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EI-2346, a Novel Interleukin-1β Converting Enzyme Inhibitor Produced by *Streptomyces* sp. E-2346

I. Taxonomy of Producing strain, Fermentation, Isolation, Physico-chemical Properties, and Biological Properties

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EI-2346, a novel interleukin-1β converting enzyme (ICE) inhibitor, was isolated from the culture broths of *Streptomyces* sp. E-2346. EI-2346 selectively inhibited the human recombinant ICE activity with an IC_{50} value of 3.9 μM, without inhibiting elastase and cathepsin B. EI-2346 also inhibited mature interleukin-1β secretion from THP-1 cells induced by LPS with an IC_{50} value of 5.2 μM.

IL-1 is the proinflammatory cytokine that contributes to the pathogenesis of several diseases including rheumatoid arthritis, septic shock and other acute and chronic inflammation[1,2]. Although the two different IL-1 genes (IL-1α and IL-1β) have been described, the proteins encoded by the two genes recognize the same cell surface receptors and share the various biological activities[3,4]. IL-1β is released from macrophage-like cells in an inflammatory situation, and is the major form of IL-1 in diseases. Although both IL-1α and IL-1β are produced as precursors in monocytes, only IL-1β is processed to its mature form by proteolytic cleavage[5]. It has been found that IL-1β converting enzyme (ICE) is responsible for the cleaves of the 31 kDa precursor form of biologically-inactive IL-1β to the 17.5 kDa mature form of biologically-active IL-1β[6,7]. From this finding, it is speculated that the inhibition of ICE by specific inhibitors could reduce the formation of mature IL-1β, in inflammatory diseases and, thus, lead to suppression of the diseases.

To discover novel anti-inflammatory drugs, we intiated a screening program to obtain ICE inhibitors from microbial sources[8~12]. In the course of this screening, we found the metabolites of *Streptomyces* sp. E-2346 inhibited ICE. In this article, we describe the taxonomy and fermentation of the producing strain and isolation, and biological properties of EI-2346.

Material and Methods

Material

Human recombinant ICE was prepared by heterologous expression of p10 and p20 subunits in *Escherichia coli* and *in vitro* refolding as described[13]. The enzyme was purified

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by ion exchange chromatography[13]. Acetyl-Tyr-Val-Ala-Asp-amino-4-methylcoumarin (Ac-Tyr-Val-Ala-Asp-AMC)[5], a fluorescent substrate was purchased from Peptide Institute, Inc., Osaka, Japan. All other chemicals were of analytical grade.

Microorganism

The producing strain E-2346 was isolated from soil collected in Mie prefecture, Japan. The strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of industrial Science and Technology, Tsukubashi, Ibaraki, Japan, as *Streptomyces* sp. E-2346 with the accession number FERM BP-5348.

Taxonomical Characterization

Culture and physiological characterization of strain E-2346 were determined by the methods of International *Streptomyces* Project[14] (ISP). Color codes were assigned to the substrate and aerial mycelial pigments according to the Color Harmony Manual[15]. Morphology of the strain was ascertained by light and scanning electron microscopy (HITACHI S-570). The temperature ranges for growth of the strain were determined after submerged cultivation using ISR No. 5 medium for 7 days. Analysis of diaminopimelic acid was performed on the hydrolysate of aerial mycelia grown on solid ISP No. 4 medium as described previously[16].

Culture and Medium Conditions

A loopful of spores of E-2346, grown on an agar slant, was inoculated into each of two 50-ml test tubes containing 10 ml of a seed medium composed of glucose 1%, soluble starch 1%, bacto tryptone 0.5%, yeast extract 0.5%, beef extract 0.3%, and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% in deionized water (pH 7.2 before sterilization). The inoculated test tubes were incubated on a reciprocating shaker for 2 days at 28°C. Five ml of the seed culture was added to a 300-ml Erlenmeyer flask containing 50 ml of the same medium. Four inoculated flasks were incubated for 2 days on a rotary shaker (220 rpm) at 28°C. Five ml of the seed culture was transferred into a 300-ml Erlenmeyer flask containing 50 ml of the 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). Forty inoculated flasks were incubated for 6 days on rotary shaker at 28°C with agitation of 220 rpm.

