糸状性,好温性ラン藻Phormidium lapideumの制限酵素

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Restriction Endonucleases from *Phormidium lapideum*, a Strain of Filamentous and Thermophilic Cyanobacteria.*

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糸状性，好温性ラン藻 *Phormidium lapideum* の制限酵素

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ABSTRACT A couple of restriction endonucleases, *Pla* I and *Pla* II, have been purified from a filametous and thermophilic cyanobacterium, *Phormidium lapideum*. *Pla* I was proved to be an isoschizomer of *Hae* III to cleavage the site GG↓CC and was a monomeric protein that had a molecular weight of about 40 kilodaltons. *Pla* II was an isoschizomer of *Nsp* (7524) V to recognize the site TTCGAA and was estimated as a heterotetrameric protein ($\alpha_2\beta_2$). *Pla* II has an apparent molecular mass of 176 kilodaltons and that of α subunit was 63 kilodaltons, β subunit 31 kilodaltons. Characteristics of *Pla* I and *Pla* II were investigated in comparison with that of the respective isoschizomers, *Hae* III and *Nsp* (7524) V.

INTRODUCTION Restrition endonucleases that recognize the specific groups of base-sequences (recognition sites) on DNA strands and break the phosphodiester bonds of the specific sites (cleavage sites) have been isolated from a wide variety of microorganisms.[1] Since *Eco* RI, a restriction enzyme from *Escherichia coli* RY 13, was documented by H. W. Boyer *et al* in 1974,[2] the restriction endonucleases have been used as requiste tools for the gene manipulation in modern molecular biology. Indeed at the moment over 100 restriction enzymes are available in commerce. Cyanobacteria are often good and interesting resources for the preparation of restriction enzymes. Moreover, cyanobacteria can work as an efficent photobioreactor to produce ATP or glutathione using light as an external energy.[3-6] In fact, intensive and systematic research during the last five years has provided us not only with a great wealth of new information about cyanobacteria but also deeper insights into their molecular biolgy and genetics. So far we have been studying molecualar breeding of *Phormidium lapideum*, a strain of thermophilic and filamentous cyanobacteria. In the lapse of our studies, the information as to the kinds of restriction enzymes existing in *P. lapideum* cells was needed in order to develop a novel host-vector system in the

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cyanobacterium. Here we report a couple of restriction endonucleases isolated from *P. lapideum*, *Pla* I and *Pla* II.

MATERIALS AND METHODS *Phormidium lapideum*, isolated from Matsue hot springs, was grown at 48 °C in 50 liter transparent bath with sterile air bubbling and with illumination from 2 X 2 fluorescent lamps placed at both sides. Enriched hot spring water[7] or Kratz-Meyers's medium[8] was used for the cyanobacterial growth. From an inoculate of 100 ml culture, 8 days were required to collect about 25 g of wet weight cells in the late logarithmic phase from 50 liter medium. *P. lapideum* cells were harvested by filtration, washed with distilled water and then stored at −80 °C until use. Sometimes we were kindly gifted frozen *P. lapideum* cells obtained through mass-cultivation process by DaiNippon Ink Kagakukogyo Co. (Tokyo, Japan). Restriction enzymes, T_4 polynucleotide kinase and relevant enzymes were all kindly gifted from Central Research Institute of Takara Shuzo Co. (Ohtsu, Japan). Chemicals used were of all reagent grade.

In order to obtain restriction enzymes from *P. lapideum*, the cells were processed with 3 cycles of freezing and thawing followed by repeated sonication in three volumes of buffer A (50 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 0.1 mM EDTA, 0.02 % 2-mercaptoethanol, 10 % glycerol). This process was requisite to extract the enzymes out of *P. lapideum* cell membranes. All procedures were done at 0 to 4 °C. For the purpose of molecular weight determination, HPLC with TSK G-3000 SW-XL gel was employed. Proteins were determined by the method of Lowry et al.[13]

Enzyme assays: Samples (1-5 μl) of column fractions were incubated at 37 °C for 1 hr in reaction mixture of 50 μl containing 0.5 μg λ-DNA. Unless otherwise noted, all procedures were directed by protocols of Takara Shuzo Co. After digestion, they were loaded onto 1.0 % agarose slab gel (in the case of *Pla* I) or 0.7 % gel (*Pla* II) in Tris-borate buffer (89 mM Tris-borate, pH 8.3, 2.5 mM EDTA). Electrophoresis was carried out at 100 V for 2 hr. The resulting electrophoregram was checked under UV light in the presence of ethidium bromide. One unit was defined as that amount of enzyme reguired to digest completely 1 μg λ-DNA in 60 min at 37 °C.

