Discovery and Purification of Enacyloxin Oxidase

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A new extracellular oxidase, which is involved in biosynthesis of ENX IIa, a congener of ENX, was found in the culture supernatant of *Frateuria* sp. W-315. The enzyme, which was secreted into the culture medium from the cells, catalyzed conversion of another congener of ENX, ENX IVa, to ENX IIa.

Abbreviations: DHase, dehydrogenase; ENX, Enacyloxin; HPLC, High performance liquid chromatography; NAD (P), Nicotinamide adenine dinucleotide (phosphate); PQQ, pyrroloquinoline quinone; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, Thin layer chromatography

Introduction

We have discovered a unique antibiotics named enacyloxin (ENX), which is a family of non-lactonic polyene antibiotics produced by *Frateuria* sp. W-315. ENX has more than 12 congeners and they are active against Gram-positive and Gram-negative bacteria.[1-5] ENX IIa has been shown to inhibit protein synthesis of *E. coli* by inhibiting binding of a.a.-tRNA to A site of ribosomes.[6,7]

In the early phase of production of the antibiotics, the main product secreted into the culture fluid was ENX IVa, while it gradually decreased in correspondence to the increase of enacyloxin IIa (ENX IIa) with the lapse of culture period. The differences in chemical structure between ENX IIa and ENX IVa (Fig. 1) is oxidative status of C-15′ suggesting that bacterial extracellular oxidase or dehydrogenase catalyzes dehydrogenation of ENX IVa. Moreover, this change seemed to be a stoichiometric conversion of ENX IVa to ENX IIa. We named the enzyme enacyloxin oxidase (ENX oxidase) tentatively.

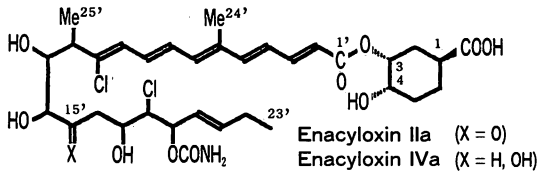

Fig. 1 Chemical Structures of Enacyloxins (ENXs)

Duine *et al.*[8] have reported that a cofactor of D-glucose DHase from *Acinetobacter calcoaceticus* was PQQ, and proposed the name of "quinoprotein" for PQQ-containing group of DHases. Several Gram-negative bacteria have quinoprotein DHases[9] in their periplasm fractions, but no extracellular quinoprotein has been reported.

In this paper, we describe a new extracellular quinoprotein which is involved in the biosynthesis of a new polyenic antibiotic, ENX IIa.

Materials and Methods

1. *Determination of ENXs and enzyme purification*

ENX IVa and ENX IIa were purified,[1,3,4] and determined by HPLC (Tosoh, CCPD 8000 system with ODS 120A column, $CH_3CN/H_2O/HCOOH$ = 1000/1200/6.6) with SIC chromatocorder 12 (System Instruments Co., Ltd, Tokyo). For purification of ENX oxidase, the culture broth was concentrated by ultrafiltration (Millipore,

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Minitan™, U.S.A.), and then ammonium sulfate was added to give 40% of saturation. Precipitate formed were centrifuged (10,000 xg, 5 min), dissolved in buffer and dialyzed overnight against buffer. The dialyzed enzyme was centrifuged (10, 000 xg, 5 min) and the supernatant was then ultracentrifuged (100,000 xg, 4 h). Precipitates formed were dissolved in buffer and were subjected to gel filtration of HPLC (TSK gel G3000SWG). Active fraction eluted from HPLC was used for enzyme assay.

2. *Enzyme assay of ENX oxidase activity*

ENX oxidase activity was determined by estimation of ENX IIa formed from ENX IVa using HPLC. One hundred μl of reaction mixture contained 20 μl of 0.1 mM ENX IVa, 10 μl of enzyme solution and 70 μl of Tris-HCl buffer (50 mM, pH7.2).

3. *Others*

Bacterial growth was measured by absorbance at 660 nm using a Hitachi U-1000 spectrophotometer. A Beckman DU-65 spectrophotometer was used for measurement of hydrogen peroxide. Changes of ENXs in the culture supernatant were measured using HPLC before and after incubation at 5°C or 30°C. Protein concentrations were determined by Lowry's method.[10] In figures, ENX IVa and ENX IIa are abbreviated to IVa and IIa, respectively. Buffer solution used in these experiments is 5 mM, Tris-HCl, pH7.2 except otherwise indicated.

