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Membrane-associated echinocandin B deacylase of *Actinoplanes utahensis*: purification, characterization, heterologous cloning and enzymatic deacylation reaction

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Aspergillus nidulans **produces echinocandin B, a neutral lipopeptide. A deacylase from** ***Actinoplanes utahensis*** **catalyzes cleavage of the linoleoyl group from echinocandin B, a key step in generating a potential antifungal agent. Virtually all (99.8%) deacylase activity was cell-associated. The deacylase was salt-solubilized, heat-treated and purified to apparent homogeneity by a 3-step chromatographic procedure. The enzyme was a heterodimer consisting of 63- and 18-to-20-kDa subunit, optimally active at pH 6.0, and at 60°C with salt. The K_m of the deacylase for echinocandin B was 50 μM and its V_{max} was 14.6 μmol cyclic hexapeptide min^{-1} mg^{-1} protein. The substrate specificity of the enzyme was broad with respect to both acyl and cyclic peptide analogues of echinocandin B. The two deacylase subunit genes were cloned and over-expressed in** ***Streptomyces lividans*****. The recombinant deacylase was purified from the culture filtrate to apparent homogeneity by a 1-step chromatographic procedure. Using the recombinant deacylase, an enzymatic deacylation of immobilized echinocandin B resulted in the generation of cyclic hexapeptide at gram-level.** *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 173–180.

Keywords: echinocandin B deacylase; substrate specificity; evolution/technology; antifungal agent

Introduction

Chemical modification of natural antifungal compounds has been used to produce a few potential therapeutic agents such as cilofungin [1,5–7]. The natural product echinocandin B (ECB) can be modified to make cilofungin by enzymatic deacylation to a cyclic hexapeptide and subsequent chemical reacylation. The enzymatic deacylation was rate-limiting for the 2-step procedure.

We were interested in development of a novel and effective enzymatic deacylation for an echinocandin or its analogue. Since penicillin G acylase catalyzes enzymatic deacylation as well as acylation under different reaction conditions [14], an echinocandin deacylase that is also capable of reversible acylation may turn out to be scientifically interesting and industrially useful. *Aspergillus nidulans* produces ECB, a neutral linoleoyl cyclic hexapeptide, which can be cleaved at an amide bond by a deacylase to generate the cyclic hexapeptide (ECB nucleus; Figure 1). Initial enzymatic deacylation was conducted with whole cells of *Actinoplanes utahensis* and *Pseudomonas* species [1,2,12]. The low conversion yield was presumably related to poor aqueous ECB solubility that limits substrate accessibility to cell-associated ECB deacylase [1]. A soluble deacylase for aculeacin A, a side-chain analogue of ECB, was purified and partially characterized previously from *A. utahensis* [20]. Also, a different membrane-associated deacylase for ECB was reported recently from *A. utahensis* [22]. For molecular and biochemical understanding

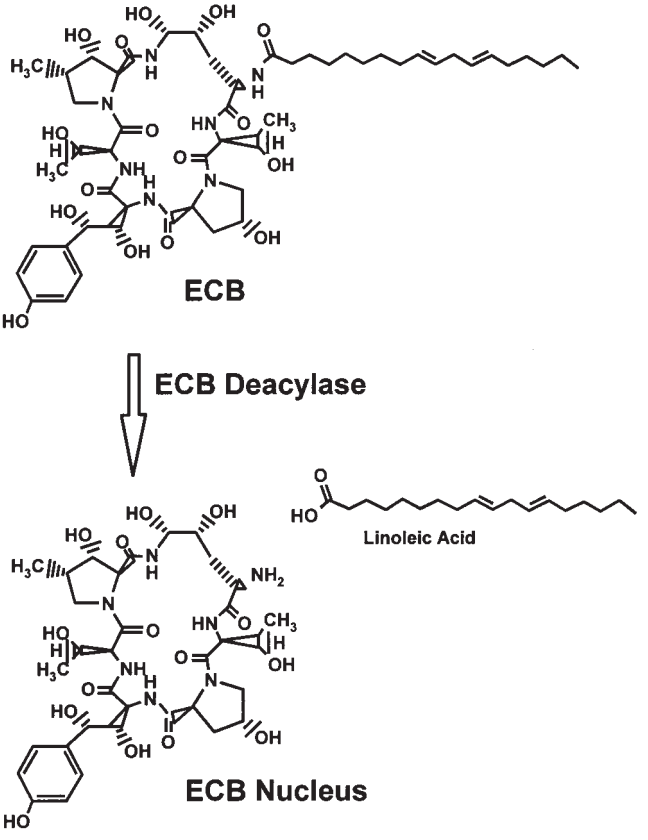

Figure 1 ECB deacylase-catalyzed reaction.

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of the latter deacylase and particularly for its potential biotechnological application, we describe here solubilization, purification and properties of membrane-associated ECB deacylase from *A. utahensis*, its heterologous gene cloning to *Streptomyces lividans*, and an effective enzymatic deacylation at gram-scale.

