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Functional Analysis of Ribonucleotide Reductase from *Cordyceps militaris* Expressed in *Escherichia coli*

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Abstract *Cordyceps militaris* produces cordycepin (3’-deoxyadenosine), which has various activities, including anti-oxidant, anti-tumoral, anti-viral and anti-inflammatory. Ribonucleotide reductase (RNR) seems to be a candidate to produce cordycepin in *C. militaris* because RNR catalyzes the reduction of nucleotides to 2’-deoxynucleotides whose structures are similar to that of cordycepin. However, the role of RNR has not been confirmed yet. In this study, cDNAs of *C. militaris* RNR (CmRNR) large and small subunits (CmR1 and CmR2) were cloned from *C. militaris* NBRC9787 to investigate the function of CmRNR for its cordycepin production. *C. militaris* NBRC9787 began to produce cordycepin when grown in a liquid surface culture in medium composed of glucose and yeast extract for 15 days. CmR1 cDNA and CmR2 cDNA were obtained from its genomic DNA and from total RNA extracted from its mycelia after cultivation for 21 days, respectively. Recombinant CmR1 and CmR2 were expressed individually in *Escherichia coli* and purified. Purified recombinant CmR1 and CmR2 showed RNR activity toward adenosine diphosphate (ADP) only when two subunits were mixed but only show the reduction of ADP to 2’-deoxyADP. These results indicate that the pathway from ADP to 3’deoxy ADP via CmRNR does not exist in *C. militaris* and cordycepin production in *C. militaris* may be mediated by other enzymes.

Keywords: *Cordyceps militaris* · Cordycepin · 2’-Deoxyadenosine · Ribonucleotide reductase · Chaperone

Introduction

Mushrooms have attracted attention as a source of biologically active compounds with various activities, such as anti-oxidant, anti-tumoral, anti-viral and anti-inflammatory activities [1]. There are more than 2000 species in nature, and most edible mushrooms seem to be medicinal. The *Cordyceps* genus includes more than 600 species and is known as a type of medicinal mushroom. In particular, *Cordyceps militaris* has been used as a tonic medicine and food in East Asia [2]. Nucleosides are among the major compounds in *C. militaris*, and more than 20 nucleosides and their analogues have been isolated from *C. militaris*. Cordycepin (3'-deoxyadenosine), one of the bioactive compounds in *C. militaris*, is a nucleoside analogue and exhibits various activities in cells, participating in apoptosis, cell cycle signaling, nucleic acid synthesis, metastasis and inflammation [3,4]. Cordycepin was isolated first from *C. militaris* culture and also from the *C. militaris* fruiting body [5]. In the case of *C. militaris* culture, most cordycepin is produced in a culture medium [3].

Recently, to investigate the cordycepin production pathway and fruiting body development of *Cordyceps*, various 'omics' analyses of *C. militaris* and *Ophiocordyceps sinensis* were performed [6–8]. Cordycepin is an adenosine analogue and adenosine lacking 3'-OH group. Cordycepin might be produced in *C. militaris* from adenosine, adenosine monophosphate (AMP) or adenosine diphosphate (ADP) through its purine biosynthetic pathway in *C. militaris* (Scheme 1) [9]. Especially, the RNR seems to be involved in cordycepin production through the similar reduction to the production of 2'-deoxyribonucleotides because ribonucleotide reductase (RNR)

catalyzes the synthesis of 2'-deoxyribonucleotides from ribonucleotides [10]. However, this process has not yet been verified.

In this study, we investigated whether RNR from *C. militaris* is involved in cordycepin production in vitro. Most RNRs are composed of large and small subunits (R1 and R2) [11]. Putative R1 and R2 genes (CmR1 and CmR2) were found in *C. militaris* CM01 [6]. In order to investigate whether RNR from *C. militaris* is involved in cordycepin production in vitro, the R1 and R2 genes from *C. militaris* NBRC9787 were cloned by reverse transcription polymerase chain reaction (RT-PCR) and genomic DNA, respectively. Then, both genes were expressed in *Escherichia coli* and an assay of cordycepin synthesis was also performed using purified RNR.