RESULTS

1. **Enzyme Purification**: After 3 times treatments of freezing and thawing (see Materials and Methods), the cells were disrupted by repeated sonication (5 min in total) in three volumes of the buffer A. After centrifugation (30,000 X g, 5 min), to the resulting supernatant was added solid ammonium sulfate to 40 % saturation. The mixture was allowed to stand in the cold, then centrifuged and to the supernatant obtained was added solid ammonium sulfate to 70 %. The resulting precipitate was collected by centrifugation, dissolved in the buffer B (10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 0.1 mM EDTA, 0.02 % 2-mercaptoethanol, 10 % glycerol) and then dialyzed against the same buffer. The enzyme solution was applied to a Heparin-Sepharose CL-6 B affinity column and the column was washed completely with 0.1 M

NaCl in the buffer B and then chromatographed with a linear gradient of 0.1 to 0.6 M NaCl in the buffer B to separate *Pla* I and II. It should be noted here that by procedure of the affinity column chromatography the respective enzymes could be free from nuisance pigments, phycobiliproteins.[9] *Pla* I activity eluted between 0.35 and 0.42 M NaCl, *Pla* II between 0.47 and 0.6 M NaCl. Each fractions with *Pla* I and *Pla* II, respectively, were dialyzed overnight against the buffer A. The enzyme solutions were applied to a DEAE-Toyopearl 650 M column and chromatographed with a linear gradient of 0 to 0.4 M NaCl in the buffer A. *Pla* I activity eluted between 0.05 and 0.1 M NaCl, *Pla* II between 0.2 and 0.3 M NaCl. Each fractions with *Pla* I and *Pla* II were pooled, respectively, and concentrated through a centricon 30. *Pla* I and II preparations thus obtained were not homogeneously purified yet, but sufficiently pure for the use of restriction enzyme reaction. No non-specific nuclease activity, as determined by agarose gel electrophoresis, was detected after incubation of 1 μg λ-DNA with around 10 units for 24 hr (over digestion). The enzymes were dissolved in the buffer B containing 20 % glycerol and stored at −20 °C for use.

Table 1. Specificities of restriction enzymes of *P. lapideum*

Enzymes	Number of cleavage sites					Specificities
	lambda	ϕX 174	pBR 322	ColEI	pUC 19	
Pla I	149	11	22	15	11	isoschizomer of *Hae* III
Pla II	7	0	0	2	0	isoschizomer of *Nsp* (7524) V

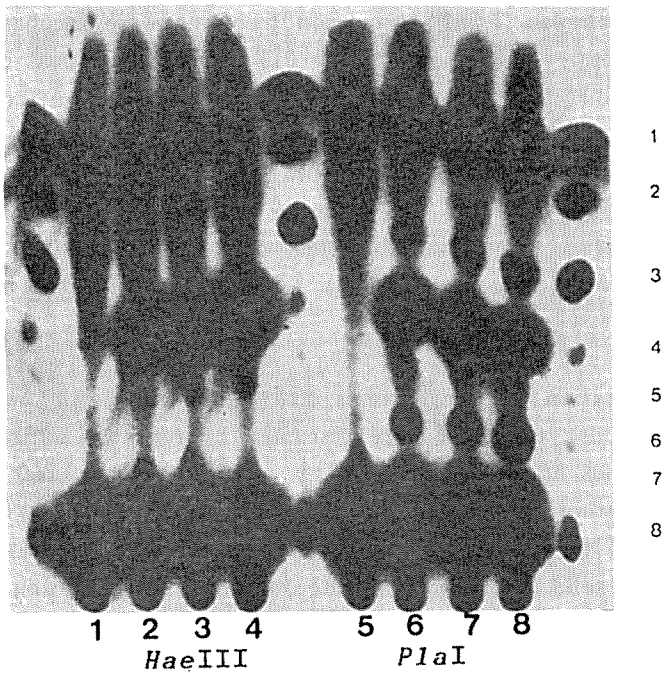


Fig. 1. Autoradiograms of oligonucleotides produced on *Hae* III and *Pla* I digestion. Incubation times were as follows: 1 and 5, 0 min; 2 and 6, 1 min; 3 and 7, 2 min; 4 and 8, 4 min.

About 2400 units of *Pla* I and 350 units of *Pla* II were obtained from 10 g of the frozen cells.

2. **Recognition Sequence of *Pla* I and *Pla* II**: Viral and Plasmid DNAs were digested with the respective enzyme and the resulting restriction fragments were compared with those of known enzymes. The results of analysis are summarized in Table 1.