Materials and methods

Chemicals

ECB is a six-membered cyclic lipopeptide (Figure 1) and daptomycin is a ten-membered cyclic peptide with three amino acids in the side-chain linked to an aliphatic moiety [22]. Highly purified (>95% by HPLC) ECB, its deacylated cyclic hexapeptide (ECB nucleus), daptomycin and its deacylated product were from Eli Lilly (Indianapolis, IN, USA). S-Sepharose, Octyl-Sepharose and Red-Sepharose, were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Enzyme source

A. utahensis NRRL 12052 was grown in a 150-L fermenter under optimized conditions described previously [2]. Cells containing a high activity of ECB deacylase (90 h after inoculation) were harvested by centrifugation, washed with 0.05 M KH_2PO_4, pH 6, and used in enzyme solubilization as described below.

Deacylase assay

A typical reaction mixture of 1 ml for the deacylase assay contained 425 μmol of ECB and 3–3000 μunits of the enzyme in 0.05 M KH_2PO_4, pH 6, in the presence of 0.68 M KCl and 15% dimethyl sulfoxide (DMSO). The enzymatic reaction was initiated by addition of the enzyme, continued for 20 min at 60°C, and interrupted by addition of H_3PO_4. After a low-speed centrifugation to remove precipitated proteins, the deacylase activity was determined by monitoring formation of the cyclic hexapeptide (ECB nucleus) at 225 nm using HPLC (Figure 2a). Alternatively, the activity was determined by using daptomycin (617 μmol) as the substrate in the absence of DMSO (Figure 2b).

Enzyme solubilization

Very little (~0.2%) deacylase activity was detected in the culture filtrate of *A. utahensis*. Less than 5% of the cell-associated deacylase activity was released by incubation of the cells at 0.01 M KH_2PO_4, pH 6, for one day. Increase of the ionic strength of this and a Tris buffer, pH 7 had a slight effect on recovery of soluble deacylase. A simple salt-treatment of the cells (~0.01 M KH_2PO_4, pH 6, in the presence of 0.8 M KCl at 4°C for one day) resulted in 60–80% recovery of soluble deacylase at 2–4-fold higher specific activity (Table 1). The salt-induced solubilization suggests that the deacylase is loosely bound to the membrane of *A. utahensis* and can be released from it by disruption of ionic interaction [4]. The solubilized enzyme was stable for its activity, however, some precipitation of the deacylase appears to occur during each step of the purification. In spite of the enzyme precipitation, which is common for membrane-associated protein [21,23], active ECB deacylase was differentially solubilized from *A. utahensis* (Table 1) and purified (Table 2).

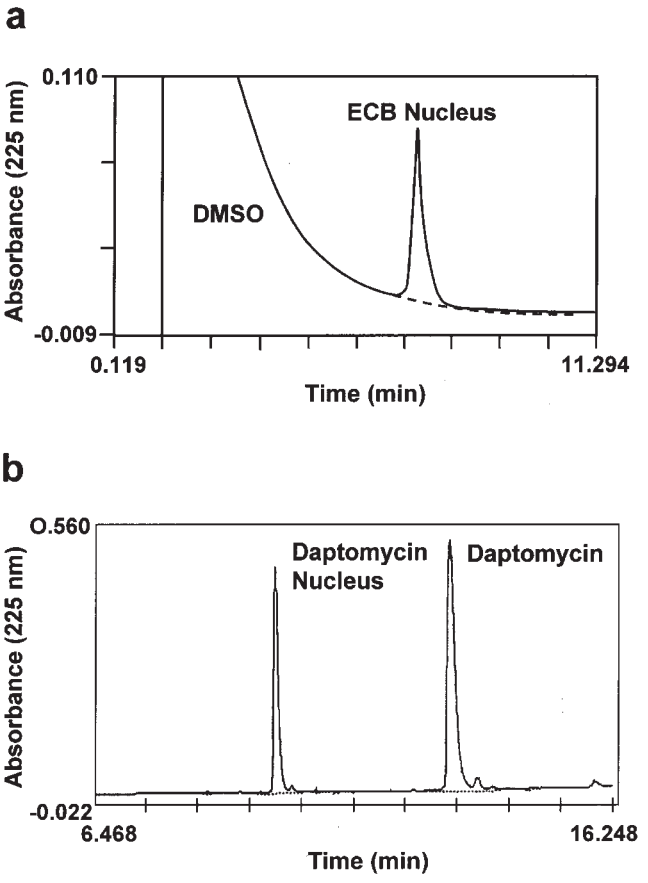

Figure 2 Activity analysis with (a) ECB and (b) daptomycin as substrate and by monitoring product formation with HPLC. Either peptide nucleus was separated by an Apex C18 column (3 μ; 4.6 × 150 mm), mobile phase of 2–90% acetonitrile in 0.1% trifluoroacetic acid, and flow rate of 1 ml min^{-1} for 20 min.

