size of the white zone formed by undegraded chitin around the disk.

Measurement of Chitinase Inhibitory Activity by the Enzyme Method

Chitinase from *Bacillus* sp. was used for the enzyme method. Fifty microliters of the chitinase solution (final concentration of 150 μg/ml) was added to the mixture of 100 μl of test sample solution and 850 μl substrate solution consisting of 1.0% squid chitin dissolved in 50 mM sodium phosphate buffer (pH 7.5), and incubated for 2 hours at 37°C. After this, the reaction mixture was

boiled for 10 minutes, and 80 μl at the supernatant was analyzed by HPLC with TSK Gel G2500 PWxL column (4.8 mm i.d. $\times$ 250 mm, Toso Mfg. Co. Ltd.). The *N*-acetylchitooligosaccharides produced by degradation of chitin were monitored at 210 nm.

The chitinase activity is expressed as the percentage inhibition of the enzyme, and was calculated by the equation: $(1-B/A)\times 100\%$, where A and B are the concentration of produced *N*-acetylchitooligosaccharides without and with the test solution, respectively.

Measurement of Xanthine Oxidase Inhibitory Activity and Trypsin Inhibitory Activity

Xanthine oxidase inhibitory activity was examined according to a modified version of NORO's method[10] and trypsin inhibitory activity was performed according to ERLANGER's method[11].

Results and Discussion

Taxonomy of the Producing Organism

Marine bacterium strain IZ208 was subjected to the standard biological and physiological tests with the results shown in Table 1. A transmission electron micrograph is shown in Fig. 1. The colony was smooth, circular and pigmented with a beige color. The strain was an aerobic Gram-negative rod (0.8×2.1 μm), and it was motile with a polar flagellum. The strain could grow at 0~7.0% NaCl concentration. The catalase and oxidase tests were both positive, and the O/F test was negative. The G+C content was 65.9 mol%. The major isoprenoide quinone was ubiquinone-9. According to BERGEY's Manual of Systematic Bacteriology, the strain was identified as *Pseudomonas* sp.

Isolation and Purification

Bioassay-guided isolation of the solution was successively performed by using the agar plate method[6]. Ten liters of the fermentation broth of the strain IZ208 was centrifuged at $18,800\times g$ for 15 minutes to provide a supernatant, which was extracted with ethyl acetate. Inhibitory activity was recovered in the aqueous layer. The active aqueous layer was applied to an activated

Fig. 1. Electron micrograph of *Pseudomonas* sp. IZ208.

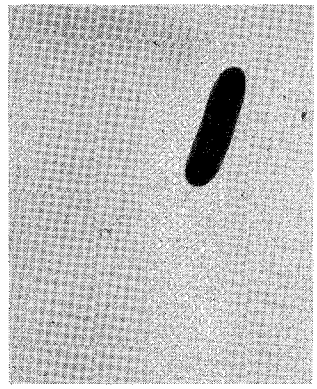


Table 1. Taxonomical studies of strain IZ208.

Characteristics		Characteristics	
Shape	Rod	Hydrolysis of Tween 80	+
Size	0.8 x 2.1 μm	Chitin	+
Pigment	-	Casein	-
Luminescence	-	starch	+
Gram reaction	-	esclin	-
Motility	+	alginate	-
Flagella	Polar	gelatine	-
Presence of spore	-	DNA	+
Aerobic growth	+	Utilization of glucose	+
Nitrate reduction to nitrite	+	mannose	-
Nitrate respiration	+	lactose	-
Production of PHB	-	maltose	+
Catalase	+	sucrose	-
Oxidase	+	galactose	-
O/F test	oxidation	fructose	+
Temperature range for growth	4-50°C	arabinose	-
Optimum growth temperature	30-37°C	inositol	-
pH range for growth	5-9	mannitol	+
Salts requirement	-	trehalose	-
Salinity for growth	0-7.0%	sorbitol	-
Gas production from glucose	-	N-acetylglucosamine	-
Indole production	-	xylose	-
H2S production	-	cellobiose	-
PHB accumulation	-	Utilization of gluconic acid	+
ß-Galactosidase	+	citric acid	+
Lecithinase	+	L-malic acid	+
Urease	-	fumaric acid	+
G+C contents	65.9%	malonic acid	-
Quinone production	ubiquinone-9	adipic acid	-

charcoal (Wako Pure Chemical Ind., Ltd., 1 liter) column, which was washed with water and eluted with EtOH - H_2O - CH_3COOH (50 : 50 : 1). The eluate was concentrated to a small volume, which was applied to a Diaion HP-20 column (Mitsubishi Chemical Ind. Ltd., 1 liter) and eluted with water. The active fraction was chromatographed on a Toyopearl HW-40 (Toso Mfg. Co. Ltd., 800 ml) column with water. Finally, the active fraction was purified by ODS-HPLC (Cosmosil C_{18} AR, Nacalai Tesque, HPLC conditions: column size, 20 × 250 mm; mobile phase, 40 minutes gradient of 0~20% CH_3CN in 0.1% TFA; flow rate 5 ml/minute; detector, UV 210 nm), to give 3 mg of the chitinase inhibitor CI-4 as a colorless powder.

