EI-1511-3, -5 and EI-1625-2, Novel Interleukin-1β Converting Enzyme Inhibitors Produced by *Streptomyces* sp. E-1511 and E-1625

I. Taxonomy of Producing Strain, Fermentation and Isolation

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EI-1511-3, -5 and EI-1625-2, novel interleukin-1β converting enzyme (ICE) inhibitors, were isolated from the culture broths of *Streptomyces* sp. E-1511 and E-1625. EI-1511-3, -5 and EI-1625-2 selectively inhibited the recombinant human ICE activity with IC_{50} values of 0.09, 0.38 and 0.2 μM, respectively. Taxonomy, fermentation of the producing strain and isolation of EI-1511-3, -5 and EI-1625-2 are described.

Interleukin-1 (IL-1) is the cytokine whose involvement in etiology of acute and chronic inflammation has been clarified with the use of IL-1 receptor antagonist (RA), soluble IL-1 receptor, and anti-IL-1 receptor antibody[1]. Two forms of IL-1 (IL-1α and IL-1β) are encoded by different genes and both IL-1 forms show biological activities through binding to their specific receptor with similar affinity[2]. Though both IL-1α and IL-1β are produced as precursors in monocytes, only IL-1β has to be processed by proteolytic cleavage to obtain biological activities[3]. The processing enzyme responsible for IL-1β maturation is interleukin-1β converting enzyme (ICE), a unique cysteine-containing heterodimeric protease, which cleaves the inactive IL-1β precursor into the biologically active IL-1β[4,5]. IL-1β is released from macrophage-like cells following inflammatory stimulation, and it is the major form of IL-1 in diseases. ICE inhibitors therefore might be useful as anti-inflammatory agents[6].

In the course of screening to obtain ICE inhibitors from microorganisms, we found the metabolites of *Streptomyces* sp. E-1511 and E-1625 inhibited ICE. In this article, we describe the taxonomy, fermentation of the producing strain and the isolation of three novel compounds, EI-1511-3, -5 and EI-1625-2. The physicochemical properties, structural elucidation and biological properties are described in accompanying papers.

Materials and Methods

Materials

Recombinant human ICE was prepared by heterologous expression in *Escherichia coli* and *in vitro* refolding as described[7]. The enzyme was purified by ion exchange chromatography[7]. Acetyl-Tyr-Val-Ala-Asp-amino-4-methylcoumarin (Ac-Tyr-Val-Ala-Asp-AMC)[4], a fluorescent substrate, was purchased from Peptide Institute, Inc., Osaka, Japan. All other chemicals were of analytical grade.

Microorganism

By evaluating ICE inhibitory activity of culture broth of many microorganisms, we found the strains E-1511 and E-1625 produces ICE inhibitory compounds. The producing strains E-1511 and E-1625 were isolated from soils collected in Shizuoka and Hokkaido prefecture, Japan, respectively. These strains have been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukubashi, Ibaraki, Japan, as *Streptomyces* sp. E-1511 with the accession number FERM BP-4792 and as *Streptomyces* sp. E-1625 with the number FERM BP-4965.

Taxonomical Characterization

Cultural and physiological characteristics of strains E-1511 and E-1625 were determined by the methods of the International *Streptomyces* Project[8] (ISP). Color codes were assigned to the substrate and aerial mycelial pigments according to the Color Harmony Manual[9]. Morphology of the strain was ascertained by light and scanning electron microscopy (HITACHI S-570). The

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temperature ranges for growth of the strain were determined after submerged cultivation for 7 days using ISP No. 5 medium. Analysis of diaminopimelic acid was performed on the hydrolysate of aerial mycelia grown on solid ISP No. 4 medium as described previously[10].

