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The secondary electron acceptor of photosystem I in *Gloeobacter violaceus* PCC 7421 is menaquinone-4 that is synthesized by a unique but unknown pathway.

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Abstract

The secondary electron acceptor of photosystem (PS) I in the cyanobacterium *Gloeobacter violaceus* PCC 7421 was identified as menaquinone-4 (MQ-4) by comparing high performance liquid chromatograms and absorption spectra with an authentic compound. The MQ-4 content was estimated to be two molecules per one molecule of chlorophyll (Chl) *a'*, a constituent of P700. Comparative genomic analyses showed that six of eight *men* genes, encoding phylloquinone/MQ biosynthetic enzymes, are missing from the *G. violaceus* genome. Since *G. violaceus* clearly synthesizes MQ-4, the combined results indicate that this cyanobacterium must have a novel pathway for the synthesis of 1,4-dihydroxy-2-naphthoic acid.

Key words: Electron acceptor, Menaquinone, Photosynthesis, Photosystem I, *Gloeobacter violaceus* PCC 7421.

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Abbreviations: Chl, chlorophyll; DHNA, 1,4-dihydroxy-2-naphthoic acid; HPLC, high performance liquid chromatography; MQ, menaquinone; P700, the primary electron donor of photosystem I; PhQ, phylloquinone; PQ, plastoquinone; PS, photosystem.

Introduction

Gloeobacter violaceus PCC 7421 (hereafter referred to as *Gloeobacter*) is an early-diverging cyanobacterium in phylogenetic trees based on 16S rRNA sequences [1]. This organism shows several unique properties; for example, it lacks thylakoid membranes in cytoplasm [2], and thus the photosynthetic system is localized on the cytoplasmic membrane where the respiratory system is also present. These two energy-fueling systems are interconnected by a pool of plastoquinone (PQ) molecules, which serves as the lipid-soluble mobile electron carrier in these systems [3]. The evolutionary uniqueness of *Gloeobacter* prompted us to investigate the structure and function of photosystem (PS) I in this organism in further detail.

In previous studies we investigated the subunit composition of PS I of *Gloeobacter*. On the basis of the whole-genome sequencing analyses and biochemical approaches, we discovered that *Gloeobacter* PS I consists of eight subunits that are conserved among cyanobacteria [4] as well as a novel subunit (PsaZ) [5], whose function is yet to be characterized. The P700/chlorophyll (Chl) *a* ratio in isolated PS I complexes was approximately 100, similar to that found in other cyanobacteria [5]. To extend our knowledge of *Gloeobacter* PS I, the biosynthesis of phylloquinone (PhQ), the secondary electron acceptor of PS I and designated as A_1, was investigated. Surprisingly, a genome-wide survey revealed that all but the genes required for the last two enzymatic steps in PhQ biosynthesis were missing in the *Gloeobacter* genome [4,6]. When PhQ is not available for PS I assembly, others have shown that PQ is incorporated into PS I, but the electron transport properties of such PS I complexes are modified [7]. Here we demonstrate that menaquinone-4 (MQ-4) (Fig. 1A) and not the more typical PhQ (Fig. 1B) [8,9] is synthesized and functions as A_1 in *Gloeobacter* PS I. On the basis of our findings, we conclude

that a unique pathway for the synthesis of 1,4-dihydroxy-2-naphthoic acid (DHNA) is present in this early-diverging cyanobacterium.

Materials and Methods

Cyanobacterial cultures and preparation of PS I complexes: *Gloeobacter violaceus* PCC 7421 and *Synechocystis* sp. PCC 6803 were grown photosynthetically in BG11 medium, and trimeric PS I complexes were isolated as described previously [5].