Synthesis of Cyclic Dipeptides

Boc-Arg(Mtr)-Pro-OBzl (*N*-α-t-butoxycarbonyl-ω-*N*-(4-methoxy-2,3,6-trimethylbenzenesulfonyl)-arginylproline benzyl ester) was prepared by the condensation of Boc-Arg(Mtr) and Pro-OBzl by the standard method using DCC[12]. Boc-Arg(Mtr)-Pro-OBzl (404 mg) was stirred in 1 ml of TFA/anisole solution (9 : 1) at 50°C for 1.5 hours. The solution was evaporated, 3 ml of water, was added and the pH was adjusted to 6.0 by addition of TEA. The solution was kept at 80°C for 16 hours. Finally, cyclo(Arg-Pro) was separated by ODS-HPLC (conditions of HPLC are described at the "Isolation" section of this report) and cyclo(Arg-Pro) was isolated as 65 mg of white powder. Three kinds of cyclo(Arg-Pro) isomers, cyclo(L-Arg–L-Pro), cyclo(L-Arg–D-Pro) and cyclo(D-Arg–L-Pro) were synthesized by using this method.

Structure Determination

The physico-chemical properties of the chitinase inhibitor CI-4 are summarized in Table 2. HR FAB-MS showed its molecular formula to be $C_{11}H_{19}N_5O_2$. The IR spectrum exhibited a strong absorption peak at 1673 cm^{-1} and no absorption at 1550 cm^{-1} which implied the existence of a diketopiperazine ring in the molecule.

All ^1H and ^{13}C signals in NMR were assigned by ^1H-^1H COSY, HSQC and HMBC experiments and are listed in Table 3. These suggested that CI-4 consisted of Arg and Pro moieties. The diketopiperazine structure

Table 2. Physico-chemical properties of chitinase inhibitor CI-4.

Appearance	White powder
$[\alpha]_D^{29}$	+42.0 (c0.24,H_2O)
Molecular formula	$C_{11}H_{19}N_5O_2$
HRFAB-MS $(M+H)^+$ (*m/z*)	
calcd:	254.1617
found:	254.1627
IR (KBr) cm^{-1}	3400, 1673, 1458, 1205, 1137
Solubility	
soluble in:	H_2O, MeOH, DMSO
insoluble in:	n-Hexane, $CHCl_3$
Rf value	0.67[a]

[a] Merck, Kieselgel 60 F_{254}: BuOH - CH_3COOH - Pyridine - H_2O (4 : 1 : 1 : 2).

Table 3. Chemical shift in the ^1H NMR and ^{13}C NMR spectra of the CI-4 and synthetic cyclo(Arg-Pro) isomers.

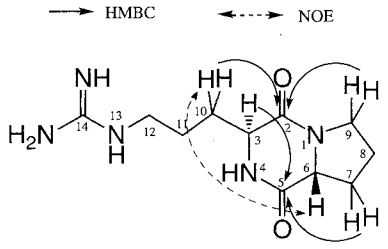
	CI-4 cyclo(L-Arg-D-Pro)	CI-4* cyclo(L-Arg-D-Pro)	cyclo(L-Arg-L-Pro)	cyclo(D-Arg-L-Pro)
H-3	4.08	3.63	4.41	4.08
4N-H		8.32		
H-6	4.43	4.12	4.38	4.42
H-7	1.99, 2.41	1.78, 2.16	2.03, 2.41	1.98, 2.42
H-8	1.99, 2.13	1.78, 1.86	2.03, 2.13	1.98, 2.14
H-9	3.62	3.42	3.62	3.62
H-10	1.92	1.63	1.97, 2.03	1.92
H-11	1.77	1.55	1.63, 1.76	1.76
H-12	3.29	3.11	3.29	3.29
13N-H		7.52		
Carbon				
2	170.2	165.4	169.6	170.2
3	59.1	56.4	57.2	59.1
5	174.0	168.4	174.9	174.0
6	60.8	57.4	61.6	60.8
7	30.8	28.5	30.4	30.8
8	24.0	21.6	24.3	24.0
9	48.3	45.0	47.9	48.3
10	32.7	30.5	28.8	32.7
11	26.4	24.8	25.5	26.4
12	43.0	40.2	43.3	43.0
14	159.4	156.6	159.3	159.3

Measured in D_2O at pD 3.0 and * in DMSO-d_6.

