A Deacylation Enzyme for Aculeacin A, a Neutral Lipopeptide Antibiotic, from *Actinoplanes utahensis*: Purification and Characterization

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An enzyme, tentatively termed aculeacin A acylase, useful in preparing deacylated peptides which are used as starting material for semisynthetic antifungal antibiotics, was purified from the culture filtrate of *Actinoplanes utahensis* NRRL12052. The purification involved ultrafiltration and column chromatographies on DEAE-cellulose, hydroxyapatite, and Butyl-Toyopearl 650M. The purified enzyme was composed of two dissimilar subunits with molecular weights of 55,000 and 19,000. The subunits were dissociated in the presence of 0.1% SDS or 6 M guanidine hydrochloride; the dissociation accompanied loss of acylase activity. The enzyme was fully active at pH 7.0 and at 60°C. Its pI was estimated to be above 10.25. The *Km* and *Vmax* for aculeacin A were 1.53 mM and 39.7 μmol/min/mg-protein, respectively.

Aculeacin A has been characterized by Mizuno et al. (1) as an antifungal antibiotic and belongs to the echinocandin type antibiotics, which are fungal metabolites produced by various *Aspergillus* species. They are presumably cyclohexapeptides with a long fatty acid side chain, consisting of threonine, hydroxyproline, and several unusual amino acids. The fatty acid constituents may be linoleic, myristic, or palmitic acid, as illustrated in Fig. 1 (2).

These antibiotics exhibit high antiyeast and antifungal activity, but easily lose their activity upon deacylation. The deacylated peptide (peptide nucleus) has been used as a starting compound for creating new and more useful antifungal agents (3). An enzyme which removes the acyl moieties of those antibiotics without affecting the peptide moieties was found in the culture medium of *Actinoplanes utahensis* NRRL12052, and its whole culture broth has been used industrially on a large scale to produce the peptide nuclei (4).

In this report we describe the purification of this acylase from the culture filtrate of the organism and some properties of the purified enzyme.

MATERIALS AND METHODS

**Chemicals**—Aculeacin A and its peptide nucleus were kindly supplied by Toyo Jozo Co. (Ohito, Shizuoka), and other antibiotics were from our laboratory stock.

**Organism and Growth Conditions**—*A. utahensis* NRRL12052 was used as the enzyme-producing organism. A loopful culture of the organism was inoculated into 10 ml of a seed medium consisting of 1.0% glucose, 1.0% dextrin, 0.5% type A hydrolysate of casein (NZ amine), 0.5% yeast extract, and 0.1% CaCO₃ (pH 6.5) in a 60-ml test tube with a spring coil and incubated for 48 h at 30°C on a reciprocal shaker operating at 250 rpm. The resulting culture was inoculated into 400 ml of the same medium in a 2-liter Erlenmeyer flask and cultivated in the same manner as above. After 48 h, 400 ml of the seed culture was transferred to a 25-liter jar-fermenter containing 20 liters of the production medium, which consisted of 3.0% sucrose, 0.5% peptone, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄·7H₂O, and 0.0002% FeSO₄·7H₂O (pH 6.5). Cultivation was carried out at 30°C for 64 h at an agitation rate of 250 rpm.

**Enzyme Assay**—The acylase activity was routinely assayed by estimating the amount of aculeacin A remaining in the reaction mixture (1 ml), which contained 0.1 M phosphate buffer (pH 7.0), 1 M NaCl, 800 nmol aculeacin A, and less than 10 mU of the enzyme. The enzyme reaction was carried out at 60°C for 30 min on a reciprocal shaker operating at 100 rpm and terminated by extraction of the remaining aculeacin A with 2 ml of *n*-butanol saturated with distilled water. The butanol layer was transferred to a new tube and dehydrated by adding anhydrous sodium sulfate. Aculeacin A in the butanol was estimated by measuring the absorbance at 278 nm. One unit of the acylase activity was defined as that required to decompose 1 μmol of aculeacin A per min at 60°C.

**Protein Determination**—Protein concentration was determined by the method of Lowry et al. (5) or of Sargent (6). The latter was mainly used for the purified enzyme. A standard curve was drawn using bovine serum albumin.

**SDS-Polyacrylamide Gel Electrophoresis**—The SDS-PAGE was carried out according to the method of Laemmli (7), and the protein in the gel was stained with Silver Stain Kanto (Kanto Chemical).