Culture and Medium Conditions

A loopful of cells from a mature slant of strain E-1511 was inoculated into each of two 50-ml test tubes containing 10 ml of seed medium composed of glucose 1%, soluble starch 1%, yeast extract (Nihon-Seiyaku) 0.5%, Bacto-Tryptone (Difco) 0.5%, beef extract (Kyokuto) 0.3%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% in deionized water (pH adjusted to 7.0 with NaOH before sterilization). The inoculated tubes were incubated on a reciprocating shaker at 28°C for 3 days. Five ml of the seed culture were added to a 300-ml Erlenmeyer flask containing 45 ml of the same medium. Four inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. Fifty ml of the seed culture were added to a 2-liter Erlenmeyer flask containing 450 ml of the same medium. Four inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. The 1.8 liters of seed culture was transferred into a 30-liter fermenter containing 17 liters of a fermentation medium composed of Diaion HP-20 10% (v/v), soluble starch 4%, soybean meal 1%, cornsteep liquor 0.5%, dry yeast (Asahi brewery) 0.5%, KH_2PO_4 0.05%, $ZnSO_4 \cdot 7H_2O$ 10 μg/ml, $CoCl_2 \cdot 6H_2O$ 1 μg/ml, $NiSO_4$ 1 μg/ml and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% in deionized water (pH adjusted to 7.0 with NaOH before sterilization). The fermentation was carried out for 6 days at 28°C with agitation of 300 rpm and aeration of 18 liters per minute. The production of EI-1511s was determined by HPLC. For this measurement, mycelia from 1 ml of the culture broth was extracted with 1 ml of MeOH and the extracts were evaporated and dissolved in 200 μl of MeOH. The concentrated extract (2 ~ 10 μl) was provided for HPLC analysis.

A loopful of cells from a mature slant of strain E-1625 was inoculated into each of two 50-ml test tubes containing 10 ml of the seed medium described above. The inoculated tubes were incubated on a reciprocating shaker at 28°C for 2 days. Five ml of the seed culture were added to a 300-ml Erlenmeyer flask containing 45 ml of the seed medium. Four inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. Five ml of the seed culture were added to a 300-ml Erlenmeyer flask containing 45 ml of the fermentation medium described above. Forty inoculated flasks were incubated for 6 days on a rotary shaker (200 rpm) at 28°C.

Determination of EI-1511s by HPLC

HPLC analysis was performed on a ODS-AQ-312 (6 mm i.d. × 150 mm, YMC Co., Ltd.). The column was eluted with 75% aqueous MeOH containing 0.1% AcOH, at flow rate of 1.0 ml per minute. The eluent was monitored at the wavelength of 220 nm. The retention times of EI-1511-3 and EI-1511-5 were 10.7 minutes and 14.4 minutes, respectively.

Assay of ICE Activity

ICE activities were measured as described by THORNBERRY *et al.*[4] with minor modification. Assay mixtures (sodium phosphate buffer: pH 6.8 10 mM, bovine serum albumin 0.2%, substrate Ac-Tyr-Val-Ala-Asp-AMC 10 μM, refolded ICE 1×10^{-12} I.U., test sample solution 1%; final volume 100 μl) were incubated for 2 hours at room temperature. One I.U. was defined as the amount of enzymatic activity which produced 1 μmol AMC per minute. Then the change in fluorescent intensity due to liberated AMC (excitation-wave length: 370 nm, emission-wave length: 440 nm) was measured.

Results

Characterization of the Producing Strain E-1511 and E-1625

Strain E-1511 and E-1625 grew well or moderately on eight agar media. The colors of aerial mycelia, substrate mycelia and soluble pigment of strain E-1511 and E-1625 are shown in Tables 1 and 2. The aerial mycelia were moderately short with simple branches and formed spiral spore chains of 10 or more. The spores were short-ellipsoidal, smooth and 0.6 to 0.8 μm by 0.7 to 0.9 μm.

Table 1. Cultural characteristics of strain E-1511.

Medium	Amount of growth	Color of:		Soluble pigment
		Aerial mycelium	Substrate mycelium	
Yeast extract-malt extract agar (ISP No. 2)	Abundant	Silver gray (3fe)	Clove brown (3pl)	None
Oatmeal agar (ISP No. 3)	Moderate	Ashes (5fe)	Golden brown (3pi)	Brown
Inorganic salt-starch agar (ISP No. 4)	Abundant	Silver gray (3fe)	Mustard brown (2pl)	Brown
Glycerol-asparagine agar (ISP No. 5)	Abundant	Silver gray (3fe)	Clove brown (3ni)	Brown
Tyrosine agar (ISP No. 7)	Abundant	Covert gray (2fe)	Mustard brown (2pi)	Brown
Sucrose-nitrate agar	Moderate	Beige gray (3ih)	Olive gray (1 1/2ig)	None
Glucose-asparagine agar	Moderate	Silver gray (3fe)	Mustard tan (21g)	Brown
Nutrient agar	Moderate	Covert gray (2fe)	Mustard brown (2pi)	None

No fragmentation of substrate mycelia was observed, and sclerotia, sporangia, or flagellated spores were not formed in cultures. The physiological characteristics of strain E-1511 and E-1625 are shown in Tables 3 and 4. Analysis of cell wall hydrolysates of the strains revealed that the cell walls contained LL-diaminopimelic acid and glycine. The predominant menaquinone type was MK-9(H8), and there was a significant amount of MK-9(H6). These taxonomic observations indicated that both strain E-1511 and E-1625 belong to the genus *Streptomyces*.

