Cutinostatin B as a New Cutinase Inhibitor Produced by Actinomycete

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Cutinostatin B was purified from a culture filtrate of a strain No. 3112-4 of an actinomycete which had been isolated from a soil sample collected in Setagaya-ku, Tokyo, Japan. The structure of this compound was determined mainly by 2D NMR experiments to be N'-(N'-[2,3-dihydroxybenzoyl]ornithyl)-N'-hydroxyornithine-ß-lactam.

Cutinostatin B inhibited the cutinase activity of Cladosporium fulvum when using ß-naphthyl caproate as the substrate with an IC₅₀ value of 28.9 μM (11.0 μg/ml), and inhibited 95% when using crude apple cutin as the substrate with a concentration of 263 μM (100 μg/ml).

Key words: cuticular layer; cutin; cutinase; enzyme inhibitor; phytopathogenic fungus

The plant cuticle plays important roles in maintaining the strength of the plant body, controlling the exchange of water or chemicals on the plant surface, and protecting the plant from infection by phytopathogenic microorganisms.

The structure of cutin, which is known to be the main component of the cuticular layer, has been reported to be a network of hydroxylated fatty acids joined by ester bonds. It is also well known that many phytopathogenic fungi produce cutinase, which is an important factor for the penetration of fungi into a plant body.

We thus considered that inhibitors of cutinase may control one aspect of plant diseases, namely, phytopathogenic infection, if they exhibit highly specific activity.

In the course of screening cutinase inhibitors from microbial metabolites, we isolated a new compound, cutinostatin B, from the culture filtrate of strain No. 3112-4 of an unidentified actinomycete.

This paper reports the isolation, physico-chemical properties, structural elucidation, and some biological activities of cutinostatin B.

Materials and Methods

Materials. Carboxyl esterase (EC 3.1.1.1) from hog liver and triglyceride lipase (EC 3.1.1.4) from Candida cylindracea were purchased from Sigma Chemical Co.

Fermentation. Strain No. 3112-4 was cultured in a 30-liter jar fermentor with 15 liter of a medium containing 1.5% glucose, 1% glycerol, 1% meat extract, 1% Polypepton, and 0.4% CaCO₃ (pH 7) at 27°C for 4 days with agitation at 400 rpm and aeration at 15 liters per min.

Structural studies. UV spectra were recorded by a Shimadzu UV-160A spectrophotometer, and IR spectra were measured with a Shimadzu IR-420 spectrophotometer. Optical rotation was recorded by a JASCO DIP-3600 polarimeter, and FAB-MS spectra were measured with a JEOL JMS-AX505S mass spectrometer. ¹H- and ¹³C-NMR spectra were measured with JEOL JNM-GX270 and JNM-GX400 spectrometers.

Assay methods. Crude cutinase for use in the assay was prepared from Cladosporium fulvum, which had been already examined as a cutinase-producing strain. This strain was cultivated on a solid medium containing 50 g of wheat bran and 30 ml of tap water at 27°C. After 15 to 20 days, the cultured material was extracted with 200 ml of a 50 mm phosphate buffer (pH 6), and the extract was dialyzed against the same buffer for one night. The same volume of glycerol was added to the resulting dialysate to prepare crude cutinase, and this solution was stored at −20°C.

The enzyme assay, using ß-naphthyl caproate as the substrate, was performed with a reaction mixture for the control experiment containing 2 ml of 500 μM ß-naphthyl caproate, 0.5 ml of a 50 mM phosphate buffer (pH 6), and 0.5 ml of the enzyme solution. When the sample for assay (0.1 ml) was added to the reaction mixture, 0.4 ml of the phosphate buffer was used. After the enzyme reaction at 37°C for 30 min, 0.5 ml of a 0.04% fast blue B solution was added to the reaction mixture, 0.5 ml of 40% trichloroacetic acid being added 5 min later to remove the proteinous materials. The resulting red-colored material, which was produced by coupling ß-naphthol with fast blue B, was extracted by 5 ml of methyl acetate, and the absorbance of the extract was measured at 540 nm.

The enzyme assay, using crude cutinase powder as the substrate, was performed with a reaction mixture for the control experiment composed of 10 mg of crude cutin powder, 2 ml of a 50 mM phosphate buffer (pH 6), and 1 ml of the enzyme solution. When the sample for assay (0.2 ml) was added to the reaction mixture, 1.8 ml of phosphate buffer was used. After the enzyme reaction at 37°C for 18h, the liberated fatty acids were extracted by ethyl ether at pH 2. The resulting extract was analyzed as a TMS ester derivative by GLC with 1.5% OV-1 (3mm x 15m) under the following conditions: column temperature, 210°C; carrier gas, N₂ (40 ml/min); and detector, FID.