HPLC analysis: Pigments were extracted by previously described procedures [10], injected onto a reversed-phase HPLC column (Kaseisorb LC-ODS 2000-3, 250 × 4.6 mm), and eluted isocratically with a degassed eluent of ethanol/water/2-propanol (100/4/1, v/v/v) (at a flow rate of 0.40 ml/min). Eluates were monitored with a JASCO UV-2070 detector (λ = 248 nm) and a Shimadzu multi-wavelength detector (SPD-M10A) in series. The amounts of MQ-4, PhQ, Chl *a* and Chl *a′* were determined by integrating the peak areas of HPLC chromatograms monitored at 248 nm and by using the molar extinction coefficients of individual compounds. The detection system had been calibrated several times with known amounts of authentic Chl *a*, Chl *a*', MQ-4 and PhQ. MQ-4, MQ-7 (Fig. 1C), and PhQ were purchased from Wako pure chemicals (Osaka, Japan). The contents of Chl *a* and Chl *a'* were also estimated with normal-phase HPLC as previously described [10].

Results

Genome survey of PhQ biosynthetic genes: On the basis of the structural similarity between PhQ and MQ, it is believed that PhQ biosynthesis in cyanobacteria occurs through an 8-step pathway similar to that for MQ-7 in *Escherichia coli* [11-13]. Consistent with the important

role of PhQ in PS I, the genes for the enzymes of this pathway, designated as *menA* through *menH*, are highly conserved in most cyanobacterial genomes sequenced to present [11,12]. However, comprehensive genome comparisons between *Gloeobacter* and other cyanobacteria as well as *E. coli* revealed that only two *men* genes, *menA* and *menG*, are encoded in the genome of *Gloeobacter* [4]. These genes encode DHNA phytyltransferase and demethylphylloquinone methyltransferase, respectively, which catalyze the last two steps of PhQ biosynthesis [6,14]. The remaining six *men* genes (*menF*, *menD*, *menC*, *menE*, *menB* and *orf241/menH*) [6,12-14] were not detectable in the *Gloeobacter* genome. These data suggest that PhQ is not synthesized at all in *Gloeobacter* or that an alternative pathway for the synthesis of DHNA might exist in this cyanobacterium.

Determination of quinone molecules in PS I complexes: To determine whether *Gloeobacter* can synthesize PhQ, we investigated the quinone species in whole cells and purified PS I complexes by reversed-phase HPLC analyses. Three authentic quinones, PhQ, MQ-4, and MQ-7, were used as standards as well as solvent extracts from the purified PS I complexes of the cyanobacterium *Synechocystis* sp. PCC 6803 and the red alga *Cyanidium caldarium*. The retention times for the authentic quinones were 38 min, 24 min, and 68 min, respectively (Fig. 2C). *Synechocystis* sp. PCC 6803 and *C. caldarium* contains PhQ and MQ-4 as the secondary electron acceptors, respectively [15]. When solvent extracts from *Gloeobacter* PS I complexes were analyzed, no peak was detectable at retention time for PhQ (38 min), but instead a peak was observed at 24 min (Fig. 2A). This retention time was identical to that of authentic MQ-4 (Fig. 2C) and that of MQ-4 isolated from *C. caldarium* (data not shown). It is noteworthy that no peak corresponding to MQ-7 was observed in *Gloeobacter*. These data suggest that *Gloeobacter* PS I

complexes contain MQ-4 instead of PhQ.

The absorption spectrum of the quinone isolated from the *Gloeobacter* PS I complexes (Fig. 3A) was compared with the spectra of the authentic samples (Fig. 3). These spectra coincided very well, with all having absorption maxima at 248 nm, 270 nm, and 332 nm. These data are expected since MQ-4 and PhQ have the same aromatic functional group (Fig. 1A vs 1B). PhQ isolated from *Synechocystis* sp. PCC 6803 and MQ-4 isolated from *C. caldarium* also had the same absorption spectra (data not shown). Mass spectrometric analyses of the quinones in whole-cell extracts from *Synechocystis* sp. PCC 6803 and *Gloeobacter* also showed that *Gloeobacter* lacks any quinoid compound with the HPLC elution properties and a m/*z* ratio of 450 characteristic of PhQ. Instead, a faster eluting component with absorption properties identical to PhQ but with a m/*z* ratio of 444, characteristic of MQ-4, was observed (data not shown). Based on all of these observations, it is concluded that *Gloeobacter* synthesizes MQ-4 instead of PhQ.