Assay of ICE Activity

ICE activities were measured as described by THORNBERRY *et al.*[5] with a slight modification. An assay mixture (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) was 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 of liberated AMC (excitation-wave length: 370 nm, emission-wave length: 440 nm) was measured.

Assay of Cathepsin B and Elastase Activities

The enzymatic activities of cathepsin B and elastase were assayed according to the method of BARRETT and KIRSCHKE[17] and that of MUMFORD *et al.*[18], respectively.

Measurement of Interleukin-1β Secretion

THP-1 cells were suspended in RPMI1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum, and were distributed into 24-well plates as inocula of 1×10^5 cells/well. The cells were differentiated with phorbol-12-myristate-13-acetate (PMA: 30 nM) for 72 hours. After the plates were rinsed with serum-free RPMI1640 medium to remove unadherent cells, adherent cells were stimulated with lipopolysaccharide (LPS: 25 μg/ml) for 4 hours in the presence of various concentrations of EI-2346. The culture media were harvested, and mature IL-1β was measured by an ELISA method using IL-1β assay kit (Amersham).

Detection of Cell Survival

The cytotoxicity of EI-2346 against THP-1 cells was examined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method[19]. THP-1 cells were suspended in RPMI1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum, and were distributed into 96-well plates as inocula of 4×10^4 cells/well. The cells were differentiated with phorbol-12-myristate-13-acetate (PMA: 30 nM) for 72 hours. After cells were stimulated with LPS (25 μg/ml) for 3 hours in the presence of various concentrations of EI-2346, MTT solution (10 μl/well; final concentration 1 mg/ml) was added to the cells and cells were incubated for 1 hour. The culture medium was removed, and dimethyl sulfoxide (50 μl/well) was added to dissolve formazan. The absorbance of formazan in DMSO solution was measured at 560 nm.

Table 1. Cultural characteristics of strain E-2346.

Medium	Amount of growth	Color of:		Soluble pigment
		Aerial mycelium	Substrate mycelium	
Yeast extract-malt extract agar (ISP No.2)	abundant	Ashes	Covert tan - covert brown	none
Oatmeal agar (ISP No.3)	abundant	Ashes	Light nustard tan - mustard brown	none
Inorganic salt-starch agar (ISP No.4)	abundant	White - silver gray	Silver gray - beige gray	none
Glycerol-asparagine agar (ISP No.5)	poor	Shell pink	Beige - dark brown	none
Tyrosine agar (ISP No.7)	abundant	White - ashes	Beige - dark brown	light brown
Sucrose-nitrate agar	abundant	Silver gray	Oat meal	none
Glucose-asparagine agar	abundant	White - silver gray	Light ivory - light brown	none
Nutrient agar	abundant	Ashes	Light mustard tan - mustard tan	none

Table 2. Physiological properties of strain E-2346.

Characteristics	E-2346
Temperature range for growth	10～45℃
Liquefaction of gelatin	positive
Hydrolysis of starch	positive
Coagulation of milk	negative
Peptonization of milk	negative
Formation melanin	positive
Utilization of	
D-Glucose	positive
L-Arabinose	positive
D-Xylose	positive
D-Fructose	positive
L-Rhamnose	positive
Sucrose	positive
Raffinose	negative
D-mannitol	positive
Inositol	positive

Results

Characterization of the Producing Strain E-2346

Strain E-2346 grew well or moderately on seven agar media, but not on glycerol-asparagine agar as shown in Table 1. The color of the aerial mycelia and substrate mycelia of strain E-2346 are shown in Table 1. Light brown soluble pigment was observed on tyrosine agar media afetr 14 days (Table. 1). The aerial mycelia were moderately short with simple branches and formed spiral spore chains of 10 or more. The spore was rod and 0.6 to 0.8 μm by 1.0 to 1.2 μm. The surface of spore was thorny. No fragmentation of substrate mycelia was observed, and sclerotia, sporangia, or flagellated spores were not formed in cultures. The physiological characteristics of strain E-2346 are shown in Table 2. Analysis of cell hydrolysates of the strain revealed that the cell walls contained LL-diaminopimelic acid and glycine. The predominant menaquinone type was MK-9 (H8) and MK-9 (H6). These taxonomic observation indicated that strain E-2346 belongs to the genus *Streptomyces*.