Results and Discussion

Isolation

As shown in Fig. 1, cutinostatin B was partially purified by column chromatography on Diaion HP 20, extraction with n-butanol, and decolorization with active carbon. The lyophilized crude material was applied to preparative PPC by using Advantech No. 51B filter paper (40 x 40 cm) and the upper layer of n-butanol–acetic acid–water (5: 1: 4) as the solvent system. The resulting active fractions were separated into two fractions A and B by preparative HPLC, using a YMC-packed ODS column (10.7 x 250 mm) and 5 mm trifluoroacetic acid–methanol (3: 1) as the solvent system. As shown in Fig. 2, fraction B exhibited a single peak by analytical HPLC, but fraction A still contained impurities.

Structural determination of cutinostatin B

The physico-chemical properties of cutinostatin B are summarized in Table 1. The trifluoroacetic acid salt of this compound was obtained as a white amorphous material, was very hygroscopic, and the color easily turned orange. FAB-MS data for the salt showed molecular ion clusters at m/z 381 [M + H]+, m/z 403 [M + Na]+, and m/z 419 [M + K]+, while an HR-FAB-MS analysis of [M + H]+ (m/z 381.1768) indicated that the molecular formula of the
The salt-free compound was C$_{13}$H$_{10}$N$_2$O$_6$. The IR spectrum of the salt indicated that this compound contained amide groups.

The $^{13}$C-NMR spectrum of the cutinostatin B trifluoroacetic acid salt showed nineteen signals consisting of three carboxyl groups, three aromatic quaternary carbons, three aromatic methines, two $sp^3$ methines, six $sp^3$ methylenes, and an additional two signals derived from the trifluoroacetic acid ion (Table II). The $^1$H-NMR spectrum of the salt in D$_2$O showed seventeen protons, and that in DMSO-$d_6$ showed twenty-five protons (Table II), indicating that eight protons were attached to oxygen and/or nitrogen in the protonated form.

The structure of cutinostatin B could be divided into three units (Fig. 3). We determined the first unit of this compound to be a 2,3-dihydroxybenzoyl residue (1) based on a coupling pattern of three aromatic protons (7.40 ppm (6-H), J = 8.0, 1.5 Hz, 6.70 ppm (5-H), J = 8.0, 8.0 Hz, 6.93 ppm (4-H), J = 8.0, 1.5 Hz) in the $^1$H-NMR spectrum that is typical of a 1,2,3-tri-substituted benzene. This was confirmed by CH-COSY and HMBC experiments. The HMBC experiment also revealed that one of the substituents of the aromatic ring was a carbonyl group (C7) attached to C1.
(116.0 ppm). The two residual substituents were estimated to be hydroxyl groups based on the chemical shift values of C2 and C3. Protons resonating at 11.83 (2-H) and 9.62 or 9.40 (3-H) ppm also suggested the presence of two or three phenolic hydroxyl groups, although coupling between these phenolic protons and aromatic carbons was not demonstrated by an HMBC experiment because of chemical exchange of these protons. The UV spectrum of the salt of cutinostatin B was identical with that of 2,3-dihydroxybenzoic acid, the presence of an o-catechol structure being supported by the coloring reaction of this compound with FeCl3 by silica gel TLC.

The second unit, an ornithine residue (2), was then determined. COSY and TOCSY experiments indicated the presence of two sets of a -(CH2)3-CH-NH (amide) sequence, one of these sequences being part of the second unit, and the other one belonging to the third unit. The COSY results indicated that 5'-H of 2 was connected with a 6'-protonated primary amine (7.72 ppm), while the results of an HMBC experiment demonstrated that 2'-H of 2 was coupled with a carbonyl carbon (C1, 170.7 ppm). These results indicate that cutinostatin B contained an ornithine residue. The existence of ornithine was also confirmed by a silica gel TLC analysis (Rf = 0.12, n-propanol-acetic acid-water = 4:1:1) on the acid hydrolysates of this compound.

A correlation peak between 7'-H (8.81 ppm) of 2 and a carbonyl carbon (C7, 168.4 ppm) of 1 in the HMBC spectrum indicate that 1 and 2 were joined together by an amide bond to give the partial structure N4-(2,3-dihydroxybenzoyl)ornithine. This structure was also supported by a NOE experiment, irradiation of 7'-H (8.81 ppm) of 2 giving a 5% NOE on 6-H (7.40 ppm) of 1.