Materials and Methods

Cultivation of *C. militaris*

C. militaris NBRC9787 was purchased from the Biological Resource Center in the National Institute of Technology and Evaluation (Tokyo, Japan). *C. militaris* NBRC9787 was maintained on potato-dextrose agarose medium (Nissui Pharmaceutical, Tokyo, Japan). For cordycepin production, *C. militaris* NBRC9787 was cultivated statically in medium A (20 g/L glucose, 7.5 g/L yeast extract, 2.5 g/L peptone, 1/10 Vogel's medium, pH 5.0) or medium B (62.6 g/L glucose, 72.5 g/L yeast extract, 1/10 Vogel's medium, pH 5.0).

Cloning of R1 and R2 genes

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valenica, CA, USA) from the mycelia of *C. militaris* NBRC9787 grown in each medium for 21 days. The extracted total RNA was treated with DNase I (Thermo Fisher Scientific K.K., Yokohama, Japan) in the presence of RNase inhibitor (Thermo Fisher Scientific K.K.), followed by the inactivation of DNase I by heating. The cDNA of *C. militaris* NBRC9787 was synthesized using PrimeScript Reverse Transcriptase (TAKARA BIO, Shiga, Japan) using oligo dT primer and random 6-mers primer. The CmR2 subunit cDNA was amplified by PCR using the *C. militaris* NBRC9787 cDNA and the primer set CmR2-F and CmR2-R (Table 1).

CmR1 cDNA was cloned from the genomic DNA of *C. militaris* NBRC9787. Its genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen K. K., Tokyo, Japan), because it was not obtained by RT-PCR. The gene has three exons (exon 1, 2 and 3) and two introns. The lengths of exons 1, 2 and 3 are 19, 158 and 2538 bp, respectively. First, exon 2 was amplified by PCR on the *C. militaris* NBRC9787 genomic DNA with primers CmR1-F1 and CmR1-R1. CmR1-F1 includes the sequence of exon 1 and the 5’ end of exon 2. CmR1-R1 includes the sequence of the 3’ end of exon 2 and the 5’ end of exon 3. Next, exon 3 was amplified by PCR using the *C. militaris* NBRC9787 genomic DNA and primer set CmR1-F2 and CmR1-R2 (Table 1). CmR1-F2 includes the 3’ end sequence of exon 2 and the 5’ end sequence of exon 3, and CmR1-R2 includes the 3’ end sequence of exon 3. Finally, the CmR1 cDNA was amplified by overlap extension PCR on the PCR-amplified exon 1–2 and 3 fragments using two primers, CmR1-F1 and CmR1-R2 (Table 1).

Each cDNA was cloned separately into the pCold I vector (TAKARA BIO) and its sequence confirmed. The resulting vectors (pCold-CmR1 and pCold-CmR2) were used for recombinant protein expression in *E. coli*.

Expression of CmR1 and CmR2 in *E. coli*

The vectors pCold-CmR1 and -CmR2 were transformed individually into *E. coli* BL21 (DE3). Each transformant was cultivated in LB medium until the optical density (OD) reached 0.6, followed by cooling on ice. After adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium, the transformant was cultivated again at 15°C for 24 h.

For the co-expression of CmR1 with various chaperones, a chaperone plasmid set (TAKARA BIO) was used. This chaperone plasmid set includes five plasmids (Table 2) containing pACYC ori, which is different from ColE1 ori in pCold, for chaperone expression in *E. coli*. Each chaperone expression plasmid was transformed into a transformant harboring pCold-CmR1. The transformant harboring two plasmids was cultivated in LB medium supplemented with arabinose and IPTG at 15°C to achieve co-expression of CmR1 with each chaperone.

SDS-PAGE and Western Blot

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% or 12% acrylamide and subsequently subjected to western blotting. After SDS-PAGE, the proteins were blotted onto a polyvinylidene fluoride

(PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After blocking in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST, pH 7.6), the membrane was incubated in 10,000-fold diluted monoclonal anti-polyhistidine-peroxidase antibody produced in mouse (Sigma Aldrich Japan, Tokyo, Japan). Detection was performed using ECL Plus Western blotting reagent (GE Healthcare Japan, Tokyo, Japan). Specific bands were detected on a Fluor-S MAX MultiImager (Bio-Rad).