Production of EI-1511-3, -5 and EI-1625-2 by Fermentation

The time courses of the the EI-1511s and other manumycin-related compounds production in 30-liter fermenter by strain E-1511 are shown in Fig. 1A and the ICE inhibitory activity in 300-ml Erlenmeyer flask by strain E-1625 are shown in Fig. 1B. Since EI-1511-5, *ent*-alisamycin (an enantiomer of alisamycin), and U-56,407 produced in culture broth of strain E-1511 had quite similar retentions time in HPLC, the amount of each compound in the culture broth could not be calculated precisely and total amount of mixture was expressed in Fig. 1A. Small amounts of EI-1511s already existed on day 3. The amount increased as cultivation progressed and reached a maximum on day 6.

The time courses of the ICE inhibitory activity in the culture broth of strain E-1625 reached a maximum on day 3 and remained by day 6.

Table 2. Physiological properties of strain E-1511.

Characteristics	E-1511
Temperature range for growth	6~38°C
Optimum temperature range	25~30°C
Liquefaction of gelatin	Positive
Hydrolysis of starch	Positive
Coagulation of milk	Negative
Peptonization of milk	Positive
Formation of melanin	Positive
Utilization of D-Glucose	Positive
L-Arabinose	Negative
D-Xylose	Positive
L-Rhamnose	Positive
Raffinose	Negative

Isolation and Purification

The isolation procedure for the EI-1511s is shown schematically in Fig. 2. The producing strain E-1511 was cultured according to the method described. Culture broth obtained (17 liters) was passed through a sieve (150 μm). The residual Diaion HP-20 resin on the sieve was washed thoroughly with water to remove mycelia. The Diaion HP-20 resin was layered on a column packed with Diaion HP-20 (1 liter). After washing with water (12 liters), the active principles were eluted with methanol-acetone (7:3) solution (10 liters). The active fractions were combined, concentrated *in vacuo* to remove acetone and diluted with water. This aqueous solution was applied to an ODS column (ODS AQ-S50, 400 ml, YMC Co., Ltd.), washed with 30% acetone-0.1% AcOH and eluted with 65% acetone-0.1% AcOH. The active fractions were again diluted with water, adsorbed on an ODS column (ODS AQ-S50, 30 mm i.d. × 500 mm), washed with 30% acetone-0.1% AcOH and eluted with 65% acetone-0.1% AcOH. EI-1511-3 was eluted before EI-1511-5. Each active fraction was diluted with water, adsorbed on an ODS column (30 mm i.d. × 500 mm, ODS AQ-S50 for EI-1511-3 and ODS-T, Nomura Kagaku Co., Ltd. for EI-1511-5). Each column was eluted with 60% acetone-0.1% AcOH, respectively. The active fractions containing EI-1511-5 were recycled, combined and then evaporated to dryness. EI-1511-5 (30 mg) was obtained as yellow powder. The active fractions containing EI-1511-3 were, on the other hand, combined, concentrated *in vacuo* and readsorbed on an ODS column (30 mm i.d. × 500 mm, ODS AQ-S50). The

Table 3. Cultural characteristics of strain E-1625.

Medium	Amount of growth	Color of:		Soluble pigment
		Aerial mycelium	Substrate mycelium	
Yeast extract-malt extract agar (ISP No. 2)	Abundant	White (a)	Tile red (5ne)	Brown
Oatmeal agar (ISP No. 3)	Abundant	Gray (g)	Rust tan (5le)	Brown
Inorganic salt-starch agar (ISP No. 4)	Abundant	Lt gray (d)	Powder rose (6ec)	Brown
Glycerol-asparagine agar (ISP No. 5)	Abundant	Cream (1 1/2ca)	Dusty yellow (1 1/2gc)	Ocher
Tyrosine agar (ISP No. 7)	Abundant	White (a)	Olive (1 1/2ni)	None
Sucrose-nitrate agar	Moderate	Pussywillow gray (5dc)	Sand (5cb)	Dark brown
Glucose-asparagine agar	Abundant	Natural (3dc)	Dusty yellow (1 1/2gc)	Yellow
Nutrient agar	Moderate	None	Lt brown (4ng)	None

Table 4. Physiological properties of strain E-1625.