Results and Discussion

In the production of ENXs by *Frateuria* sp. W-315, ENX IVa was detected first in the culture supernatant and it decreased gradually in accordance with the increase of ENX IIa after a prolonged culture. To confirm this phenomenon more precisely, an aliquot of the culture was centrifuged (10,000 xg, 10 min) to remove the cells and incubated at 5°C. Two big peaks, ENX IVa and ENX IIa were seen (Fig. 2). After 1 h of incubation, profiles of two ENXs changed greatly and this change proceeded further with the lapse of incubation time (3 h). Peak areas in Fig. 2 were integrated and the amounts of ENX IIa and ENX IVa were estimated. The results showed that the sum of ENX IVa and ENX IIa was kept constant during 3 h of incubation (Table I). These results, together with the chemical structures of both ENXs suggest that two atoms of hydrogen were withdrawn from ENX IVa and transfered to some materials to produce ENX IIa stoichiometrically. This reaction was completely inactivated by treatment of the culture supernatant at 90°C for 2 min, suggesting that bacterial oxidase or dehydrogenase would participate in this reaction. When the culture supernatant was incubated at 30°C, sum of ENXs was not constant, probably because an enzymatic cleavage of carbamoyl moiety of ENXs or other reactions would take place to form chemically changed ENXs in the culture supernatant. These side reactions seemed to be negligible at 5°C.

During the production of antibiotics, when measured from 30 h to 38 h as shown in Fig. 3, ENX IVa and ENX IIa increased gradually with the lapse of the culture period. Since these

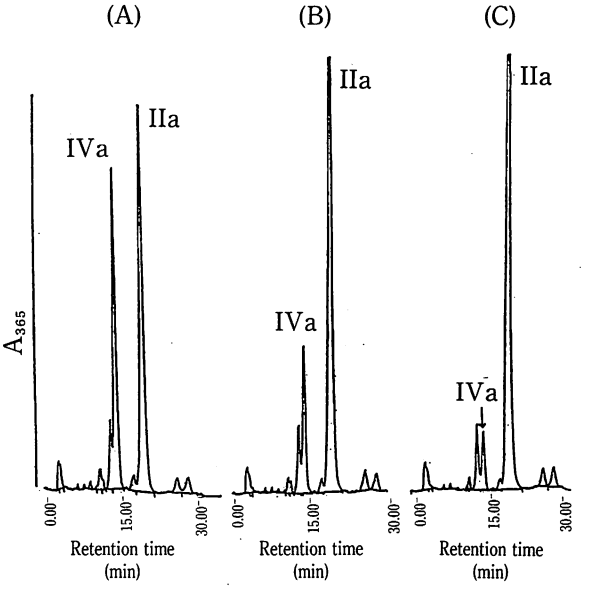

Fig. 2 HPLC Profiles of ENXs in the Culture Supernatant

(A) The supernatant of the culture (38 h).
(B) The supernatant after incubated for 1 h at 5°C.
(C) The supernatant after incubated for 3 h at 5°C.

Table I Determination of ENX IVa and ENX IIa from Peak Areas in HPLC

Name	A_{365} unit				
	(A)	(B)	(A)−(B)	(C)	(A)−(C)
ENX IVa (a)	12.0	5.8	6.2	2.2	9.8
ENX IIa (b)	18.7	24.9	−6.2	28.6	−9.9
(a)+(b)	30.7	30.7		30.8	
Others	7.3	7.0	6.5		
Total	38.0	37.7	37.3		

The amounts of ENXs integrated by SIC chromatocorder 12 were shown as A_{365} units. One A_{365} unit corresponds to 1 ng of each ENX IIa and ENX IVa. (A), (B) and (C) correspond to HPLC profiles incubated for 0, 1 and 3 h at 5°C.

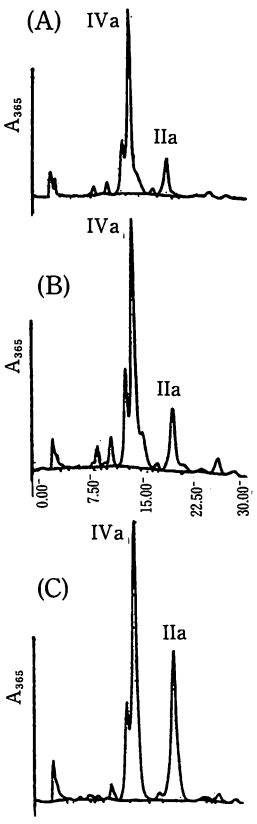

Fig. 3 Changes of ENX Profiles during the Cultivation of *Frateuria* sp. W-315

(A) HPLC profiles of the supernatant from 30 h-culture.
(B) HPLC profiles of supernatant from 34 h-culture.
(C) HPLC profiles of supernatant from 38 h-culture.