Production of EI-2346 by Fermentation

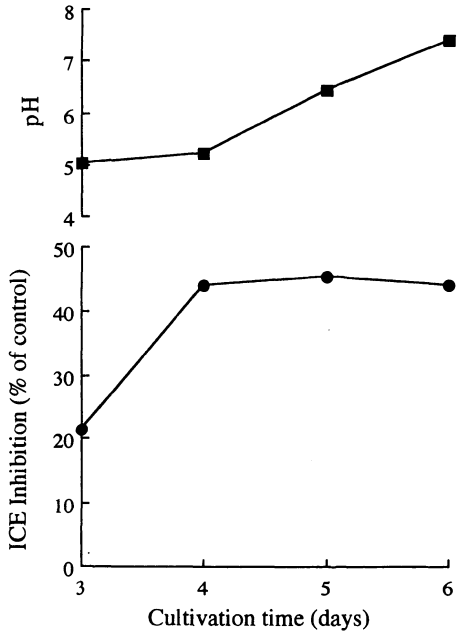
E-2346 was cultured in 300-ml Erlenmeyer flasks containing the fermentation medium described in Material and Methods. The time course of the EI-2346 production and pH of the medium are shown in Fig. 1. The production of active materials in the culture broth initiated on day 4, and the amount of active materials reached maximum on day 5. Active materials were produced in mainly mycelia.

Isolation and Purification

The isolation procedure for the EI-2346 is shown schematically in Fig. 2. The producing strain E-2346 was cultured according to the method described in the Materials and Methods. EI-2346 was purified from mycelia obtained by filtration of fermentation broth. The mycelia cake was extracted with methanol. The extract was diluted with 8 liters of deionized water and applied on a Diaion HP-20 column (400 ml). After washing the column with 50% aqueous methanol (1.6 liters), the active principles were

eluted from the column with methanol (1.6 liters). Active fractions were concentrated *in vacuo* to give a crude material. This material was dissolved with chloroform. 20 g of Radiolite #600 (Showa Kagaku Kogyo Co., Ltd.) was added to this solution, and the solution was concentrated *in vacuo*. This residual material adsorbed on Radiolite was applied onto a silica gel column (200 ml) equilibrated with chloroform, and eluted with chloroform, chloroform-methanol 99.5:0.5, 99:1 and 98:2 (200 ml each), sequentially. EI-2346 was eluted with chloroform and chloroform-methanol 98:2. Active fractions containing EI-2346 were pooled and concentrated *in vacuo* to yield crude EI-2346 (66.4 mg). This crude EI-2346 was dissolved in methanol and purified by preparative HPLC using a column (SH-343 5AQ, 20 i.d.×250 mm) with 70% aqueous methanol as elution solvent. Active fractions containing EI-2346 were evaporated, and EI-2346 (8.0 mg) was obtained as orange oil.

Fig. 1. Time course of E-2346 production in a 2-liter fermenter.



Inhibition % of ICE activity by culture media (●) and pH of the culture broth (■) were indicated.

Physico-chemical Properties

Physico-chemical properties of EI-2346 are summarized in Table 3. EI-2346 was readily soluble in methanol and acetonitrile. The structure of EI-2346 was determined and shown in Fig. 3 on the basis of HRFAB-MS, ^{1}H and ^{13}C NMR spectral data. This compound was found to be a novel compound. Details of structural elucidation studies and chemical properties of EI-2346 will be reported elsewhere.

Inhibition of ICE

EI-2346 inhibited the enzymatic activities of human recombinant ICE in a concentration-dependent manner;

Fig. 2. Purification procedure for EI-2346.

Fermentation broth (2 liters)
↓ filtered
Mycelia
↓ extracted with methanol (2 liters)
↓ filtered
MeOH extract
↓ diluted with water
Diaion HP-20 column (400 ml) chromatography
↓ eluted with methanol
↓ concentrated *in vacuo*
Silica gel column (200 ml) chromatography
↓ eluted with chloroform : methanol = 100:0, 99.5:0.5, 99:1 and 98:2 (stepwise)
↓ concentrated *in vacuo*
Preparative HPLC (YMC SH-343 5AQ)
↓ eluted with 70% methanol
↓ concentrated *in vacuo*
EI-2346 (8.0mg)

Table 3. Physico-chemical properties of EI-2346.