Characteristics	E-1625
Temperature range for growth	5.5~46.5°C
Optimum temperature range	25~30°C
Liquefaction of gelatin	Negative
Hydrolysis of starch	Negative
Coagulation of milk	Negative
Peptonization of milk	Negative
Formation of melanin	Negative
Utilization of D-Glucose	Positive
L-Arabinose	Positive
D-Xylose	Positive
D-Fructose	Positive
L-Rhamnose	Positive
Sucrose	Negative
Raffinose	Negative
D-Mannitol	Positive
Inositol	Positive

Fig. 1. Time course of EI-1511s production in a 30 liter fermenter (A) and EI-1625 production in 300-ml Erlenmeyer flask (B).

○ pH, △ mixture of *ent*-alisamycin, U-56,407 and EI-1511-5, ● manumycin G, □ EI-1511-3, ▲ Inhibition of ICE (%)

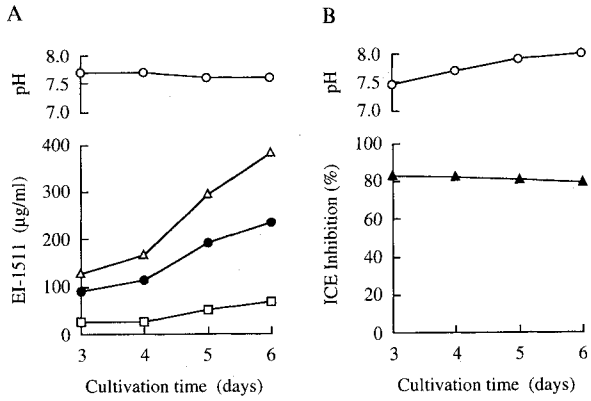
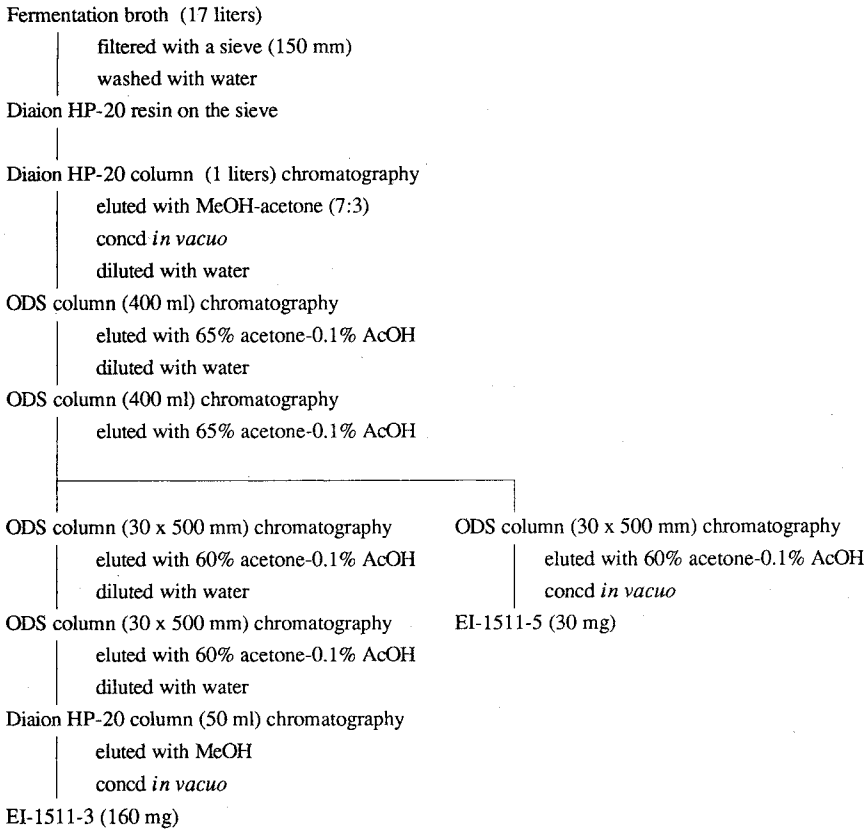


Fig. 2. Purification procedure for EI-1511-3 and -5.



column was eluted with 60% acetone-0.1% AcOH. The active fractions containing EI-1511-3 were combined, diluted with water and then adsorbed on an HP-20 column (50 ml). The active fractions were eluted with acetone and the evaporated to dryness. EI-1511-3 (160 mg) was obtained as yellow powder.