**High Performance Liquid Chromatography**—HPLC was on a JASCO Model VL-614 chromatograph; the eluates were monitored with a UVIDEC-100-V detector at 280 nm. A YMC-Pack diol 120 (8 φ × 500 mm) column for gel filtration and a YMC-Pack A-302 (4.6 φ × 150 mm) column
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Fig. 1. Structures of echinocandin-type antibiotic. The arrow indicates the amide bond which is hydrolysed by aculeacin A acylase.

for the reverse-phase system were used.

Determination of Molecular Weight—The molecular weight of the enzyme was estimated by SDS-PAGE and HPLC as described above.

Determination of pI by Isoelectric Focusing—Flat-bed isoelectric focusing was performed on 5.0% polyacrylamide gel containing 5% Pharmalyte (Pharmacia) pH 8-10.5 (8). Electrophoresis was carried out at 200 V for 5 h at 10°C using buffers of 0.25M HEPES (anolyte) and 1M NaOH (catholyte). After electrofocusing, the gel was sliced into 2.5-mm sections and a half of each piece was transferred to a test tube containing 0.5 ml of 0.1M phosphate buffer (pH 7.0) and then shaken for 2 h at 4°C. The acylase activity of the extract from each piece was measured as described above. The other piece showing acylase activity was extracted with distilled water and its pH was estimated using a pH meter. A parallel gel was stained with Coomasie Brilliant Blue G-250 (9). A high pI calibration kit (pI 5.0-10.5) was used as pI marker proteins (Pharmacia).

RESULTS

The enzyme production and the growth rate of the organism were tested in a temperature range of 25-55°C. As shown in Fig. 2, cultivation at 45°C was the most favorable for mycelial growth, while the production of acylase in the culture medium was maximal at 35 to 40°C. At all the temperatures tested, the acylase activities accumulated in the cells were less than half of those secreted in the culture medium (data not shown). Therefore, we used the culture medium as the enzyme source in the subsequent work. In our preliminary experiment we found that some proteins which were difficult to remove in the purification steps described below were produced at cultivation temperatures higher than 30°C (data not shown). For this reason, cultivation for enzyme production was set at 30°C. The enzyme activity, under these conditions, reached a plateau after 60 h of cultivation and maintained this level for at least 12 h. The cultivation was terminated after 64 h of cultivation and the filtrate was used for the following purification of acylase. Unless otherwise stated, all purification steps were carried out at 4°C.

The culture filtrate (18 liters) was concentrated to 450 ml with a hollow-fiber ultrafiltration apparatus (model AIL-2011, Asahi Chemical Industry). The concentrate was applied to a DE52 column (76 x 130 mm) equilibrated with 10 mM phosphate buffer (pH 6.5) and developed with the same buffer. The effluents fractions with activity were pooled and dialysed against 5 mM phosphate buffer (pH 7.0).

The dialysed enzyme solution was loaded on a hydroxyapatite column (32 x 110 mm) previously equilibrated with 5 mM phosphate buffer (pH 7.0). The fractions containing the activity were eluted with 5 mM phosphate buffer (pH 7.0) containing 2.5M NaCl and were pooled and dialysed against 0.1 M phosphate buffer (pH 7.0). To the dialysed enzyme solution, ammonium sulfate was added to 15% saturation; it was then loaded on a Butyl-Toyopearl 650M column (15 x 26 mm) previously equilibrated with 0.1 M phosphate buffer (pH 7.0) 15% saturated with ammonium sulfate. After washing the column with the same

TABLE I. Purification of aculeacin A acylase from Actinoplanes utahensis NRRL 12052.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total volume (ml)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth filtrate</td>
<td>37,600</td>
<td>18,000</td>
<td>490.6</td>
<td>0.0130</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>8,610</td>
<td>450</td>
<td>445.5</td>
<td>0.0517</td>
<td>90.8</td>
<td>3.98</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>743</td>
<td>2,100</td>
<td>107.8</td>
<td>0.145</td>
<td>22.0</td>
<td>11.2</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>69.4</td>
<td>430</td>
<td>107.8</td>
<td>1.55</td>
<td>22.0</td>
<td>119.2</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650M</td>
<td>9.35</td>
<td>32</td>
<td>88.9</td>
<td>9.51</td>
<td>18.1</td>
<td>731.5</td>
</tr>
</tbody>
</table>