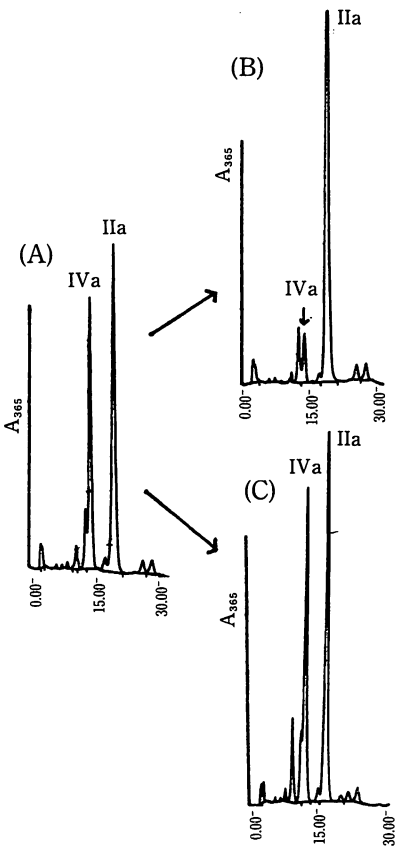

Fig. 4 Effects of Cells on ENX Oxidase Activity

(A) HPLC profiles of the supernatant from 38 h-culture.
(B) HPLC profiles of the supernatant after prolonged incubation at 30°C for 3 h without cells.
(C) HPLC profiles of the supernatant after prolonged incubation at 30°C for 5 h with cells.

changes were quite different from those of Fig. 2, we attempted to analyze these phenomena employing the culture supernatant of 38 h by prolonged incubation with or without the cells at 30°C (Fig. 4). The prolonged incubation with the supernatant only for 2 h showed a big decrease of ENX IVa like those of Fig. 2, whereas the prolonged incubation with both the supernatant and

the cells for 5 h showed only a slilght increase of both ENXs, and this result resembled to that of Fig. 3. One of the explanation for these differences in results of the presence and absence of the cells seemed to be due to dissolved oxygen in the supernatant. To reveal the role of oxygen in the changes of ENXs more clearly, oxygen in the incubation tube containing the culture supernatant was replaced by argon gas *in vacuo* several times. When oxygen was removed (Fig. 5-B), ENXs did not change significantly as compared to Fig. 5(A), whereas when oxygen was not removed, ENX IVa decreased greatly (Fig. 5-C). In the logarithmic phase of the culture, most of the dissolved oxygen would be used by the cells and enough oxygen would not be supplied to ENX oxidase, and therefore, the profiles of ENXs in Fig. 2 (without cells) would be different from those in Fig. 3 (with cells).

From these results, an oxidase or dehydrogenase would participate in the conversion of ENX IVa to ENX IIa. In general, there are two kinds of enzymes which catalyze dehydrogenation; one is dehydrogenase and the other is oxidase. The former requires mainly NAD (P) or flavin as electron acceptor and the latter can directly or indirectly react with molecular oxy-

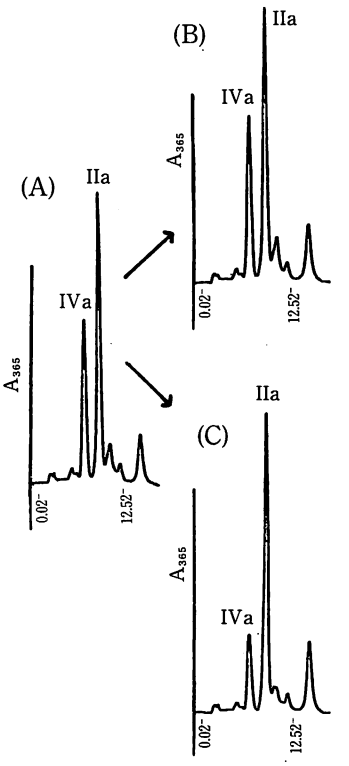

Fig. 5 Effects of Oxygen on ENX Oxidase Activity

(A) HPLC profiles of the supernatant from 38 h-culture.
(B) HPLC profiles of the supernatant after prolonged incubation at 30°C for 1 h without oxygen (Gas phase in the incubation tube was replaced by argon gas).
(C) HPLC profiles of the supernatant after prolonged incubation at 30°C for 1 h with oxygen (Gas phase in the incubation tube was air).

Table II Summarized Results of Purification of ENX Oxidase.

Purification step	Total protein	Total activity	Specific activity	Purification	Yield
	(mg)	(Kat)*	(Kat×10^{-7}/kg)	(fold)	(%)
Culture supernatant	1200	474	3.95	1	100
$(NH_4)_2SO_4$ precipitate (0—40%)	180	270	15.0	3.8	57
Ultracentrifuge (100,000 xg, 4h)					
supernatant fr.	158	178	11.27	2.8	37.5
precipitate fr.	20	99	49.3	12.5	21
Gel filtration (HPLC)**					
fraction 1	0.76	6	82.9	21.0	1.3
fraction 4	0.95	10	105.3	26.6	2.1

* One Kat was defined as the amount of enzyme forming 1 mol of ENX IIa per sec at 5°C.
**Elution buffer contains 0.2% Triton X-100.

gen to form hydrogen peroxide. As the enzyme described in this report required molecular oxygen, we named the enzyme enacyloxin oxidase (ENX oxidase) tentatively.