Stoichiometry and function of MQ-4 in PS I complexes: The stoichiometries of MQ-4/P700 and PhQ/P700 in PS I complexes isolated from *Gloeobacter* and *Synechocystis* sp. PCC 6803, respectively, were estimated by using Chl *a'* as an internal standard, because Chl *a'* is known to be present in the ratio of one molecule per one P700 [16]. The Chl *a'* peak was well resolved and clearly detectable in both *Gloeobacter* and *Synechocystis* sp. PCC 6803 (Fig. 2). The Chl *a'* content in PS I complexes was determined to be one per 91.7 ± 0.8 Chl *a* and 88.5 ± 1.5 Chl *a* for *Gloeobacter* and *Synechocystis* sp. PCC 6803, respectively. The stoichiometry of MQ-4/Chl *a'* in *Gloeobacter* was 2.00 ± 0.10, and the PhQ/Chl *a'* ratio in *Synechocystis* sp. PCC 6803 was 1.94 ± 0.13. Thus, the quinone/P700 ratio is almost equal to 2.0 for the PS I complexes

of both cyanobacteria. Validating the methods used here, the value obtained for *Synechocystis* sp. PCC 6803 is consistent with previous results for PS I complexes of this organism [17] and spinach chloroplasts [9], which contain two molecules of PhQ as A_1.

Under light-saturating conditions (2,600 μE/(m^2•s)), the electron transfer activities of PS I complexes as measured by the Mehler reaction were found to be 360 ± 35 and 750 ± 70 μmole O_2/((mg Chl *a*)•h) for *Gloeobacter* and *Synechocystis* sp. PCC 6803, respectively. Under light-limiting conditions (130 μE/(m^2•s)), the activities were 160 and 240 μmole O_2/((mg Chl *a*)•h), respectively. If MQ-4 was merely loosely bound to the PS I complexes as a contaminant, and if no other quinone were present to function as A_1, the overall electron transfer activity would be expected to be much lower than that observed. Therefore, we conclude that *Gloeobacter* PS I complexes contain two MQ-4 molecules and that MQ-4 functions as A_1.

Discussion

Presence of quinone molecules: In most oxygenic photosynthetic organisms PhQ is the secondary electron acceptor of PS I [7-9]. Since electron transfer from A_0 (Chl *a*) to PhQ is very rapid [18], the charge-separated state between $P700^+$ and PhQ^- is stably formed, and charge recombination is generally not observed in PS I, leading to the absence of delayed fluorescence in PS I [19]. *Gloeobacter* cells and their isolated PS I complexes showed no delayed fluorescence even at 77 K (data not shown). Therefore, it seemed unlikely that the PS I complexes of *Gloeobacter* lacked the A_1 even if the genes encoding the biosynthetic enzymes of PhQ were not identifiable in its genome. It is demonstrated here that MQ-4, with a stoichiometry of two molecules per one Chl *a',* was present in the PS I particles of *Gloeobacter.*

Distribution of MQ-4 biosynthesis in oxygenic phototrophs: Five genes (*menA*, *menB*, *menD*, *menE* and *menG*) out of eight genes have been directly shown to encode for enzymes in PhQ biosynthesis in *Synechocystis* sp. PCC 6803 [6,11-13]. The involvement of *menB*, *menF*, and *menG* in MQ-4 biosynthetic pathway was also confirmed in *Synechococcus* sp. PCC 7002 [11,12]. Thus, the PhQ and MQ-4 biosynthetic pathways in cyanobacteria follow the same enzymatic steps as the MQ biosynthetic pathway in *E. coli*. As shown in this study, MQ-4 also replaces PhQ in the PS I complexes of *Gloeobacter*. Together these observations indicate that the pathway that leads to the synthesis of MQ-4 in *Gloeobacter* must be unique. Because the missing genes are required for the synthesis of the intermediate DHNA, we hypothesize that *Gloeobacter* has a novel pathway leading to the synthesis of DHNA and that it uses its conserved MenA and MenG activities to convert DHNA to MQ-4 as in other cyanobacteria.