The isolation procedure for the EI-1625-2 is shown schematically in Fig. 3. The producing strain E-1625 was cultured according to the method described. Culture broth obtained (2 liters) was filtered with the aid of diatomaceous earth. The mycelia was extracted with MeOH (2 liters) and filtered with the aid of diatomaceous

Fig. 3. Purification procedure for EI-1625-2.

Fermentation broth (2 liters)
↓ filtered
Mycelia
↓ extracted with MeOH (2 liters)
↓ filtered
MeOH extract
↓ diluted with water
Diaion HP-20 column (400 ml) chromatography
↓ eluted with MeOH-acetone (7:3)
↓ concd *in vacuo*
Diaion HP-20SS column (200 ml) chromatography
↓ eluted with 20~100% MeOH-0.1% AcOH
↓ concd *in vacuo*
Preparative HPLC (YMC ODS-AQ S-50)
↓ eluted with 75% MeOH-0.1% AcOH
↓ concd *in vacuo*
EI-1625-2(20 mg)

earth. The filtrates were diluted with 4 liters of water and adsorbed on a Diaion HP-20 column (400 ml). After washing with 20% aqueous MeOH (1.6 liters), the active principles were eluted with methanol-acetone (7:3) solution (1.6 liters). The active fractions were combined, concentrated *in vacuo* to remove acetone and diluted with water. Half of this aqueous solution was applied to a Diaion HP-20SS column (200 ml), eluted with a MeOH gradient elution (from 0 to 880 minutes from 20 to 100%; from 880 to 1000 minutes at 100%, flow rate 5 ml per minute) in the presence of 0.1% AcOH. The active fractions containing EI-1625-2 were combined, dried *in vacuo* to dryness, dissolved in 5 ml of 75% MeOH and adsorbed on an ODS column (ODS AQ-S50, 30 mm i.d. × 500 mm). The column was eluted with 75% MeOH-0.1% AcOH. The active fractions were combined and dried *in vacuo* to dryness. EI-1625-2 (20 mg) was obtained as yellow powder.

Discussion

We have isolated the novel compounds, EI-1511-3, -5 and EI-1625-2, from the culture broths of *Streptomyces* sp. strain E-1511 and strain E-1625. Three isolated compounds were structurally related to, but different from, known manumycin group compounds such as manumycin A[11], asukamycin[12], colabomycin A[13], alisamycin[14], nisamycin[15], U-62,162[16], U-56,407[17], and manumycin B, C, D, E, F and G[18,19]. These compounds are active antibiotics against Gram-positive bacteria. Although polymorphonuclear leukocyte elastase inhibitory activities and Ras farnesyltransferase inhibitory activities were also reported in some of the manumycin-related compounds[20], ICE inhibitory activities have not yet been described to date, During isolation of the compounds we found that *Streptomyces* strain E-1511 also produced manumycin G, *ent*-alisamycin and U-56,407 and *Streptomyces* strain E-1625 produced manumycin B and C. These manumycin group antibiotics also shared ICE inhibitory properties as described in an accompanying paper.

Several peptide-related compounds derived from ICE recognition amino acid sequence have been reported as ICE inhibitors[4,21], and the role of ICE in the pathology of inflammation has been clarified with these inhibitors. EI-1507s[22], benz[α]anthracene-related compounds, and L-741,498[23], though less potent, are the known ICE inhibitors isolated previously from microbial sources. EI-1511-3, -5 and EI-1625-2 had different chemical skeletons from these compounds that different stability and distribution of ICE inhibitors *in vivo* and new insight to the design of ICE inhibitor could be expected, although, these inhibitors were shown to have activities comparable to those of EI-1507-1 and -2.

IL-1β, generated by ICE, is implicated in the pathophysiology of various diseases[1], but details of this implication are still unclear. EI-1511-3, -5 and EI-1625-2 would be useful for clarifying the true pathophysiological and physiological roles of ICE.

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