Fig. 2. Comparison of aculeacin A acylase level with mycelial growth at the various cultivation temperatures. A. utahensis was cultured in 5 ml of the production medium described in the text in a Monod tube for 60 h at the indicated temperature on a Monod shaker operating at 60 rpm. Then the culture was centrifuged at 3,000 rpm for 10 min at 4°C. The enzyme activity of the supernatant (open bar) was measured as described in "MATERIALS AND METHODS." The mycelial growth (solid bar) was measured gravimetrically by washing the broth solids twice with deionized water and then drying to constant weight.
Fig. 3. Reverse phase HPLC profiles of the reaction product from aculeacin A and the authentic peptide nucleus. Under the standard conditions described in the text, 800 μg of aculeacin A was hydrolysed in 1 ml of the reaction mixture containing 20 milliunits of the purified aculeacin A acylase for 1 h at 60°C. A part (80 μl) of the mixture was applied to a YMC-Pack A-302 column pre-equilibrated with distilled water. Then the column was developed at a flow rate of 1 ml/min with a mixture of water, acetonitrile, acetic acid, and pyridine (96:2:1:1). The authentic sample (ca. 60 μg) was chromatographed as described above. The eluents were monitored at 280 nm. I, authentic nucleus; II, reaction product from aculeacin A.

Fig. 4. Gel filtration by HPLC and SDS-PAGE of the purified aculeacin A acylase. A: HPLC profile on a YMC-Pack diol 120 column with 0.1 M phosphate buffer (pH 7.0) as the mobile phase. B: SDS-PAGE on 12.5% gel containing 0.1% SDS. The standard proteins and their molecular weights were as follows (from the top): bovine serum albumin (68,000); ovalbumin (43,000); carbonic anhydrase (30,000); trypsin inhibitor (20,100); and lysozyme (14,000). The gel was stained for protein with Silver Stain Kanto.

Table II. Aculeacin A acylase activity of subunits separated in the presence of guanidine hydrochloride. The purified aculeacin A acylase (9.51 units/mg of protein) was gel-filtrated as described in Fig. 5. Portions of each peptide were separately dialysed against 100 volumes of 0.1 M phosphate buffer (pH 7.0) with changing three times to new buffer for every 2 h at 4°C; these were used in Experiments 1-3. In Experiment 3, both peptides were mixed after dialysis and incubated for 3 d at 4°C. Other portions of each peptide were mixed immediately after separation at the indicated concentrations in this table (Experiments 4-6), and then dialysed as described above. After dialysis, these mixtures were incubated for an additional 3 d at 4°C. Aculeacin A acylase activities of these samples were estimated as described in "MATERIALS AND METHODS."

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Subunit (μg/ml)</th>
<th>Aculeacin A acylase activity (units/ml)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>11.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>11.5</td>
<td>4.0</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>23.0</td>
<td>4.0</td>
<td>5.4</td>
</tr>
<tr>
<td>6</td>
<td>46.0</td>
<td>4.0</td>
<td>9.8</td>
</tr>
</tbody>
</table>

To clarify the role of these peptides on the acylase activity, they were separated by HPLC in the presence of 6 M guanidine hydrochloride. The separation profile of the subunits on HPLC and their SDS-PAGE are shown in Fig. 5, A and B. The migration distances of peaks I and II on SDS-PAGE were identical with those of peptides A and B, respectively, as shown in Fig. 4B. During dialysis of the solution containing peptide A alone, a turbid precipitate was formed, suggesting that the solubility of peptide A of 55,000 and 19,000 on SDS-PAGE (Fig. 4, A and B). These species were termed peptides A and B, respectively. Similar results were obtained on SDS-PAGE under non-reducing conditions (data not shown). Several denaturants and detergents were tested for separation of these peptides by HPLC: 0.1% SDS and 6 M guanidine hydrochloride were effective, but 6 M urea and 1% sodium cholate were not. These results indicate that the acylase consists of two subunits which are associated by a bond other than disulfide bond.

To check the purity of the enzyme, the final enzyme preparation was submitted to HPLC and SDS-PAGE. A single protein peak was found on HPLC, while there were two bands which were estimated to have molecular weights of 55,000 and 19,000 on SDS-PAGE (Fig. 4, A and B).
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The acylase from A. utahensis NRRL12052 described here has two peculiar properties. One is that the optimum temperature is rather high in comparison with the suitable growth temperature of the producing-organism. The role of this acylase in the organism is thus a point of interest. The growth temperature of the producing-organism. The role of this acylase in the organism is thus a point of interest. The temperature is rather high in comparison with the suitable growth temperature of the producing-organism.