Table 1 Salt-solubilization of ECB deacylase from *A. utahensis*

Conditions	Activity distribution (%)	Sp. act. (U mg^{-1})
KCl/KH_2PO_4 with sonication		
Supernatant fraction	80	0.1
Pellet fraction	20	–[a]
KCl/KH_2PO_4		
Supernatant fraction	60–80	0.2–0.4
Pellet fraction	20–40	–
KH_2PO_4 with sonication		
Supernatant fraction	80	0.1
Pellet fraction	20	–
KH_2PO_4		
Supernatant fraction	<5	–
Pellet fraction	>95	–

[a]Not determined.

Purification of native deacylase

After the salt-treatment a soluble extract was obtained by filtration through Whatman No. 1 paper and by concen-

Table 2 Purification of ECB deacylase from *A. utahensis*

Step	Protein (mg)	Activity[a] (U)	Sp. act. (U mg^{-1})	Recovery (%)
Soluble extract	6897	2185	0.317	100
Heat-treated extract	2120	1343	0.633	61
S-Sepharose eluate	150	418	2.79	19
Octyl-Sepharose eluate	35	406	11.6	19
Red-Sepharose eluate	15	219	14.6	10

[a]ECB was used as the substrate.

tration to 2.6 L using an Amicon DC10 spiral cartridge concentrator with a YM 10 000 MW cut-off cartridge. The material was then heated to 60°C for 1 h, precipitated proteins were removed by filtering the suspension through Whatman paper and then a 0.45-μM pore size filter. The salt-extracted heat-treated enzyme preparation was then diluted with buffer A (pH 7), concentrated and diluted repeatedly using an Amicon spiral cartridge concentrator. The dia-filtered enzyme preparation was concentrated to a final volume of 2.4 L in 0.05 M KH_2PO_4, pH 7, 0.05 M KCl. The filtrate was loaded onto an S-Sepharose column (500 ml), previously equilibrated with 0.05 M KH_2PO_4, pH 7 (buffer A). The column was washed with buffer A and bound proteins were eluted with a linear gradient of 0–2 M KCl in buffer A. The fractions having a higher specific activity than that of the heat-treated extract were pooled as S-Sepharose eluate. The eluate was adjusted to 1.0 M KCl, 14% $(NH_4)_2SO_4$, pH 7. The material was centrifuged at 38 000 × *g* for 1 h, the supernatant fraction was loaded onto an Octyl-Sepharose column (200 ml), previously equilibrated with 0.05 M KH_2PO_4, pH 6.5, 1 M KCl and 14% $(NH_4)_2SO_4$ (buffer B). The column was washed with buffer B and bound proteins were eluted with a linear gradient of 14% $(NH_4)_2SO_4$/1 M KCl–0 M KCl/0% $(NH_4)_2SO_4$ in 0.05 M KH_2PO_4, pH 6.5. The fractions containing higher specific activity than that of S-Sepharose eluate were pooled as Octyl-Sepharose eluate. The latter eluate was loaded onto a Red-Sepharose column (100 ml), previously equilibrated with buffer A. The column was washed with buffer A and bound proteins were eluted with a linear gradient of 0–3.0 M KCl in buffer A. The fractions having a higher specific activity than that of Octyl-Sepharose eluate were pooled as Red-Sepharose eluate.

Heterologous cloning and over-expression of deacylase genes

The ECB deacylase gene was isolated from *Actinoplanes utahensis* NRRL 12052 using standard methods [17]. Radiolabelled degenerate oligonucleotides were synthesized based on the N-terminal amino acid sequences of the purified ECB deacylase (as described under Results and Discussion) and used to probe restriction digests of *A. utahensis* genomic DNA. An approximately 4-kb *Pst*I fragment was observed following southern hybridization. The hybridizing fragment was gel isolated from low melting point agarose (New England Biolabs, Beverly, MA, USA) and cloned into the pUC19 cloning vector (Life Technologies, Gaithersburg, MD, USA). The resulting correct plasmid, pSHP100 (Figure 3a) was sequenced and shown to contain the entire ECB deacylase coding region with an additional approximately 1 kb of DNA upstream and approximately 0.5 kb of DNA downstream of the ECB deacylase coding region.

Increased expression of the ECB deacylase gene was achieved using an autonomously replicating *Streptomyces* plasmid, pSHP150 (Figure 3b). This plasmid was constructed by insertion of the approximately 4-kb *Pst*I-*Bam*HI fragment of pSHP100 containing the ECB deacylase gene into an approximately 5.8-kb *Pst*I-BglII fragment of the autonomously replicating streptomyces cloning vector pIJ702 [9]. The resulting expression vector was introduced into *Streptomyces lividans* TK23 using PEG-mediated transformation of protoplasts as described elsewhere [9]. Thiostrepton-resistant transformants were subsequently tested for activity. Expression of the ECB deacylase gene by pSHP150 is regulated by the native *A. utahensis* 5′ and 3′ regulatory sequences.