Summarized results of the purification shown in Table II indicate that the highest purification was achieved after gel filtration. Specific activity of fraction 4 was higher by 26.6 fold than that of the culture broth, but it still contained more than two bands in SDS-PAGE (data not shown). In the following experiments, we employed this partially purified fraction as the enzyme preparation. In order to know whether molecular oxygen was required as electron acceptor in ENX oxidase reaction more precisely, detection of both hydrogen peroxide and ENX IIa were performed. Namely, after ENX oxidase was incubated with ENX IVa at 30°C, both formations of hydrogen peroxide and ENX IIa were measured by the method of Teranishi *et al.*[11] and by HPLC, respectively as shown in Fig. 6. Similar amounts of hydrogen peroxide and ENX IIa were formed during incubation of 90 min as shown in Fig. 6. Enzyme preparation employed in these experiments showed only a slight dark color but it had no red color and no appreciable absorbance from 350 nm to 700 nm by scanning (data not shown), suggesting that it presumably would not have cytochromes and then molecular oxygen would accept two atoms of hydrogen directly from the enzyme. We can conclude that ENX oxidase requires molecular oxygen as electron acceptor and this reaction is coupled to ENX IVa oxidation.

To identify the cofactor required for ENX oxidase reaction, cofactor such as NAD (P), FAD and PQQ were added separately to the reaction mixture containing ENX IVa and ENX oxidase in buffer, and incubated. Any stimulative effect of each cofactor on the enzyme reaction, however, was not observed, suggesting that the cofactor of ENX oxidase would remain in the partially purified enzyme preparation (data not shown). Since carbonyl reagent is known as an inhibitor of quinoprotein by its reaction with carbonyl group of PQQ, effect of hydroxylamine on ENX oxidase was examined (Fig. 7). The culture supernatant incubated for 1 h with hydroxylamine (Fig. 7-B) showed a big decrease of ENX oxidase activity as compared to that of Fig. 4(B), suggesting that the enzyme would have PQQ or PQQ-like substanace as its cofactor.

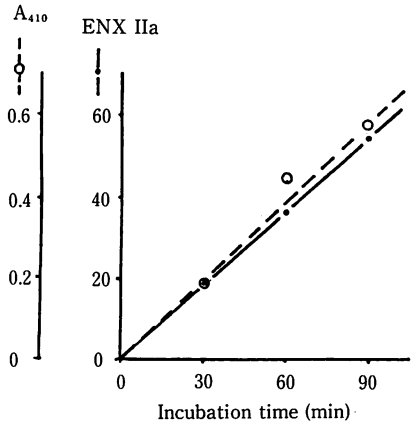

Fig. 6 Hydrogen Peroxide Production Correspondent to ENX IIa Formation

Reaction mixture contained 40 μl of 0.1 mM ENX IVa, 50 μl of ENX oxidase and buffer in a total volume of 100 μl. After the reaction was terminated as indicated in figure, it was divided into two portions. One is used for estimation of ENXs and the other is to measure hydrogen peroxide. For estimation of hydrogen peroxide, 5 μl of the reaction mixture was added with 20 μl of Ti reagent and 15 μl of distilled water. Absorbance at 410 nm was recorded.

Symbols -○- and -•- indicate production of hydrogen peroxide and formation of ENX IIa (per cent of ENX IIa against ENX IVa plus ENX IIa), respectively.

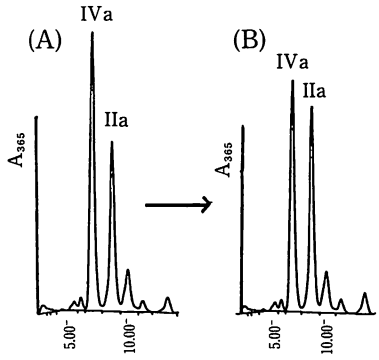

Fig. 7 Inhibitory Effect of Carbonyl Reagent on ENX Oxidase Activity

(A) HPLC profiles of the supernatant from 38 h-culture just after addition with 10 mM of hydroxylamine HCl (HA).

(B) HPLC profiles of the supernatant incubated for 1 h at 30°C with HA.

Addenda

This work was carried out at The Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai 981, Japan.

Among the studies of enacyloxin and ENX oxidase, structural elucidation and mode of action of enacyloxins were presented in the Congress of International Union of Microbial Society held in Osaka, Japan, 1990 and the 3rd International Conference of Biotechnology of Microbial Products held in San Francisco, U.S.A., 1993. Results including ENX oxidase were presented in the 2nd International Symposium of PQQ and Quinoprotein held in Yamaguchi, Japan, 1991.

Acknowledgements

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