NOTES

# A New Enzyme, Edeine $B_1$ Amidinohydrolase, from *Bacillus brevis* TT02-8

## Purification and Determination of the *N*-terminal Amino Acid Sequence

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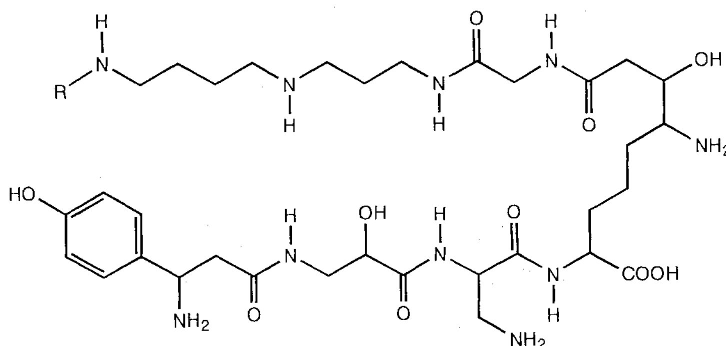
Edeine $A_1$ and $B_1$ are peptide antibiotics produced by *Bacillus brevis* TT02-8 which inhibit growth of Gram-positive and Gram-negative bacteria, mycoplasmas, fungi and mammalian neoplastic cells in tissue culture[1]. The mechanism of action of these antibiotics is *via* inhibition of DNA and protein synthesis in both prokaryotic and eukaryotic cells. As shown in Fig. 1, edeine $A_1$ and $B_1$ are linear oligopeptides, which are composed of five amino acid residues and an organic base; spermidine for edeine $A_1$ and guanylspermidine for edeine $B_1$.[1]

During our studies on microbial metabolites from culture filtrates of *B. brevis* TT02-8, we observed that production of edeines was dependent on components of culture medium; edeine $A_1$ was mainly produced when cultured in Borowska medium,[1] whereas edeine $B_1$ was yielded when manganese ion ($Mn^{++}$) was depleted from the medium. It is assumed that an enzyme with amidinohydrolase activity participates in the conversion of edeine $B_1$ to $A_1$, based on the chemical structures of these two compounds, and is likely to be $Mn^{++}$-dependent, since edeine $A_1$ was produced from edeine $B_1$ by incubation with cell lysate as well as cell suspension from *B. brevis* TT02-8 when $Mn^{++}$ was added.

Arginase activity in the form of arginine amidinohydrolase is present in cell free extract of *B. brevis* TT02-8. Thus, we first predicted that the arginase from this bacteria participates in the synthesis of these antibiotics, in particular conversion of edeine $B_1$ to $A_1$. We confirmed in a previous paper that purified arginase from *B. brevis* TT02-8 could hydrolyze edeine $B_1$ to $A_1$. The hydrolytic efficiency of this enzyme for edeine $B_1$, however, was only 0.8% of the rate of hydrolysis for L-arginine, a major substrate for arginase.[2] Furthermore, we observed the presence of a second protein with amidinohydrolase activity in cell free extract of *B. brevis* TT02-8.[2] When

Fig. 1. Structures of edeine $A_1$ and $B_1$.



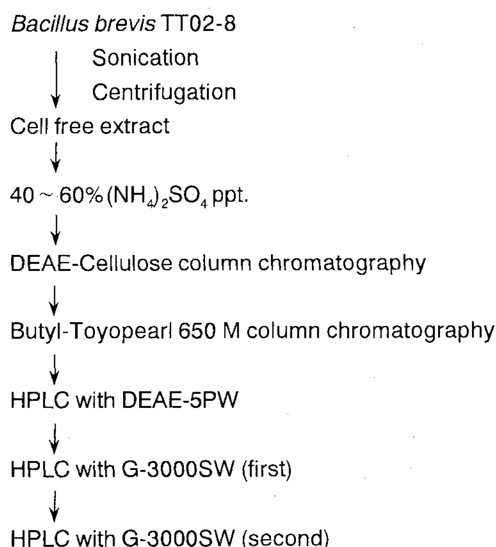
Edeine $A_1$ : R= H—

Edeine $B_1$ : R= $H_2N$(HN)C—

the cell lysate was fractionated by hydrophobic column chromatography, the protein was eluted in a different fraction to arginase. This protein requires $Mn^{++}$ for amidinohydrolase activity.