Activity analysis and subunit stability

The deacylase activities from the culture filtrates of *S. lividans* and *S. griseus*, the latter cloned and expressed similarly to the former as described above, were analyzed by using ECB as the substrate (Figure 2a). The soluble activity from either recombinant *S. lividans* or *S. griseus* was 180-fold of that from *A. utahensis* (Table 3). Although the soluble activity from recombinant *S. griseus* was comparable to that reported previously, the soluble activity from recombinant *S. lividans* was 20-fold higher (Table 3; [10]). Also, as analyzed by Western-Blot with antisera against purified deacylase subunits (ie, both antisera were combined), the two subunits from *S. lividans* appeared more stable to proteolytic degradation than those of *S. griseus* (Figure 4). Antisera were prepared from separate rabbits against each subunit of native deacylase, using about a 650-μg subunit that was electro-eluted from a preparative SDS-PAGE gel (by previously Hazleton Research Products and currently HRP, Denver, PA, USA). From *S. lividans*, step-wise processing of the small subunits (from 20- to 18-kDa) was observed. Thus, due to its high activity and protein stability, the soluble deacylase from *S. lividans* was our preferred source for its purification and enzymatic deacylation.

Purification of recombinant deacylase

The two genes encoding ECB deacylase subunits from *A. utahensis* were cloned and over-expressed, as described above, by *S. lividans* as soluble extracellular proteins. The recombinant *S. lividans* was grown in a 10-L fermenter under the conditions previously described [10]. The culture, after 2-day growth, was filtered through Whatman No. 1 filter paper. The resulting culture filtrate was loaded onto a S-Sepharose column (200 ml), previously equilibrated with 0.05 M KH_2PO_4, pH 6.5 (buffer A). The column was washed with buffer A and bound proteins were eluted with a linear gradient of 0–1.5 M KCl in buffer A. The fractions containing higher specific activity than that of the soluble filtrate were pooled as S-Sepharose eluate.

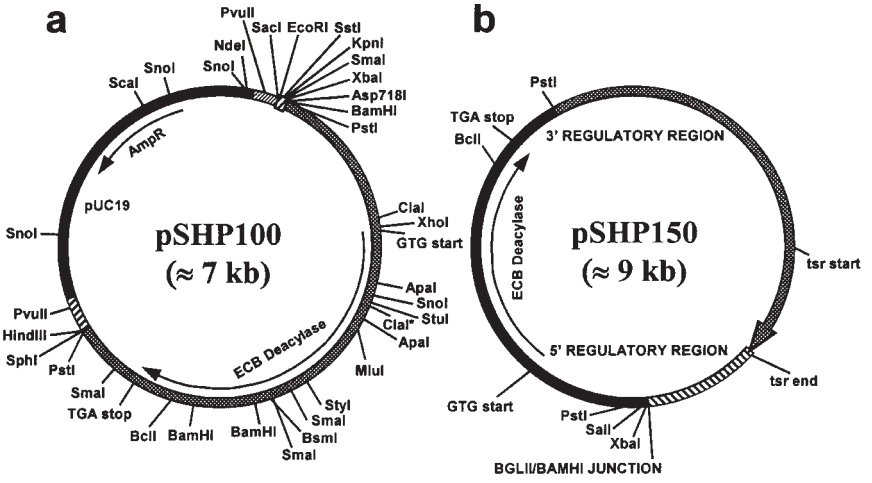

Figure 3 Restriction enzyme site and function maps of (a) plasmid pSHP100 and (b) plasmid pSHP150.

Table 3 Activity comparison of native *vs* cloned ECB deacylase

Enzyme source	Maximum ECB deacylase activity (mU ml^{-1} culture filtrate)	
	This study	Reference [15]
A. utahensis	2[a]	10
S. lividans	360	15
S. griseus	360	240

[a]From soluble portion; most of ECB deacylase activity was insoluble.

Enzymatic deacylation of immobilized ECB

The enzymatic deacylation process is based on increased accessibility of soluble deacylase from recombinant *S. lividans* to partially purified ECB, which is immobilized to HP20. Three major process steps are described below:

(a) ECB immobilization: Insoluble ECB can be selectively extracted from *A. nidulans* by an organic solvent such as acetone. The extracted ECB is about 50% pure. Further chromatographic purification of ECB is determined by overall process economy. Partially purified ECB is dissolved in an organic solvent such as methanol or acetone (either solvent appears more compatible environmentally than DMSO), and mixed with a type of non-functional polystyrene HP20 resin such as HP20SS pre-wetted with methanol. Slow addition of water to the mixture to about 25% methanol and adjustment of the pH to about 4.0 with continuous stirring causes complete binding of ECB to the resin, as confirmed by checking for ECB, with HPLC from the filtrate of ECB/resin. ECB-bound resin was washed with water to remove any residual methanol. The binding capacity of HP20 for ECB is 100–200 mg ml^{-1}; this ECB concentration is more than 100-fold greater than that solubilized at 15% DMSO (ie, at this concentration, the deacylase remained active).

(b) Deacylase preparation: Crude deacylase from recombinant *S. lividans* can be used: fermentation broth, culture filtrate or filtrate concentrate. No cofactor or metal ion is required for, or stimulatory to, the deacylase. However, inclusion of a salt such as KCl or NaCl (at about 0.8 M) or just increasing the ionic strength of the buffer (eg, to 200 mM KH_2PO_4) increased the activity/stability of the enzyme, thus improving the enzymatic deacylation of ECB. The deacylase activity (in milliunits) was determined in a total reaction volume of 1 ml with 617 μmol daptomycin, in 50 mM potassium phosphate and 0.8 M KCl, pH 6, at 60°C for 10 min (Figure 2b).