Purification of Recombinant Subunit

E. coli cells were suspended with phosphate-buffered saline (pH 7.4) containing 5 mM 2-mercaptoethanol (2-ME) and 0.1% Triton X-100, then disrupted by sonication. The homogenate was centrifuged at 20,000 × g for 30 min to remove insoluble materials, and the supernatant was applied to TALON Metal Affinity resin (TAKARA BIO) chromatography. Unbound proteins were washed out with wash buffer (50 mM sodium phosphate buffer, 5 mM 2-ME, 300 mM NaCl, 0.1% Triton X-100, 10 mM imidazole, pH 7.4). Each expressed subunit was eluted with elution buffer (50 mM sodium phosphate buffer, 5 mM 2-ME, 300 mM NaCl, 0.1% Triton X-100, 200 mM imidazole pH 7.4) and dialyzed with buffer (50 mM Tris-HCl, 100 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 5% glycerol, pH 7.6) before the RNR assay.

The protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific K.K.)

RNR Assay and Cordycepin Measurement

The RNR assay was conducted as described in the previous paper [12]. In brief, the reaction was performed in reaction buffer (100 mM potassium acetate, 20 mM magnesium acetate, 2 μM $FeCl_3$, 20 mM dithiothreitol, 1 mM ADP, 3 mM ATP, 0.1 mM dATP and 20 mM HEPES-KOH, pH 7.3). First, 10 μM purified CmR1 and CmR2 was added to the reaction buffer. The reaction mixture was incubated at 25°C, and the reaction was stopped by heating. The reaction products were separated by TSK-gel ODS-80TM column (4.6 × 25 cm, Tosoh, Tokyo, Japan) with running buffer (10 mM phosphoric acid, 10 mM triethylamine, 2% methanol, pH 6.8) using the LC10AD VP system (Shimadzu, Kyoto, Japan) at 254 nm. To detect cordycepin or 2'-deoxyadenosine based on recombinant RNR, the RNR assay was conducted, followed by treatment with calf intestinal alkaline phosphatase for 12 h to remove the phosphate groups of the reaction products. Cordycepin and 2'-deoxyadenosine were separated by TSK-gel ODS-80TM column (4.6 × 15 cm, Tosoh) with running buffer (0.1% methanol: phosphoric acid = 98: 2) using the LC10AD VP system (Shimadzu) at 254 nm.

Results

Cordycepin Production in *C. militaris*

C. militaris NBRC9787 was cultivated in a liquid surface culture using medium A and B (Fig. 1). Cordycepin was produced earlier in medium A than in medium B. However, the amount of cordycepin produced in medium B was higher than in medium A. In

medium A, the addition of glycine and adenine, which are important compounds for purine synthesis, enhanced the production of cordycepin. These results suggest that the purine nucleotide metabolism is involved in cordycepin production in *C. militaris*.

Expression of Recombinant CmR1 and CmR2 in *E. coli*

To clone the cDNAs of CmR1 and CmR2 from *C. militaris* NBRC9787, the total RNA was extracted from its mycelia. Based on the sequences of CmR1 and CmR2 from *C. militaris* CM01, we cloned CmR1 and R2 cDNA from *C. militaris* NBRC9787. The CmR2 cDNA was amplified by RT-PCR on the *C. militaris* NBRC9787 total RNA, but we could not obtain CmR1 cDNA. Therefore, we prepared CmR1 cDNA from genomic DNA of *C. militaris* NBRC9787. In the CmR1 cDNA, nucleotide substitution was found at 19 sites, but only one amino acid substitution was found compared to *C. militaris* CM01 (^{548}S→A). In CmR2, nucleotide substitution was found at 7 sites, but only one amino acid substitution was observed in the CmR2 cDNA from *C. militaris* NBRC9787 compared to *C. militaris* CM01 (^{64}V→A). These cDNAs were inserted separately into the pCold I vector. The recombinant CmR1 and CmR2 were expressed separately in *E. coli* BL21(DE3). Recombinant CmR2 (approximately 50 kDa) was expressed in both the soluble and insoluble fractions. However, most of recombinant CmR1 (approximately 105 kDa) was expressed in the insoluble fraction (Fig. 2).