To determine which amidinohydrolase biologically converts edeine $B_1$ to $A_1$ in *B. brevis* TT02-8, we purified the second protein with amidinohydrolase activity. The purified protein had higher catalytic activity than arginase for the edeine $B_1$ to $A_1$ reaction, and had characteristic features of amidinohydrolase activity such as metal ion requirement. Thus, we termed this enzyme edeine $B_1$ amidinohydrolase (EB-hydrolase). We have determined the *N*-terminal amino acid sequence of the protein, towards characterizing the gene for this enzyme.

Fig. 2. Procedures for purification of EB-hydrolase from *Bacillus brevis* TT02-8.

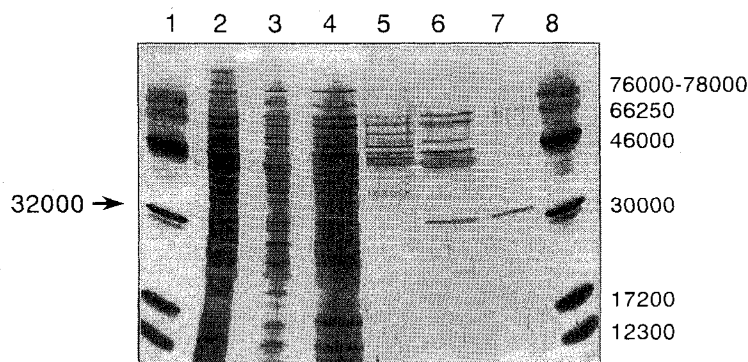



Starting from 200 g of wet *B. brevis* TT02-8 cells, we purified EB-hydrolase by following the procedure used to purify the arginase from this bacteria established by us with slight modification. The summary of the stepwise purification procedure is shown in Fig 2. As EB-hydrolase was not as stable with heat as the arginases from *B. brevis* TT02-8,[2] rat liver or *Neurospora crassa*,[3,4] the treatment step in which the enzyme-containing cell lysate was incubated at 60°C for 10 minutes was skipped.

Both activities of EB-hydrolase and arginase were not resolved sufficiently by ion exchange chromatography with DEAE-cellulose, and the most efficient separation of these activities was achieved by hydrophobic column chromatography using Butyl-Toyopearl 650M (Tosoh, Tokyo). EB-hydrolase was eluted in the eluate of around 0% $(NH_4)_2SO_4$ and arginase at around 13% $(NH_4)_2SO_4$. Since the enzymatically active fraction from the hydrophobic column chromatography was still contaminated with many other proteins, the following purification steps were included for further purification of this enzyme as shown in Fig. 2. Finally EB-hydrolase was purified to electrophoretic homogeneity from *B. brevis* TT02-8. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis the protein had a molecular mass of 32 kilo Dalton (kDa) (Fig. 3).

However, EB-hydrolase activity eluted on a TSK gel G-3000 SW column had an apparent molecular weight of 82-kDa (data not shown). Thus, the EB-hydrolase

Fig. 3. SDS-PAGE of purified EB-hydrolase.




Protein bands were stained with 2D-Silver Stain II reagent (Daiichi, Tokyo). 1, 40 ~ 60% $(NH_4)_2SO_4$; 2, DEAE-cellulose fraction; 3, Butyl-Toyopearl fraction; 4, HPLC TSK gel DEAE-5PW; 5, HPLC TSK gel G-3000 SW (first); 6, HPLC TSK gel G-3000 SW (second); 7, size markers.