Table 4. Optical rotations of CI-4 and the synthetic cyclo(Arg-Pro) isomers.

	CI-4	cyclo(L-Arg-D-Pro)	cyclo(L-Arg-L-Pro)	cyclo(D-Arg-L-Pro)
$[a]_D^{29}$ (H_2O)	+42.0° (c 0.24)	+42.1° (c 0.46)	-70.9° (c 0.40)	-43.0° (c 0.67)

Fig. 2. Coupling diagram of HMBC and NOE experiments in DMSO-d_6 of CI-4.



was confirmed by the observation of the correlations between C-5 (carbonyl carbon of Pro) and H-3 (α-proton of Arg), and C-2 (carbonyl carbon of Arg) and H-9 (δ-proton of Pro) in the HMBC spectrum (Fig. 2). These data suggested the plenary structure of cyclo(Arg-Pro) for CI-4.

The NOE experiment of CI-4 exhibited a cross peak between H-6 (α-proton of Pro) and H-10 (β-proton of Arg) (Fig. 2), which suggested that the α-carbons of these two amino acids had opposite configurations. The stereochemistry of CI-4 was confirmed by the comparison of spectral data and optical rotations of CI-4 with that of the L-L, L-D and D-L synthetic stereoisomers. The NMR chemical shifts of CI-4 and synthetic cyclo(L-Arg–D-Pro) were completely identical. The optical rotation of CI-4 (+42.0°) was also identical with that of synthetic cyclo(L-Arg–D-Pro) (+42.1°) (Table 4). Thus, the structure of CI-4 was determined to be cyclo(L-Arg–D-Pro) shown in Fig. 2.

Among the diketopiperazine compounds of bacterial origin, only one compound, cyclo(L-Pro–D-Pro), containing a D-amino acid had been reported; CI-4 is a second example.

Biological Activity

By the agar plate method, cyclo(L-Arg–D-Pro) (CI-4) and cyclo(L-Arg–L-Pro) exhibited strong chitinase inhibitory activity at a concentration of 50 μg/disk, which was almost similar to that of the reference, $CuSO_4$, as shown in Table 5. Inhibitory activity of cyclo(D-Arg–L-Pro) was weaker than $CuSO_4$. L-Arg, D-Arg, L-Pro, D-Pro and cyclo(Gly-Gly) did not inhibit chitinase.

Table 5. Chitinase inhibitory activity by the agar plate method.

Sample	Concentration (μg/disk)	Activity
L-Arginine	50	-
D-Arginine	50	-
L-Proline	50	-
D-Proline	50	-
Cyclo(Gly-Gly)	50	-
Cyclo(L-Arg-L-Pro)	50	++
Cyclo(L-Arg-D-Pro) (CI-4)	50	++
Cyclo(D-Arg-L-Pro)	50	±
$CuSO_4$, positive control	50	++

++: strong activity, +: normal activity, ±: weak activity, −: no activity.

Table 6. Chitinase inhibitory activity by the enzyme method.

Sample	Concentration (mM)	Inhibitory Activity(%)
L-Arginine	1.0	0
D-Arginine	1.0	0
L-Proline	1.0	0
D-Proline	1.0	0.1
L-Glysine	1.0	0
Cyclo(Gly-Gly)	1.0	3.2
Cyclo(L-Arg-L-Pro)	1.0	18.4
Cyclo(L-Arg-D-Pro) (CI-4)	1.0	17.2
Cyclo(D-Arg-L-Pro)	1.0	4.9
$CuSO_4$, positive control	1.0	25.9

Cyclo(L-Arg–D-Pro) and cyclo(L-Arg–L-Pro) also showed 18% inhibitory activity by the enzyme method, which was slightly weaker than $CuSO_4$ (Table 6). Cyclo(D-Arg–L-Pro) and cyclo(Gly-Gly) were weaker than CI-4. Neither L- nor D-Arg and L- nor D-Pro showed any inhibiting activity against chitinase. These results of enzyme method were comparable to that of the agar plate method.

CI-4 and cyclo(L-Arg–L-Pro) did not show inhibitory activity against other enzymes such as trypsin and xanthine oxidase.

CI-4 and cyclo(L-Arg–L-Pro) exhibited no antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Vibrio costicola*, *Bacillus subtilis* and *Candida albicans* at the concentration 50 μg/disk by the paper disk agar diffusion method and no cytotoxic activity against B/Hras, B/Normal and HeLa S3 cells at

the concentration of 10 μg/ml.

As CI-4 shows no toxic characteristics, it might find applications as biochemical tools for the development of new types of drugs. Morphological studies against fungus and yeast by cyclo(Arg-Pro) are now under investigation.

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