MQ-4 is the secondary electron acceptor in the photosynthetic reaction centers of a few species: for example, in the Type II reaction centers of a small number of purple sulfur bacteria [20] and in the type I reaction centers of the red alga *C. caldarium* [15]. Recently the cyanobacterium *Synechococcus* sp. PCC 7002 was also found to synthesize MQ-4 and to utilize it instead of PhQ as A_1 [21]. Given the widespread occurrence of MQ biosynthesis in bacteria, it is logical to assume that PhQ biosynthesis evolved from MQ biosynthesis. The fact that *Gloeobacter* and a few other oxygenic phototrophs synthesize MQ-4 leads us to speculate that early cyanobacteria synthesized MQ and that PhQ biosynthesis developed later from MQ biosynthesis during the evolution of cyanobacteria. This is consistent with the fact that PhQ biosynthesis is restricted to oxygenic phototrophs. If this is true, one can imagine that MQ-4 synthesis and utilization in PS I may be more common and widely occurring among oxygenic phototrophs than currently established. A systematic analysis of diverse cyanobacteria and

eukaryotic algae is required in order to test this hypothesis.

Acknowledgments

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photosystem I. Characterization of a *menB rubA* double deletion mutant in *Synechococcus* sp. PCC 7002 devoid of F_X, F_A, and F_B and containing plastoquinone or exchanged 9,10-anthraquinone. *J. Biol. Chem.* 280, 12371-12381.

Figure legends

Fig. 1 Molecular structures of (A) MQ-4, (B) PhQ, and (C) MQ-7.

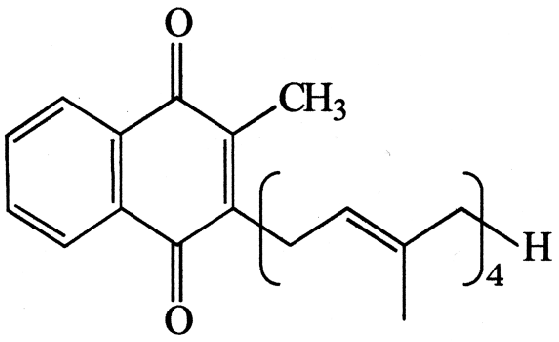
Fig. 2. HPLC elution profiles of extracts of PS I complexes.

(A) Extracts from PS I complexes of *Gloeobacter*, (B) PS I complexes of *Synechocystis* sp. PCC 6803, and (C) a mixture of authentic MQ-4, PhQ, and MQ-7. Detection wavelength was 248 nm.

Fig. 3. Absorption spectra of quinone molecules on HPLC.

(A) Extracts of *Gloeobacter*, (B) authentic MQ-4, (B) authentic MQ-7, and (C) authentic PhQ.

(A)



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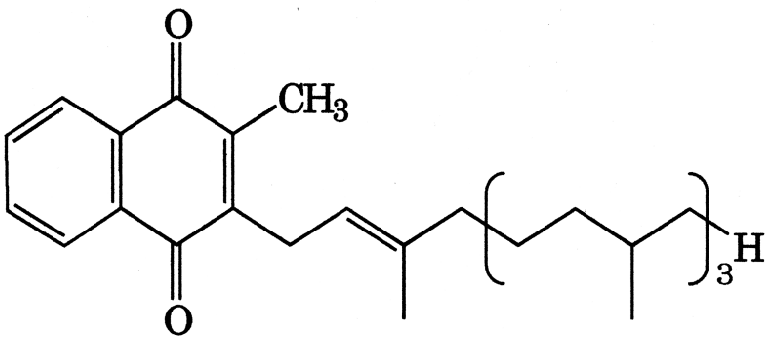