The acylase was determined by incubation at various temperatures in 0.1 M phosphate buffer (pH 7.0). No loss of activity could be observed at 60°C for 24 h, but rapid loss was found at temperatures higher than 70°C. The effect of pH on the stability of the enzyme was examined under standard assay conditions after incubation for 4 h at 30°C in properly buffered solutions in the pH range of 2–9. The acylase retained its initial activity at the pH range of 4–8, but lost it completely at pH 2. The optimal pH and temperature for the acylase activity were 7.0 and 60°C, respectively.

The effect of substrate concentration on the activity of the purified acylase was examined in 0.1 M phosphate buffer (pH 7.0) at 60°C. The Km and Vmax for aculeacin A were calculated to be 1.53 mM and 39.7 μmol/min/mg protein, respectively.

No significant effect on the enzyme activity was observed by addition of Mg2+, Mn2+, Cu2+, Co2+, Ca2+, or Zn2+ to the reaction mixture. EDTA did not affect the activity at concentrations up to 10 mM. However, NaCl and KCl increased the enzyme activity 1.69- and 1.47-fold at 1 M, respectively. Based on this result, 1 M NaCl was added to the standard assay solution.

The amidohydrolytic activity of the purified enzyme on compounds with an amide bond besides those illustrated in Fig. 1 was examined. No significant activity was found with ampicillin, tunicamycin, colistin, lankacidin C or blasticidin S.

DISCUSSION

The acylase from A. utahensis NRRL12052 described here has two peculiar properties. One is that the optimum temperature is rather high in comparison with the suitable growth temperature of the producing-organism. The role of this acylase in the organism is thus a point of interest. The other is the high pl value. It is assumed that the large discrepancy between the molecular weight estimated by HPLC and that estimated by SDS-PAGE is due to the particularly high pl of this enzyme. The molecular weight of lysozyme, with a pl of 10.5, was calculated as 6,000 from the calibration curve (Fig. 6), although the actual value is 14,300. Based on this result, it was concluded that the molecular weight of acylase should be 74,000, the sum of the values of the two subunits obtained by SDS-PAGE (Fig. 4B).

SDS-PAGE of the purified enzyme revealed two subunits focusing to be more than 10.25 as shown in Fig. 7.

The heat stability of the acylase was determined by incubation at various temperatures in 0.1 M phosphate buffer (pH 7.0). No loss of activity could be observed at 60°C for 24 h, but rapid loss was found at temperatures higher than 70°C. The effect of pH on the stability of the enzyme was examined under standard assay conditions after incubation for 4 h at 30°C in properly buffered solutions in the pH range of 2–9. The acylase retained its initial activity at the pH range of 4–8, but lost it completely at pH 2. The optimal pH and temperature for the acylase activity were 7.0 and 60°C, respectively.

The effect of substrate concentration on the activity of the purified acylase was examined in 0.1 M phosphate buffer (pH 7.0) at 60°C. The Km and Vmax for aculeacin A were calculated to be 1.53 mM and 39.7 μmol/min/mg protein, respectively.
with molecular weights of 55,000 and 19,000; these subunits could be separated by gel filtration in the presence of guanidine hydrochloride. It was assumed that both peptides are required for acylase activity (Table II). We speculate that the reason why activity was not observed when both species were mixed after separate dialysis was that the conformation of peptide A was changed during dialysis and the two subunits could not reform the originally active form. The occurrence of some conformational changes in peptide A was naturally inferred from the observation that a turbid precipitate was formed during dialysis of the solution containing peptide A alone.

It has been reported that a penicillin acylase from *Escherichia coli* ATCC 11105 consists of two dissimilar subunits with molecular weights of 69,000 and 20,500 (11). It is quite different from our acylase in biochemical, kinetic, and other properties, as well as substrate specificity.

Boeck et al. have recently reported that the same strain used in this work produces another deacylating enzyme, most of whose activity (94%) is present in the mycelia (12). This enzyme possesses deacylating activity against A21978C, an acidic lipopeptide antibiotic complex. Each member of this family has the same thirteen amino acid peptide nucleus, with the fatty acid acyl group attached at the N-terminus of the nucleus (13). Although detailed characteristics of this enzyme have not been reported, it is probably different from our enzyme for the following reasons. The first is the location of the enzyme; our enzyme is an exoenzyme and theirs an intracellular enzyme. The second is the different substrate specificity. For identification of both enzymes, we must wait until their enzyme is purified or more detailed information is published.

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REFERENCES