To improve the soluble fraction of recombinant CmR1 in *E. coli*, the recombinant CmR1 was co-expressed with each chaperone, and the soluble fraction was detected using TALON metal affinity resin (Fig. 3). Purified recombinant CmR1 with some minor proteins and GroEL was used for the RNR assay because recombinant CmR1

was a main band in the eluted fraction (Fig. 4A). After the purification of each recombinant subunit using TALON metal affinity resin, highly purified CmR2 was obtained and used for the RNR assay (Fig. 4B).

RNR Assay of Recombinant CmR1 and CmR2

It is known that the RNR large subunit can form a complex with the RNR small subunit in the presence of its substrate and ATP [13]. ADP was used as a substrate of recombinant CmRNR to confirm cordycepin synthesis by CmRNR. RNR catalyzes the conversion of 2'-deoxyADP from ADP. No new peak was detected in the reaction mixture using only CmR1 or CmR2 between 20 and 25 min in the HPLC chromatogram, nor was there any reaction in the presence of both recombinant subunits (Fig. 5). However, a new peak, which corresponds to 2'-deoxyADP, was detected after 1 h reaction in the presence of both recombinant CmR1 and CmR2. To confirm the involvement of CmRNR in cordycepin (3'-deoxyadenosine) synthesis, alkaline phosphatase was added to the RNR reaction mixture after allowing the RNR reaction to proceed for 1 h (Fig. 6C, D) to produce deoxyadenosine from deoxyADP. Alkaline phosphatase dephosphorylates nucleotides to nucleosides. Therefore, the cordycepin peak appears after the RNR reaction if CmR1 and CmR2 produce 3'-deoxyADP. 2'-Deoxyadenosine was detected at 9.2 min, and cordycepin was not detected at 10.4 min. These results indicate that recombinant CmRNR does not catalyze the synthesis of 3'-deoxyADP from ADP, which indicates that ADP is not precursor of 3'-deoxyADP in cordycepin production in *C. militaris* (Scheme 1).

Discussion

Cordycepin was first isolated from *C. militaris* culture in 1950, but its biosynthetic pathway in *C. militaris* has not yet been determined. However, using various "OMICS" strategies, the biosynthetic pathway has been deduced [6–8]. Cordycepin seems to be produced by the reduction of ADP, which is similar to the RNR reaction. RNR catalyzes the reduction of nucleotides to 2'-deoxynucleotides, which are involved in DNA synthesis and DNA repair [14]. Therefore, RNR is regarded to be able to produce cordycepin in *C. militaris*. In this study, the ability of CmRNR from *C. militaris* NBRC9787 to synthesize cordycepin was investigated. Recombinant CmRNR expressed in *E. coli* showed RNR activity against ADP but did not produce 3'-deoxyADP from ADP.

In our RNA-seq experiment on *C. militaris*, the expression levels of CmR1 and CmR2 in the liquid surface culture of *C. militaris* were almost the same as in the submerged culture with shaking, but cordycepin was not produced in the submergd culture (data not shown). This result indicates no involvement of CmRNR with cordycepin production in *C. militaris*. Cordycepin is found only in *C. militaris* and *O. sinensis*. Zheng et al. states that CmRNR would not be involved in cordycepin production because *C. militaris* has only one set of RNR subunits [7]. By contrast, *O. sinensis* has two sets of RNR subunits [8]. Our study shows that CmRNR is not involved in cordycepin production in *C. militaris*, which suggests that *C. militaris* may have a different cordycepin biosynthetic pathway from *O. sinensis*.