Pla I and *Pla* II were found to be an isoschizomer of *Hae* III from *Haemophilis aegyptius*,[10] and *Nsp* (7524) V from *Nostoc* species PCC 7524,[12] respectively.

Fig. 1 shows an autoradiograms of oligonucleotides produced on *Hae* III and *Pla* I digestion of a synthetic oligonucleotide ^{32}P-TTGGCCAA.[11] Thus, the cleavage site of *Pla* I was detected to be

5'--GG↓CC--3'
3'--CC↑GG--5',

the same as that of *Hae* III.

One experiment was done in order to detect the cleavage site of *Pla* II: λ-DNA (1 μg) was digested by *Pla* II and the resulting DNA fragments were separated as precipitates by adding ethanol to the reaction mixture. By using the precipitates as the substrate of the ligation reaction (66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP, and 10 units of T_4DNA ligase, at 16 °C, from 0 to 120 min), ligated products were detected as the function of reaction period of time (Fig. 2). With the lapse of time, gradual ligation was observed in the reaction mixture of *Pla* II and of *Nsp* (7524) V, respectively, but not observed of *Pla* I and of *Hae* III. This result indicates the cleavage site by *Pla* II was a type of "cohesive" ends and probably the same as that of *Nsp* (7524) V, 5'--TT↓CGAA--3'
3'--AAGC↑TT--5', (see Discussion).

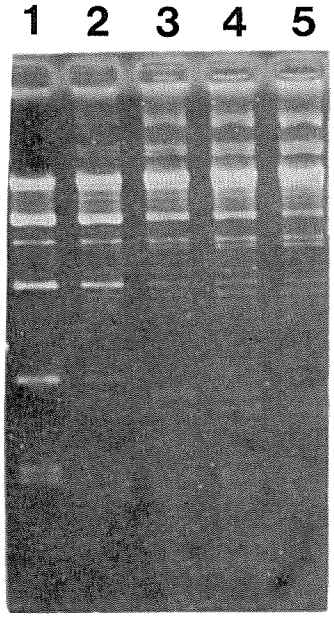


Fig. 2. Ligated patterns of *Pla* II fragments.
Ligation times were as follows: 1, 0 min; 2, 15 min; 3, 30 min; 4, 60 min; 5, 120 min.

3. **Molecular Weight and Subunits**: The molecular weights of the enzymes, *Pla* I and *Pla* II, were estimated as 40 kilodaltens (*Pla* I) and 176 kilodaltons (*Pla* II), respectively, from the results of TSK G-3000 SW-XL gel filtration as shown in Fig. 3A. SDS polyacrylamide gel electrophoresis for *Pla* I gave a single protein band, while the electrophoresis for *Pla* II showed that *Pla* II molecule consisted of 2 kinds of subunits corresponding to molecular weight of approximately 63 kilodaltons (α)

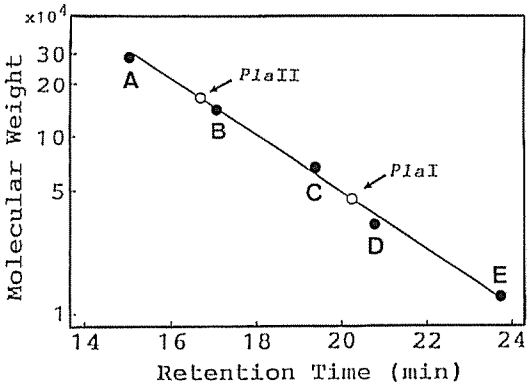


Fig. 3 A. Molecular weight measurement by HPLC. See the text for details. A, Glutamate dehydrogenase (290,000); B, Lactate dehydrogenase (142,000); C, Enolase (67,000); D, Adenylate kinase (32,000); E, Cytochrome c (12,400).

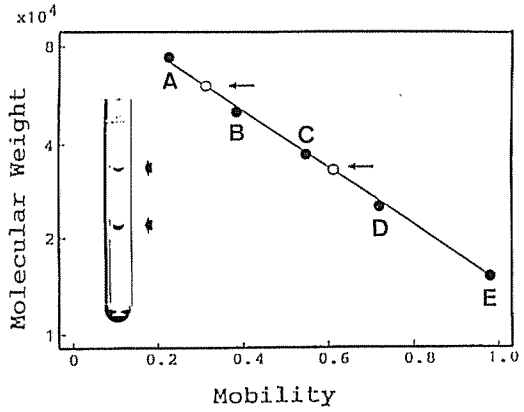


Fig. 3B. Molecular weight measurement and SDS polyacrylamide gel electrophoresis (inset) of the purified *Pla* II.
A, Cytochrome c hexamer (74,400); B, Tetramer (49,600); C, Trimer (37,200); D, Dimer (24,800); E, Monomer (12,400).

and 31 kilodaltons (β), respectively (Fig. 3B). These results show that *Pla* II has an apparent molecular mass of 176 kilodaltons and is a novel heterotetrameric protein, $\alpha_2\beta_2$. This is so far a unique circumstance as for the type II restriction endonucleases.