(c) Enzymatic deacylation: Conversion of immobilized ECB to soluble ECB nucleus was achieved by passing the soluble deacylase slowly through ECB-bound HP20 resin (column-mode) at pH 6.0 and room temperature. Formation of the soluble ECB nucleus was monitored by HPLC and confirmed by NMR and mass spectroscopy (data not shown). After the enzymatic reaction, the remaining ECB was eluted, thus minimizing operational ECB loss. Further reaction optimization improved the conversion efficiency, as described below.

Results and discussion

Enzyme purity: native and recombinant

The purification of ECB deacylase from *A. utahensis* is summarized in Table 2. SDS-PAGE of the purified native deacylase showed apparent homogeneity of the enzyme with one large subunit and one small subunit (Figure 5a). A laser densitometric scan showed a ratio of about 3 to 1 for the staining intensities of the large to the small subunit. Native-PAGE for the purified deacylase at pH 7 and 9 did not provide resolution of the enzyme. The purification of ECB deacylase from *S. lividans* is summarized in Table 4. SDS-PAGE of the purified recombinant deacylase also showed apparent homogeneity of the enzyme with one large subunit and two small subunits (Figure 5b).

Physical, catalytic and kinetic properties

The subunit sizes of the enzyme, as determined by SDS-PAGE, were 63 kDa and 18- to -20 kDa (Figure 5a). The relative staining intensities of the large to small subunits

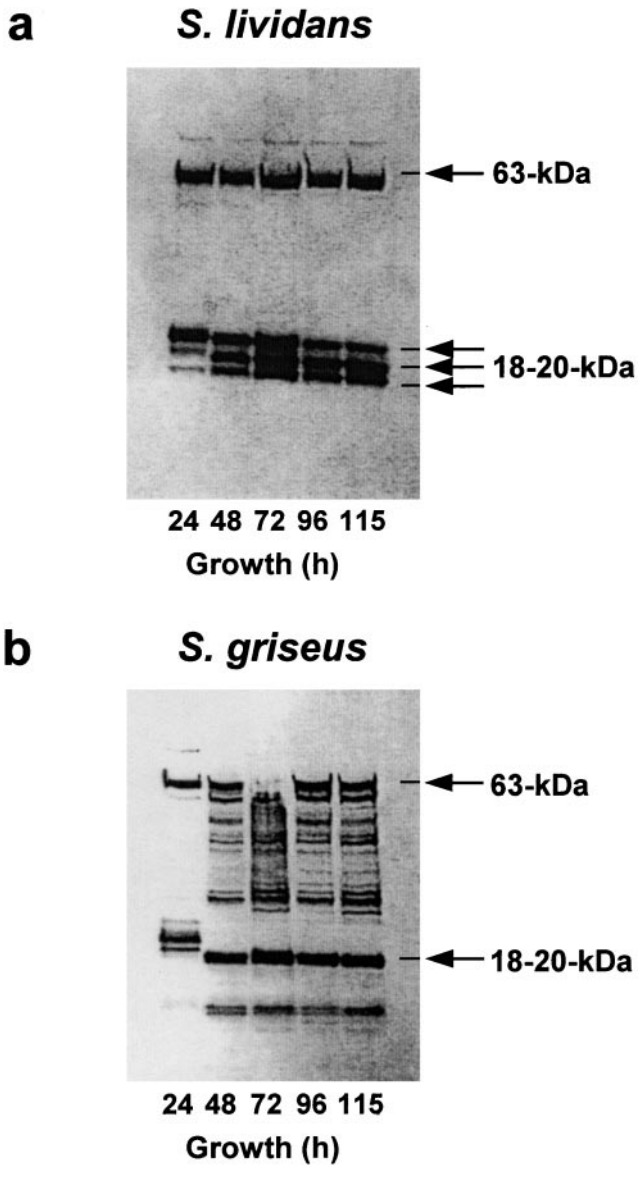

Figure 4 Western-blot of the culture-filtrate of (a) *S. lividans* and (b) *S. griseus* using the antisera prepared against purified ECB deacylase subunits (two antisera were combined).

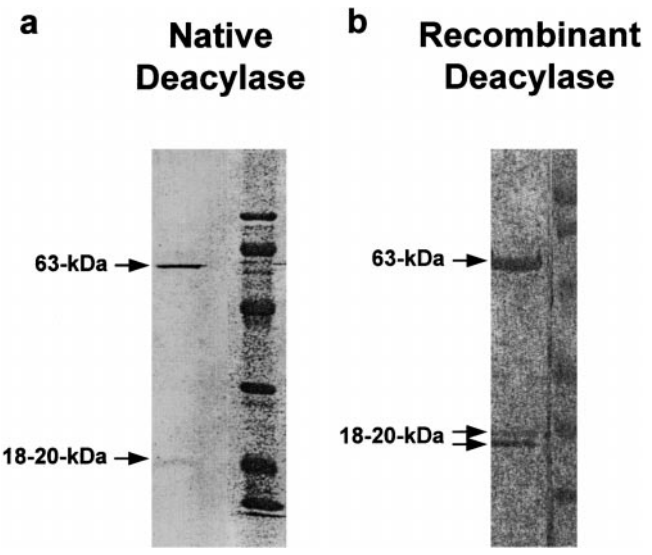

Figure 5 SDS-PAGE of purified ECB deacylase. (a) Native enzyme (5 μg) and (b) recombinant enzyme (20 μg). Both gels were stained with Coomassie blue.