Table 1. Substrate specificity of EB-hydrolase and arginase from *Bacillus brevis* TT02-8.

| Substrate | Relative activity (%) | |
|---|---|---|
| | EB-hydrolase | Arginase[2] |
| Edeine $B_1$ [a] | 100 | 0.8 |
| Blasticidin S[b] | 0.1 | 0.7 |
| Canavanine | 0.1 | 25.6 |
| Guanidinobutyric acid | 0 | 0.4 |
| Creatine | 0 | 0 |
| Glycocyamine | 0 | 0.5 |
| L-Arginine | 14.9 | 100 |

Substrate concentration was 25 mM, [a] 6.3 mM or [b] 11 mM (final concentration). EB-hydrolase activities were shown relative to that for edeine $B_1$ as the substrate. Arginase activities were shown relative to that for L-arginine as the substrate.

Table 2. Effects of metal ions on EB-hydrolase activity.

| Metal ion | EB-hydrolase activity (%) |
|---|---|
| None (control) | 100 |
| LiCl | 106 |
| KCl | 95 |
| NaCl | 89 |
| $BaCl_2$ | 142 |
| $CoCl_2$ | 345 |
| $CuCl_2$ | 20 |
| $FeCl_2$ | 88 |
| $MgCl_2$ | 99 |
| $MnCl_2$ | 723 |
| $NiCl_2$ | 5 |
| $ZnCl_2$ | 9 |
| $AlCl_3$ | 52 |
| $FeCl_3$ | 77 |
| $MnCl_2$ + EDTA | 11 |

Metal ions were added to the reaction mixture at a final concentration of 1 mM and the mixtures were incubated at 37°C for 10 minutes. Residual activity was assayed as described in Exprimental. EDTA was added to the reaction mixture including $Mn^{++}$ at a final concentration of 1 mM.

activity is likely to be composed of three 32-kDa subunits.

This purified enzyme was then further characterized. The guanidino-compounds listed in Table 1 were tested as potential substrates for the enzyme. The enzyme showed high specificity for edeine $B_1$, but hydrolyzed L-arginine at 15% of the rate for edeine $B_1$. Arginase from this organism hydrolyzed edeine $B_1$ at 0.8% of the rate for L-arginine.[2] In general, the secondary metabolic enzyme is not as efficient as the first metabolic enzyme for a substrate. The Michaelis constant for edeine $B_1$, calculated from a Lineweaver-Burk plot, was 5.56 mM. The enzyme showed the highest activity at pH 10~10.5. Maximal enzyme activity was observed at 50~55°C.

The effect of metal ions on the enzyme activity was analyzed. After incubating the enzyme with the various metal ions listed in Table 2 at 37°C for 10 minutes, the enzyme activity was measured. $Mn^{++}$ was required for all enzyme activity. $Co^{++}$ could replace $Mn^{++}$ to retain enzyme activity. No significant activation or inhibition was found upon the addition of metal ions such as $Li^+$, $Na^+$, $K^+$, $Fe^{++}$, and $Mg^{++}$ as chloride compounds, but $Cu^{++}$, $Ni^{++}$, and $Zn^{++}$ were inhibitory to the enzyme (80, 95, and 90% inhibition). Addition of 1 mM EDTA to the mixture of the enzyme plus $Mn^{++}$, reduced the enzyme activity to 1.5% of the maximum activity for $Mn^{++}$ added. Arginases[5~13] from various organisms, guanidinoacetate amidinohydrolase,[14,15] guanidinobutyrate amidinohydrolase,[16,17] and proclavaminate amidinohydrolase (PAH)[18] are activated by $Mn^{++}$ and other divalent cations and are optimal at alkaline pH (pH 9.0~10.5). In the case of agmatine ureohydrolase (AUH)[19] and creatine amidinohydrolase (CAH),[20] the numerous cationic ions did not affect their activities and the optimal pH for activity was 7.3 and 7.5, respectively. In this context, EB-hydrolase is likely to be a divalent metal-requiring enzyme and more similar to arginase in this context than to the other amidinohydrolases such as AUH and CAH.