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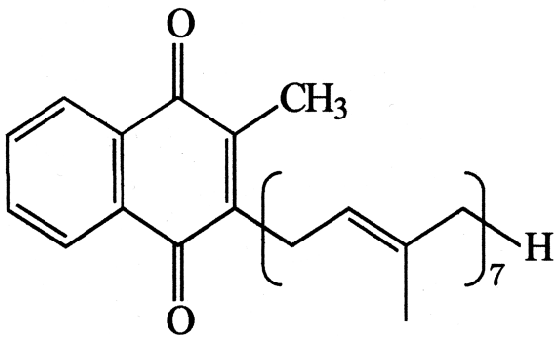
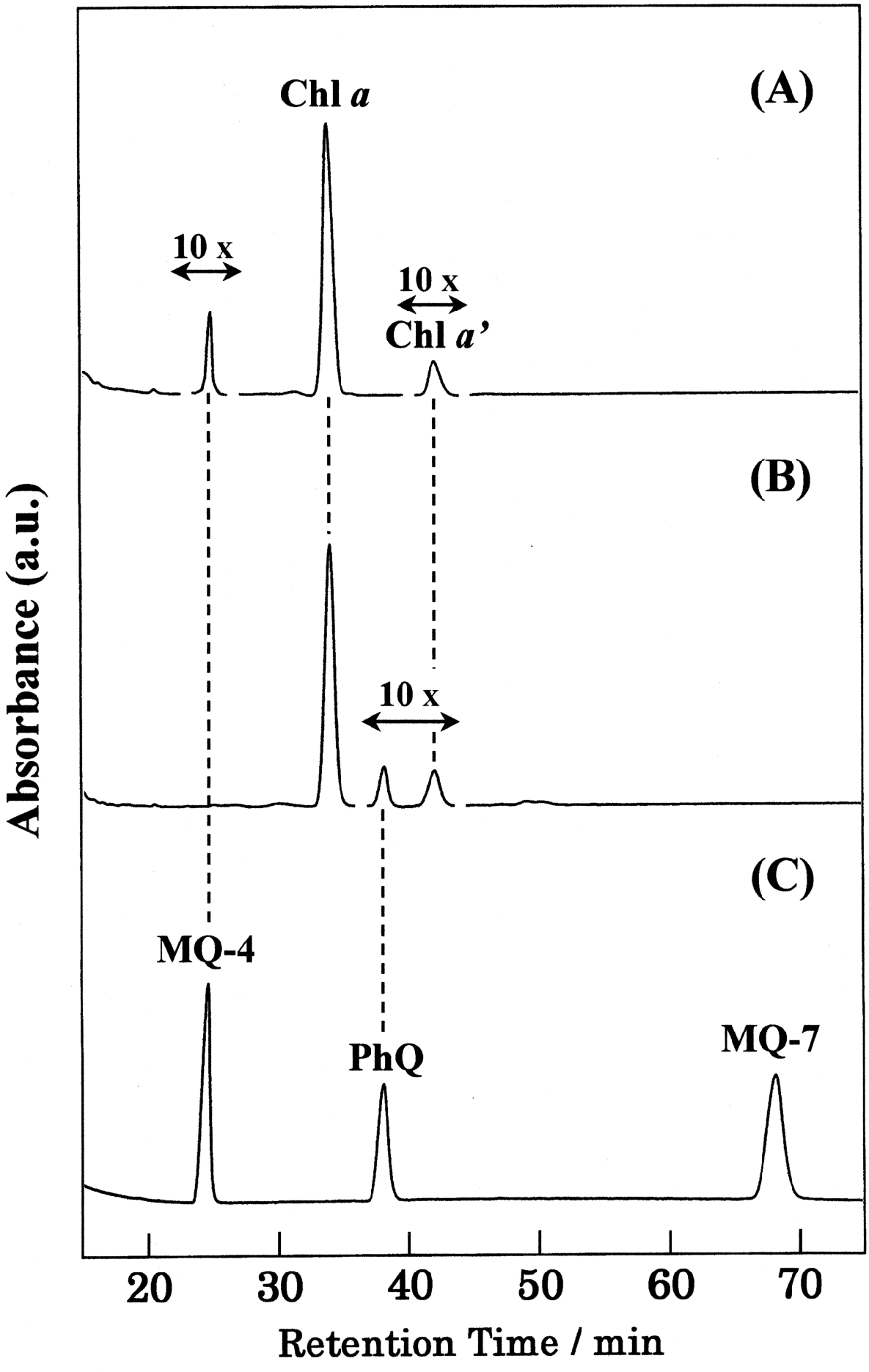