Table 4 Purification of ECB deacylase from *S. lividans*

Step	Protein (mg)	Activity[a] (U)	Sp. act. (U mg^{-1})	Recovery (%)
Soluble filtrate	4392	5735	1.3	100
S-Sepharose eluate	110	1660	15.1	29

[a]Daptomycin was used as the substrate.

Table 5 Enzymatic deacylation of immobilized ECB

Substrate/product	Quantity (g)
Starting ECB	2.5
Consumed ECB	1.6
Formed ECB nucleus	1.2[a]
Recovered ECB	0.9

[a]On a molar basis, the substrate/product conversion was 64%.

were about 3 to 1. Amino-terminal sequencing of the purified deacylase (from the first eight cycles) showed a 1 to 1 molar ratio of the two subunits (data not shown). Thus, the deacylase was a heterodimer. The lack of resolution for the deacylase by native-PAGE at pH 7 and 9 indicates that the isoelectric point of the enzyme was above 9.

The purified ECB deacylase required no external cofactor, metal ion or reducing agent for maximal activity. Also, the activity was not affected by addition of a metal chelator or sulfhydryl reagent. The substrate, ECB, was practically insoluble in aqueous solutions. Among several organic solvents tested, DMSO at a low concentration (~15%) was compatible with the deacylase-catalyzed reaction and a subsequent enzyme activity analysis with HPLC (Figure 2a). Of four buffers examined, the deacylase was optimally active at pH 6 in 0.05 KH_2PO_4 and 60°C (data not shown). A stimulation of the deacylase up to 3-fold was observed by addition of a salt (Figure 6) and the salt stimulation was general with respect to several mono- and di-valent metal salts examined (data not shown). The deacylase was at least twice as active when the reaction was initiated with the enzyme rather than when it was initiated with the substrate. This suggests that the substrate might protect the deacylase from its subunit dissociation at a low enzyme concentration. Under our optimized reaction conditions, a slight degradation (<10%) of ECB and possibly also of the cyclic hexapeptide product occurred (not shown in Figure 2a). The K_m of the enzyme for ECB, as determined by the Lineweaver–Burk method, was 50 μM. The V_{max} for the deacylase-catalyzed reaction was ~15 μmol of the cyclic hexapeptide formed min^{-1} mg^{-1} protein.

Product confirmation and reaction stoichiometry

The putative cyclic hexapeptide, one of the two deacylase-catalyzed cleavage products from ECB (Figure 1), was

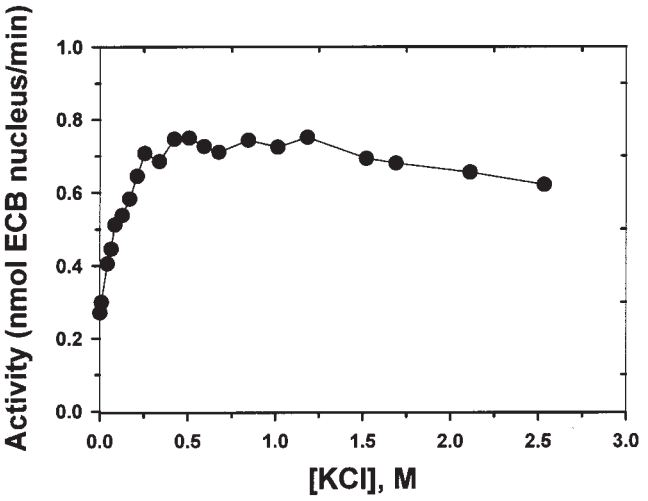

Figure 6 Stimulation of ECB deacylase by KCl. Purified native enzyme (Red-Sepharose eluate, Table 2) was dialyzed with 0.05 M KH_2PO_4, pH 7 to remove KCl and then KCl was added to the reaction mixture as shown in the figure.

identified by HPLC (Figure 2a) as well as by NMR/MS using authentic cyclic hexapeptide standard. The molar ratio for the peptide formation/ECB disappearance during a 2-h deacylase-catalyzed reaction was in the range of 0.8–0.9. Such 0.1–0.2 deviation from an expected molar ratio of 1.0 might be caused by a slight degradation of the cyclic hexapeptide as mentioned above. The conversion of ECB to the cyclic hexapeptide was 43% complete under the optimized reaction conditions (0.05 mM KH_2PO_4, pH 6.0, 0.8 M KCl, and 60°C) with enzyme excess. Further reaction optimization would be needed to improve the enzymatic conversion, as described below.