4. **Effect of NaCl and KCl on the Enzyme Activity**: Restriction enzyme, in general, requires the presence of NaCl or KCl in order to express its maximum activity. We investigated the effect of NaCl and KCl concentration on *Pla* I, *Pla* II and their respective isoschizomers (table 2). As for *Pla* I, the optimum concentration of NaCl was found between 0 and 40 mM, and in the presence of 100 mM NaCl *Pla* I activity was completely inhibited. Contrary yet, *Hae* III, an isoschizomer of *Pla* I, needed 40 to 100 mM NaCl for the maximum activity. This situation may be an interesting problem from the viewpoint of comparative protein stereochemistsry. *Pla* II's behavior to NaCl concentration was similar to that of *Nsp* (7524) V: optimum was found between 0 to 60 mM. *Pla* I, *Pla* II and their isoschizomers all disliked the presence of KCl.

5. **Thermal Properties and pH Profiles**: By using the enzyme solution preincubated at various temperature (37 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C) for 10 min, the enzyme reaction was performed for 30 min, respectively, at the same temperature employed at the preincubation. Thus resulting electrophoregram was observed. Table 2 shows optimum reaction conditions of *Pla* I, *Pla* II and their respective isoschizomers. *Pla* I and *Pla* II, both obtained from thermophilic cyanobacteria, showed a little more heat-tolerancy than their isoschizomers. For pH profile determination, the similar procedures were used. Table 2 also shows the effects of pH on the enzyme activities.

Table 2. Parameters of optimal reaction conditions of *Pla* I, *Hae* III, *Pla* II and *Nsp* (7524) V

	Pla I	*Hae* III	*Pla* II	*Nsp* (7524) V
NaCl(mM)	0-40	40-100	0-60	40
KCl(mM)	0	0	0	0
Temperature(°C)	45-55	37	45-55	37
pH	7.5-8.0	7.5	8.0-8.5	8.0

6. Fffect of Divalent Cations and Sulfhydryl Reagents: Seven milimolar $MgCl_2$ was needed for activity expression of the restriction endonucleases as usual. However, the activities of *Pla* I and *Pla* II were expressed with 5 mM Mn^{2+} cation in place of Mg^{2+} ion without any expression of star activity.[15] On the other hand, an isoschizomer of *Pla* II, *Nsp* (7524) V did not work with any metal ion when Mg^{2+} ion was absent. Sometimes in the presence of 5 mM $MnCl_2$, *Pla* I preparation gave a clearer restriction pattern on slab gel electrophoregram than that with $MgCl_2$, probably because here contaminating other endonuclease (s) could not work with Mn^{2+}. Interestingly in addition, *Pla* I worked a little even in the presence of 0.1 mM $BaCl_2$ without any $MgCl_2$. In fact, when Mg^{2+} ion was present, $CaCl_2$ and $BaCl_2$ did not prevent the enzymic reaction of *Pla* I and of *Pla* II, respectively. Recently we have found that activities of both DNA polymerase (P pol I) and RNA polymerase, which were purified from *P. lapideum* cells, were more than doubled in the presence of Ba^{2+} ion. Moreover, growth of *P. lapideum* was also encouraged by 2 fold in the presence of barium acetate in the culture medium.[14] These are unique circumstances as compared to the general experience with other living things. Other metalic ions were all inhibitory to the enzyme reactions of *Pla* I and *Pla* II (table 3).

Sulfhydryl reagent (0.5 mM each) was added to the reaction mixture in the absence of DTT or 2-mercaptoethanol. The results are shown in table 3. Mercuric chloride strongly inhibited *Pla* I, *Pla* II and their respective isoschizomers at all. PCMB and DTNB inhibited *Pla* II and *Nsp* (7524) V but not *Pla* I and *Hae* III. Iodoacetate and NEM of such a concentration as 0.5 mM did not exerted any effect on *Pla* I, *Pla* II and their isoschizomers. Consequently, *Pla* II, an oligomeric enzyme was more sensitive to sulfhydryl reagents than a monomeric protein, *Pla* I.