Appearance	Orange oil
Optical rotation	$[\alpha]_D^{27}$ +250° c 0.35, MeOH
Molecular formula	
FAB-MS *m/z*	435 $(M+H)^+$
HR FAB-MS Found.	435.1671 $(M+H)^+$
Calcd.	435.1655
UV λ_{max} (MeOH) (ε)	220 (32,000), 253 (12,000), 431 (3,200)
IR ν_{max} (KBr)	3444, 1647, 1614, 1394 1288, 1255, 1092, 1034
TLC (Rf)	
$CHCl_3$-MeOH (9 :1)[a]	0.49
Color reaction	
Positive	I_2, H_2SO_4
Solubility	
Soluble	MeOH, acetonitrile

[a] Silica gel $60F_{254}$ plate (Merck)

Fig. 3. Structure of EI-2346.

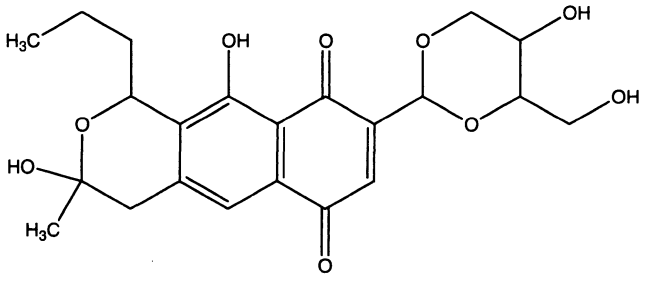
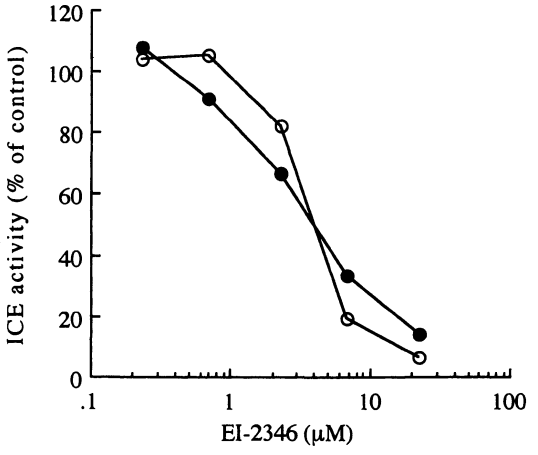


Fig. 4. Inhibition of ICE by the EI-2346.



The assay mixture was incubated for 2 hours at room temperature in the absence or presence of 2 mM DTT. Then the fluorescent intensity (excitation-wave length: 370 nm, emission-wave length: 440 nm) of the assay mixtures was measured. Symbols indicate the ICE inhibition % in the absence of DTT (●) and in the presence of 2 mM DTT (○).

IC_{50} value was calculated to be 3.9 μM. EI-2346 also inhibited ICE activity in the presence of 2 mM DTT (Fig. 4). The enzyme specificity of EI-2346 was examined by testing for inhibition of cathepsin B (another cysteine protease) and elastase. EI-2346 was inactive against these two enzymes at concentrations up to 23 μM. These data showed the selective inhibitory activities of EI-2346 against ICE and are summarized in Table 4.

Biological Properties

In order to determine whether EI-2346 was efficacious in intact cells, we investigated the effects of EI-2346 on the extracellular release of IL-1β from THP-1 cells. EI-2346 inhibited IL-1β secretion in a concentration-dependent manner (Fig. 5); IC_{50} value of EI-2346 was calculated to be 5.2 μM. On the other hand, this inhibitor affect the cell survival with IC_{50} value of 55 μM (as shown in Fig. 5).

However, cell viability at a concentration of 12 μM, at which EI-2346 completely inhibited IL-1β secretion from THP-1 cells were more than 90%, showing that EI-2346 inhibited IL-1β secretion from THP-1 cells induced by LPS in the concentration ranges where cell viability was not affected. These data indicated that EI-2346 inhibited mature IL-1β secretion from THP-1 cells without cell toxicity at concentrations lower than 12 μM.