Substrate specificity

Enzymatic deacylation of acyl side-chain and peptide nucleus analogues of ECB was examined with the purified native deacylase under reaction conditions optimized with ECB, as described above. Unless specified, the deacylation reaction was conducted in the presence of DMSO (for solubility of ECB). The deacylation rate for an ECB analogue was analyzed by both substrate disappearance and product formation with HPLC. Aculeacin, a biological product that differs from ECB by a palmitoyl (instead of linoleoyl) side-chain, was cleaved effectively [22]. No cleavage of cilofungin, a chemical reacylation product from the cyclic hexapeptide of ECB, was detected. However, a moderate to slight deacylation was observed for several side-chain analogues of cilofungin [22]. A21978-C1, –C2 and –C3 (biological origin) and daptomycin (from chemical reacylation) are side-chain and nucleus analogues of ECB [1]. In the presence of DMSO, the deacylase catalyzed an efficient cleavage of the four analogues [22]. In the absence of DMSO, the enzymatic deacylation rates for the analogues increased 0.4- to 3-fold [22], suggesting DMSO inhibition of the deacylase. No enzymatic cleavage for four other structurally distinctive side-chain/nucleus analogues (from chemical reacylation) of daptomycin was detectable [22]. The deacylase-catalyzed cleavage was also observed with teicoplanin [18] and pseudomycin A (Lilly patent pending). However, no enzymatic deacylation was observed for the following β-lactam compounds tested: penicillin N, penicillin G, penicillin V, ampicillin, deacetoxycephalosporin C, deacetylcephalosporin C, and cephalosporin C.

ECB nucleus generation

An initial enzymatic deacylation of immobilized ECB was conducted with a culture filtrate of recombinant *S. lividans*. A 60% molar conversion was observed (Table 5). Remaining ECB was eluted, thus minimizing operational loss of ECB (Table 5). With multiple enzyme additions in a batch mode, near complete conversion was observed (Figure 7). The overall deacylation process can be further optimized by improving recovery of the product as well as reuse of the enzyme and resin.

ECB nucleus recovery

At room temperature (about 25°C) or above, the ECB nucleus is stable at pH 6.0 or below (data not shown). Lowering the pH of the resulting eluent from the enzymatic reaction to about pH 4.0 can increase stability of the ECB nucleus and also its binding to a second column of HP20 resin, potentially improving purity and recovery of the ECB nucleus from the resin by elution with an organic solvent such as methanol.

Deacylase/resin reuse

Reuse of the deacylase will depend on its stability and economic justification. If desirable, enzyme reuse may be achieved by ultrafiltration with a suitable membrane. Reuse of HP20 resin can easily be achieved by washing the used resin with an organic solvent such as methanol.

With some modification, this immobilized ECB may also

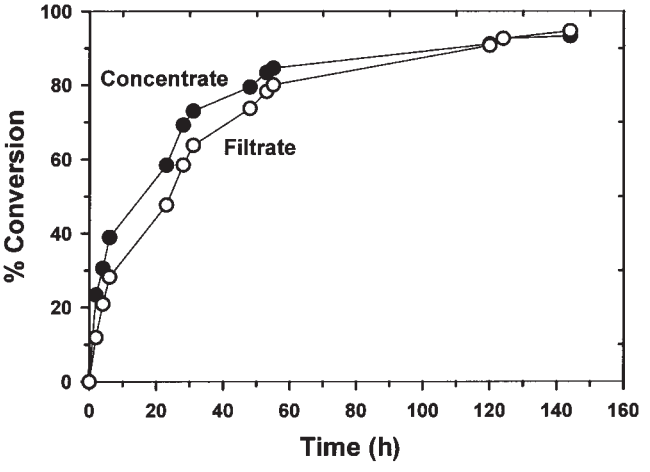

Figure 7 Enzymatic deacylation of immobilized ECB: using filtrate *vs* concentrate. ECB (95 mg) was bound to HP20SS (3.4 g), mixed with 50 ml of the culture filtrate or of its 5×-concentrate in 250 mM potassium phosphate, pH 6, and the conversion was conducted at 30°C. At a specific reaction time, as shown in the figure for each sample, the mixture was centrifuged and the supernatant fraction was removed and monitored for ECB nucleus formation by HPLC. A fresh 50-ml filtrate or concentrate was added to remaining ECB-bound resin for continuous conversion until 140 h, at which time any residual bound-ECB was removed by methanol for quantitation. With either the filtrate or concentrate each % conversion was the average value from duplicate runs.

be applicable to other insoluble hydrophobic substrates. Modification of this immobilized ECB process may include specific binding resins for enzymatic conversion and for product recovery.

ECB deacylase and aculeacin A acylase: structure/function relationship

Both ECB deacylase and aculeacin A acylase were purified from *A. utahensis* ([20]; this report), however, whether they are from a single bacterial strain remains unclear. They share similar catalytic properties with regard to optimal pH and temperature, salt stimulation as well as lack of requirement or stimulation by cofactor, metal ion or reducing agent (Table 6; [20]). They also appear to catalyze deacylation with similar substrate specificities [20,22]. However, ECB deacylase can be differentiated from aculeacin A acylase by the enzyme location, salt-induced solubilization and stimulation extent as well as the large-subunit size (Table 6). The amino-terminal sequences for the two subunits from ECB deacylase, as shown below, are different.