DISCUSSION

A couple of restriction endonucleases, *Pla* I and *Pla* II, were purified from *Phormidium lapideum* cells, a strain of thermophilic and filamentous cyanobacteria isolated from Matsue hot springs. *Pla* I and *Pla* II were both supposed to be membrane-bound enzymes because it was difficult to extract the enzymes by simple sonication or homogenization of the cells. We identified *Pla* I to be an isoschizomer of a known restriction enzyme *Hae* III (table 1). Both recognition site and cleavage site of *Pla* I were detected to show the same as those of *Hae* III (Fig. 1). *Pla* I has

Table 3. Inhibitory effect of divalent cations and sulfhydryl reagents on *Pla* I, *Hae* III, *Pla* II and *Nsp* (7524) V in the presence of 7 mM $MgCl_2$

Cations*	*Pla* I,	*Hae* III	*Pla* II,	*Nsp* (7524) V
Mn	−	±	−	+
Co	+(0.5)	−	+(2)	+(0.1)
Ni	±	+(0.5)	+(0.5)	+(0.5)
Ca	−	−	−	+(0.1)
Ba	−	−	−	+(0.1)
Zn	+(0.5)	+(0.1)	+(0.1)	+(0.1)
Cu	+(0.5)	+(0.1)	+(0.1)	+(0.1)
Cd	+(0.5)	+(0.1)	+(0.1)	+(0.1)
Hg**	+	+	+	+
Reagents**				
NEM	−	−	−	−
Iodoacetate	−	−	−	−
PCMB	−	−	+	+
DTNB	−	−	+	+

*A variety of concentrations between 0 and 5 mM were used to test the effect. The figure in parenthesis shows minimum concentration (mM) to inhibit the enzyme reaction.
+ : inhibitory, − : not inhibitory.
** The concentration used was 0.5 mM.

a molecular weight of about 40 kilodaltons, and was estimated to be a monomeric protein (Fig. 3). *Pla* I was rather unstable on storage even in the presence of 20 % glycerol at −20 °C. The presence of 50 % glycerol may be necessary for keeping the activity.

Pla II was an isoschizomer of *Nsp* (7524) V that was isolated from a filamentous cyanobacterium, *Nostoc* species PCC 7524. At the moment were present seven isoschizomers of *Nsp* (7524) V including *Asu* II (isolated from *Anabaena subcylindrica*)[16] and *Mla* I (from *Mastigocladus laminosus*)[17], all of which recognize the following hexanucleotide sequence of TT↓CGAA and break the site indicated by arrow. *Nostoc* species, *A. subcyrindrica* and *M. laminosus* are all filamentous cyanobacteria. Thus, we suppose that *Pla* II also recognize the same hexanucleotide sequence and introduce cleavage at the indicated position. This idea should be also supported by the results of ligation experiments described above (Fig. 2), because it is known that unless the cleavage site is a type of "cohesive" ends the ligating reaction could not be performed under such reaction conditions as employed in our experiments. We found *Pla* II as an oligomeric protein ($\alpha_2\beta_2$) having an apparent molecular mass of 176 kilodaltons. α subunit was estimated as 63 kilodaltons and β was 31 kilodaltons. In comparison with a monomeric protein *Pla* I, *Pla* II was more sensitive to such sulfhydryl reagents as PCMB and DTNB. Such tendency as *Pla* II is the case of oligomeric protein in general. Nothing is reported so far about oligomeric type II restriction

endonucleases. Therefore, it is a new finding that *Pla* II is a heterotetrameric enzyme and it is an important problem to know functions of each α and β subunit in the recognition as well as cleavage reaction on DNA strands. Now that we have had informations about *Pla* I and *Pla* II, we believe that molecular breeding experiments on *P. lapideum* should be performed in near future.

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摘　　要

糸状性，好温性ラン藻 *Phormidium lapideum* より2種類の制限酵素 *Pla* I, *Pla* II を精製しその性質を調べた．塩基配列既知の λDNA, Col EI, pBR 322, φX 174 などに対する制限パターンの検討結果，及び合成 DNA－ホモクロマト法，ligation 実験の結果などからそれぞれに，*Pla* I は *Hae* III, *Pla* II は *Nsp* (7524) V のIsoschizomer であると判断された．ゲル濾過および SDS ゲル電気泳動による分子量測定の結果，*Pla* I は分子量 40,000のモノマーであった．一方，*Pla* II は分子量 176,000で，サブユニット分子量63,000と31,000のそれぞれ2個づつよりなる新規なヘテロテトラマーであると推定された．さらにそれぞれの Isoschizomer 間に於ける酵素化学的挙動についての比較研究を行い，本ラン藻制限酵素の特性，意義について考察した．