EI-2346 had weak antimicrobial activities against *Enterococcus hirae*, *Staphylococcus aureus* subsp. *aureus*, and *Bacillus subtilis*. MIC values were 92 μM (Table 5).

Discussion

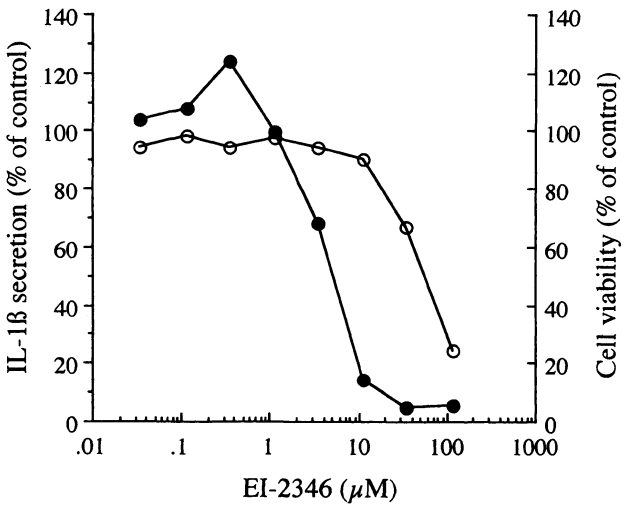
We have isolated a novel compound, EI-2346, from the culture broths of *Streptomyces* sp. E-2346, and demonstrated that this compound is an ICE inhibitor. EI-2346 is a new compound, the structure of which is close to that of naphthopyranomycin[20], exofoliamycin[21,22] and K1115B1[23]. It has been reported that naphthopyranomycin showed cytotoxicity against P388 leukemia cells with IC_{50} value of 0.3 μg/ml[20]. However, it remains unknown whether or not these compounds inhibit ICE activities. Naphthopyranomycin and exofoliamycin showed weak antimicrobial activity against Gram-positive bacteria. EI-2346 had weak antimicrobial activities against Gram-positive bacteria. Among ICE inhibitors from microbial sources, EI-1507-2, EI-1511s and EI-1625-2 and EI-1941-1 also had weak antimicrobial activities against Gram-positive bacteria.

We have demonstrated that EI-2346 inhibited the enzymatic activity of ICE, without inhibiting cathepsine B and elastase, showing ICE selective inhibition. Some

Table 4. Effect of EI-2346 on various enzyme activities.

Enzyme	IC_{50} value (μM)
ICE	3.9
Elastase	>23
Cathepsin B	>23

Fig. 5. Effect of EI-2346 on IL-1β secretion from LPS-stimulated THP-1 cells and on cell viabilities of THP-1 cells.



Symbols indicate the IL-1β secretion (●) and percentage of viable cells (○).

Table 5. The antibiotic activities of EI-2346.

Strains	MIC (μM)
Candida albicans ATCC10231	>184
Enterococcus hirae ATCC10541	92
Pseudomonas aeruginosa BHM No.1	>184
Staphylococcus aureus subsp. *aureus* ATCC6538P	92
Escherichia coli ATCC26	>184
Bacillus subtilis No. 10707	92
Proteus vulgaris ATCC6897	>184
Shigella sonnei ATCC9290	>184
Klebsiella pneumoniae ATCC10031	>184

members of ICE inhibitors which have epoxy structure in their molecule failed to inhibit ICE acitivity in the presence of DTT[11,12]. On the other hand, EI-2346, which has a different chemical skelton from these ICE inhibitors, inhibits ICE activity in the presence of DTT. Therefore, ICE inhibitory activity of EI-2346 could be due to a mechanism other than that of DTT-sensitive ICE inhibitors. We demonstrated also that EI-2346 inhibited IL-1β secretion from LPS-induced THP-1 cells. This inhibition was not due to toxic effects of the EI-2346 as shown in Fig. 5. These results suggest that inhibition of IL-1β secretion from THP-1 cells could be due to ICE inhibition.

In this paper, we described that EI-2346 inhibited human recombinant ICE together with IL-1β secretion from THP-1 cells induced by LPS. EI-2346 would be a useful tool to understand the physiological and pathophysiological roles of ICE.

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