EB-hydrolase is a member of the evolutionarily divergent arginase family of enzymes. To clone the gene for this enzyme, we determined the *N*-terminal amino acid sequence of the *B. brevis* EB-hydrolase by automated Edman degradation using an Applied biosystems Model 477A sequencer. The resulting *N*-terminal amino acid sequence of the first 25 residues of EB-hydrolase was [M-R-F-D-E-A-Y-R/S-G-N-V-F-I-(R)-S-(H)-G-N-Y-E-E-S-Q-A-V]. Efforts are now focused on cloning the gene to analyze the entire structure of the enzyme.

## Experimental

Purification of EB-hydrolase

*B. brevis* TT02-8 was cultivated at 27°C for 48 hours in modified Borowska medium, containing 2% glycerine, 1% yeast extract, 0.25% L-asparagine, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% $KH_2PO_4$, 0.05% $K_2HPO_4$, and saturating amounts of $Ca(H_2PO_4)_2 \cdot H_2O$ (pH 7.2). The culture was centrifuged and approximately 200 g of wet cells were suspended in 150 ml of 50 mM Tris-HCl (pH 8.0) (buffer A). The suspension was sonicated for 10 minutes with an ultrasonic disintegrator, and centrifuged at 143,000 × *g* for 30 minutes to remove cell debris. The supernatant solution (250 ml) was adjusted to 40% saturation with solid $(NH_4)_2SO_4$ and stirred for 30 minutes. The suspension was centrifuged and the pellet was discarded. The supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$ and spinned after stirring for 30 minutes, and the pellet was retained. The pellet (27 g) was resuspended in buffer A (30 ml) and dialyzed two times in 4 liters of the same buffer.

The dialyzed solution was applied to a 3.7 × 50 cm column of DEAE-cellulose (Whatman, England) equilibrated with buffer A. The column was thoroughly washed with 75 ml of buffer A, and EB-hydrolase was eluted with a 2,400 ml linear gradient of 0～0.5 M NaCl in the same buffer. The fractions containing enzymatic activity were combined and adjusted to 30% saturation with solid $(NH_4)_2SO_4$ and applied to a 2.2 × 20 cm column of Butyl-Toyopearl 650M (Tosoh, Tokyo) equilibrated with 30% $(NH_4)_2SO_4$ in 20 mM Tris-HCl (pH 8.0) (buffer B). The column was washed with buffer B containing 30% $(NH_4)_2SO_4$ and then eluted with buffer B supplemented with $(NH_4)_2SO_4$ in a linear concentration gradient from 30% to 0%. In this last step EB-hydrolase activity was separated from arginase activity. EB-hydrolase was eluted at around 0% $(NH_4)_2SO_4$ and arginase at around 13% $(NH_4)_2SO_4$.

The fractions with EB-hydrolase were collected. The fraction was concentrated and desalted with Centricon 10 (Amicon, USA). The diluted sample was applied to a 7.5 mm i.d. × 7.5 cm TSK gel DEAE-5PW column (Tosoh, Tokyo) equilibrated with buffer A. The column was washed with 5.2 ml of the same buffer, and proteins were eluted with a 100 ml linear gradient of equilibration buffer containing 0.2 to 1 M NaCl. Fractions containing EB-hydrolase activity were collected, desalted, and concentrated to a small volume with an Amicon Centricon 10.

The enzyme preparation obtained by the TSK gel DEAE-5PW column chromatography was further purified by gel filtration using a TSK gel G-3000 SW HPLC column (Tosoh, 7.5 mm i.d. × 60 cm), equilibrated with 0.2 M phosphate buffer (pH 6.8). Fractions containing EB-hydrolase activity were combined, concentrated, and run again on the same column. The active fraction was kept at 4°C. EB-hydrolase activity was measured by the method described by J. M. GEYER *et al.*[21] with a slight modification. One unit was defined as 1 μmole of urea liberated per minute from edeine $B_1$. Protein was assayed by the Bradford method using bovine serum albumin as a standard.

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