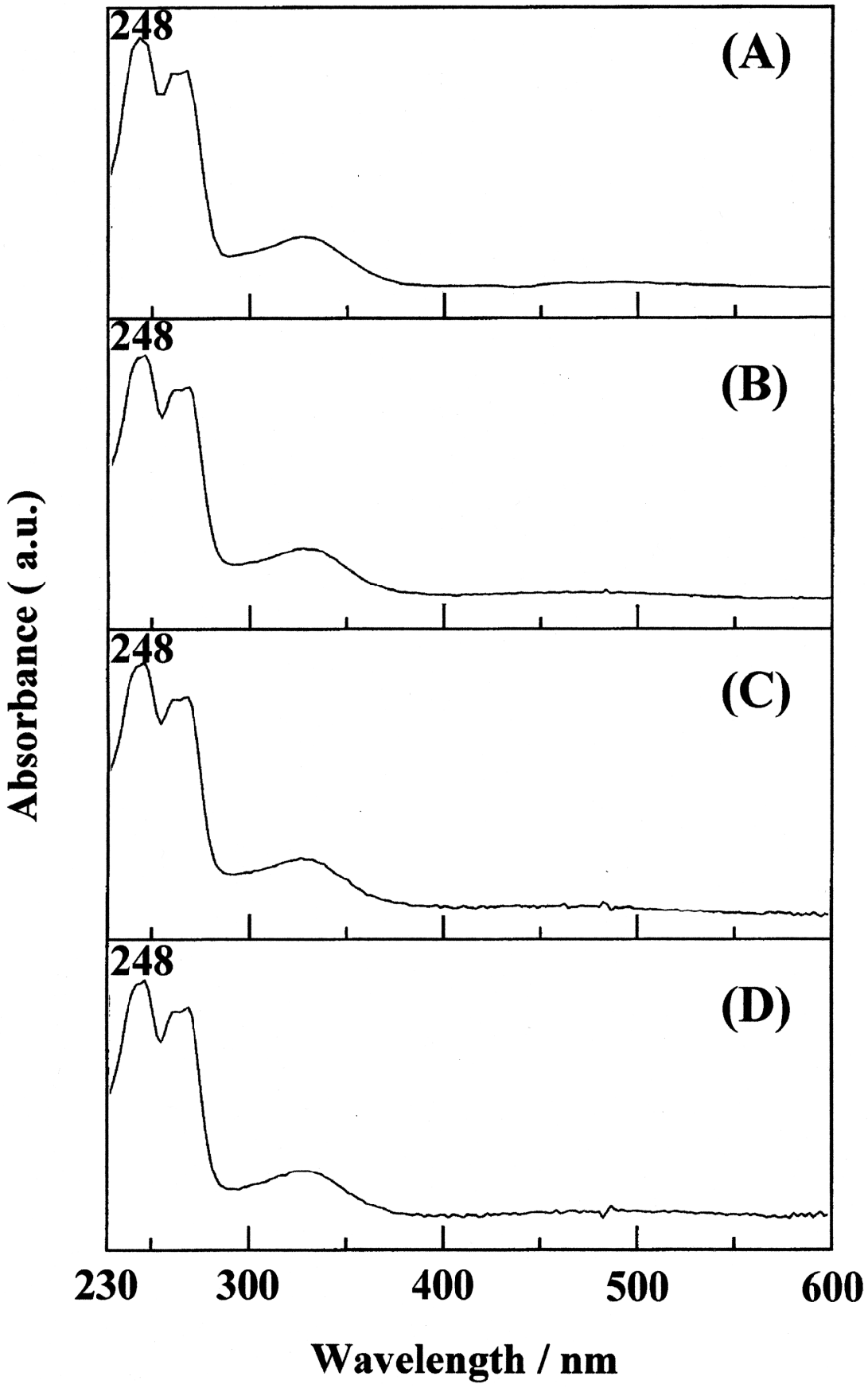


Mimuro et al. Fig. 1



Mimuro et al. Fig. 2



Mimuro et al. Fig. 3