The third unit was N4'-hydroxyornithine-δ-lactam (3). The sequence N-(CH2)3-CH-NH (amide) in the lactam was demonstrated by the results of COSY and TOCSY experiments and by chemical shift values. A correlation peak between 7'-H (8.35 ppm) and a carbonyl carbon (C1', 170.7 ppm) in the HMBC spectrum indicate that 3 was joined to 2 by an amide bond, this also being supported by NOE (2%) on 2'-H when 7'-H was irradiated. An HMBC experiment also indicated that a carbonyl carbon (C1', 164.7 ppm) was joined to the sequence to give a δ-lactam. Although no correlation between carbonyl carbon C1' and 3'-H (1.75 and 1.85 ppm), 4'-H (1.90 ppm) or 5'-H (3.46 ppm) was apparent, no other structure than a δ-lactam was possible. A residual exchangeable proton at 9.62 or 9.40 ppm and an abnormal chemical shift value for C5' (51.3 ppm) in lactam 3 are explained by the presence of a hydroxyl group on the ring nitrogen. This N-hydroxyl group was also suggested by the coloring reaction, one of the hydrolysst products of cutinostatin B being detected as a blue spot (Rf = 0.28) with K3[Fe(CN)6]-FeCl3 reagent by silica gel TLC (n-propanol-acetic acid-water = 4:1:1).

The structure of cutinostatin B was, hence, determined to be N4'-[N4-(2,3-dihydroxybenzoyl)ornithyl-N4'-hydroxyornithine-δ-lactam] (Fig. 4).

**Enzyme inhibition**

Carboxyl esterase and triglyceride lipase were also used in addition to cutinase for the assay.

When β-naphthyl caproate was used as the substrate, the specific activities of cutinase, carboxyl esterase, and triglyceride lipase were 2.4 × 10^4 u, 2.3 × 10^5 u, and 5.6 × 10^2 u, respectively. As shown in Table III, cutinostatin B inhibited cutinase and carboxyl esterase, the IC50 values against both enzymes being 28.9 μM and 21.0 μM, respectively. Furthermore, only triglyceride lipase hydrolyzed triolein, but this enzyme could not be inhibited by the compound (Table III). The Lineweaver–Burk plot shown in Fig. 5 indicates that the apparent mode of inhibition of this compound against cutinase was competitive.

When crude cutin power was used as the substrate, the hydrolyzate from cutinase (Fig. 6(2)) exhibited a peak at tR 15.1 min, similar to the case of the alkaline hydrolysate (Fig. 6(1)). Analytical data by 1H-NMR and GC-MS of this peak component indicated it to be a mixture of at least three to five substances, which may have been mono, di-, and or tri-hydroxyl fatty acids of C14 to C32. On the other hand, no peak was found in the hydrolysates by carboxyl esterase (Fig. 6(3)) and triglyceride lipase (Fig. 6(4)). This means that cutin could not be a substrate for carboxyl esterase and triglyceride lipase.
Fig. 6. GLC Analyses of the Cutinase Hydrolysis Products from Crude Cutin.

(1) Alkali hydrolysate, (2) cutinase hydrolysate, (3) carboxyl esterase hydrolysate, (4) triglyceride lipase hydrolysate, and (5) cutinase hydrolysate in the presence of cutinostatin B.

Table IV. Antimicrobial Activity of Cutinostatin B

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td>Bacillus cereus</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Cochliobolus miyabeanus</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Pyricularia oryzae</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

The growth inhibition zone was determined by the paper disc method.

As shown in Fig. 6(5), the addition of cutinostatin B to the reaction mixture decreased the peak area of crude cutin hydrolysate by cutinase, 263 µM of this compound inhibiting 95% of cutinase. These results indicate that cutinostatin B actually inhibited the action of cutinase against crude cutin. The result that cutinostatin B also inhibited carboxyl esterase when using β-naphthyl caproate as a substrate lead us to considered that the specificity of this compound as a cutinase inhibitor still needs further study.

As shown in Table IV, cutinostatin B did not exhibit any inhibitory activity against any of tested microorganisms up to a concentration of 100 µg/ml by the paper disc-agar method.

Köller et al. have reported that the IC₅₀ values for ebelacontone A were 0.08 and 4.0 µg/ml, and those for ebelacontone B were 0.67 and 0.013 µg/ml against cutinase from Venturia inequalis and Rhizoctonia solani, respectively. In comparison with these data, the inhibitory activity of cutinostatin B seems to have been lower than that of ebelacontones A and B. However, it seems that direct comparison of these data is difficult, because the origins of the enzymes are different, and the inhibition of ebelacontones against cutinase when using cutin as a substrate were not examined. As already mentioned, the mode of inhibition of cutinase by cutinostatin B was competitive. In this respect, cutinostatin B differs from ebelacontones A and B, which seems to irreversibly bind with a hydroxyl group at the active center of the enzymes.

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References