Large subunit: SNAYGLGAQATVNGSG
MVLANPHFP(W)Q–AE(R)FY
Small subunit: HDGGYAALIRRASYGV(P)HITADDF–
SLGFGVVYVQAE
(): tentative residue; –: residue not identified

The two sequences are identical to those reported for aculeacin A acylase except that the first two residues of the small subunit of ECB deacylase are absent from that of aculeacin A acylase [10]. It is of great interest to determine whether ECB deacylase and aculeacin A acylase are structurally slightly different or they merely represent two (membrane-bound and soluble) isozymes [8,11] which might be attributable to slightly different ionic conditions for growth of *A. utahensis* [2,20] and/or proteolytic processing.

Table 6 Comparison of ECB deacylase *vs* aculeacin A acylase

Parameter	ECB deacylase	Aculeacin A acylase[a]
Producing organism	*A. utahensis*	*A. utahensis*
Enzyme location	Cell-associated	Extracellular
Substrate		
Cyclic peptide	6-Membered	6-Membered
Acyl side-chain	Linoleoyl	Palmitoyl
Effect of salt (KCl or NaCl)		
Enzyme solubility	Required	Not required
Enzyme stimulation	3-Fold	0.6-Fold
Oligomeric structure	Dimer	Dimer
Large subunit	63-kDa	55-kDa
Small subunit	18 to 20-kDa	19-kDa
Amino-terminal sequence		
Large subunit	SNAYG	SNAYG
Small subunit	HDGGY	GGY

[a]From References [10] and [20].

ECB deacylase and penicillin acylase: evolutionary implication

ECB deacylase is an 81 to 83-kDa heterodimer consisting of 63- and 18-to-20-kDa subunits. Penicillin G acylase from *Escherichia coli* is an 87-kDa heterodimer with 65- and 22-kDa subunits [3]. For comparison, cephalosporin acylase from a *Pseudomonas* strain is an 83-kDa heterodimer consisting of 57- and 26-kDa subunits [16]. The essential absence of any external catalytic requirement, cofactor stimulation or product inhibition of ECB deacylase is also an intrinsic property of penicillin acylase [13]. The amino-terminal sequences from the two subunits of ECB deacylase show no detectable similarity. It is not yet known whether the two subunits are products of two different genes, or both subunits derive from intracellular processing of a single pre-protein as described for penicillin acylase [3], or, possibly, the small subunit is a processing or degradation product from the large subunit. From a preliminary search of a protein data bank, a moderate sequence similarity (~48%) has been observed between the small subunit of ECB deacylase and a penicillin acylase as shown below. This sequence similarity, detected from the available but limited amino-terminal sequences of the large and small subunits, suggests an evolutionary relationship between ECB deacylase and penicillin G acylase.

ECB deacylase small subunit:	9	IRRAS	YGVPH	ITADD
Penicillin acylase:	36	IVRDE	YGMPI	YAADA
ECB deacylase small subunit:	29	F-SLG	FGVVY	VQAE
Penicillin acylase:	56	TWHLF	YGYGY	VVAQ

The broad substrate specificity of penicillin and cephalosporin acylases using 3-membered β-lactam peptides as substrates [14,15,19] has allowed them to play a critical industrial role in generation of key intermediates for manufacturing of therapeutic antibacterial β-lactam compounds. Although ECB deacylase has some biochemical and structural similarities to penicillin/cephalosporin acylases, the deacylase has a broad independent substrate specificity for 6-membered and more complex cyclic peptides. Thus, ECB deacylase may impact, by its enzymatic deacylation and acylation, the development of therapeutic antifungal cyclic peptides. The broad substrate specificity of the native deacylase may be further extended, eg in acquiring a novel deacylation or acylation, by enzyme engineering. Toward this direction, future elucidation of the active site of ECB deacylase appears desirable for potential industrial application.

Conclusions

The membrane-associated ECB deacylase of *A. utahensis* can be salt-solubilized, heat-treated and purified to apparent homogeneity by a 3-step chromatographic procedure. The deacylase is a heterodimer consisting of 63- and 18-to-20-kDa subunits. Except for salt-stimulation, the deacylase has no external cofactor or metal-ion requirement and has broad substrate specificity for 6-member and more complex cyclic

peptides. The deacylase genes were cloned, over-expressed and stabilized in *S. lividans*. The recombinant deacylase has been used with immobilized ECB for generation of a gram-quantity of ECB nucleus. ECB deacylase is similar to, in structure/function, but can be differentiated from aculeacin A acylase. Also, ECB deacylase shares a sequence similarity with penicillin G deacylase. Thus, the recombinant ECB deacylase and its yet to-be-improved versions, eg, by enzyme engineering, can be potentially useful for biotechnological applications.

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