RNR activity is regulated allosterically by 3'-deoxynucleoside triphosphates (dNTPs), altering its substrate specificity [15]. In addition, eukaryotic cells have

mechanisms that regulate RNR activity tightly to maintain balanced pools of dNTPs [16]. In yeasts, Sml1 inhibits RNR activity by its association with the N-terminal domain of the R1 subunit to hinder the accessibility of the C-terminal domain of the R1 subunit [17]. Dif1 seems to control the subcellular localization of RNR R2 subunit in yeast [18]. Kuo et al. reported that the absence of an RNR inhibitor may lead to cordycepin accumulation [10]. In this study, we should that CmRNR catalyzes 2'-deoxyADP synthesis from ADP and the cordycepin production pathway via CmRNR does not exist in *C. militaris*. It suggests that other targets should be investigated for its cordycepin production including the RNR inhibitors which may control the RNR activity if they are in *C. militaris*.

Acknowledgment

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Conflict of interest

The authors report no conflict of interest.

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Table 1 Primers used.

Name	5’ to 3’
CmR1-F1	CGCGAATTCATGTACGTGAAGAAGAGAGATGGACGCCAGGAGCG
CmR1-F2	GGCGGTCTCGGCAGCGAGATCATCAAGTTGAATAG
CmR1-R1	CAACTTGATGATCTCGCTGCCGAGACCGCCGCCTA
CmR1-R2	TTACTGCAGCTAACCGCTGCACATG
CmR2-F	CACGAATTCATGGCTTCCCAAACAAC
CmR2-R	CGCCTGCAGTTAAAAGTCGTCATCAAAGCTGAAG

Table 2 Chaperones used.

Plasmids	Expressed chaperones
pTf16	Tig
pG-Tf2	GroES-GroEL-Tig
pKJE7	DnaK-DnaJ-GrpE
pGro7	GroES-GroEL
pG-KJE8	DnaK-DnaJ-GrpE, GroES-GroEL

Figure legends

Scheme 1 Predicted cordycepin production pathway in *C. militaris*. In most of organisms, 2'-deoxyadenosine is produced through 2'-deoxyADP produced by RNR from AMP. It is predicted that cordycepin may be produced from adenosine, AMP or ADP in *C. militaris*. RNR may be involved in cordycepin production through the synthesis of 3'-deoxyADP from ADP if ADP is a precursor of cordycepin. ADEK: adenylate kinase; ADK: adenosine kinase; ADP: adenosine diphosphate; AMP: adenosine monophosphate.

Fig. 1 Cordycepin production by the static cultivation of *C. militaris* NBRC9787 in medium A and B with/without Vogel's medium (A) and in medium A supplemented with adenine and glycine (B). (A) A - v: Medium A, A + v: Medium A supplemented with one-tenth Vogel's medium, B - v: Medium B, B + v: Medium supplemented with one-tenth Vogel's medium (B) A: Medium A, A + g·a: Medium A supplemented with 1 g/L adenine and 16 g/L glycine.

Fig. 2 Recombinant CmR1 and CmR2 in *E. coli*. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (CBB). "S" and "I" indicate the soluble and insoluble fraction, respectively.

Fig. 3 Investigation of the level of soluble recombinant CmR1 in chaperone (Table 2)-co-expressing *E. coli*. Soluble CmR1 was separated from the supernatant of chaperone-co-expressing *E. coli* homogenate using TALON metal affinity resin. Proteins in eluted fractions were separated by SDS-PAGE and stained with CBB. F:

Flow-through fractions, W1 and W2: Wash fractions, E: eluted fractions. Arrows denote expected molecular weight of recombinant CmR1.

Fig. 4 Purification of recombinant CmR1 and CmR2 from *E. coli*. (A) Purification of recombinant CmR1 co-expressed with GroES-GroEL in *E. coli*. (B) Purification of recombinant CmR2 expressed in *E. coli*.

Fig. 5 Chromatograms of RNR reaction products in RNR reaction mixtures.

Fig. 6 Chromatograms of RNR reaction products followed by alkaline phosphatase treatment. AP denotes alkaline phosphatase. (A) Commercial 2’-deoxyadenosine, (B) Commercial cordycepin, (C) AP-treated products before RNR reaction (D) AP-treated RNR reaction products.

Scheme 1, Kato et al.

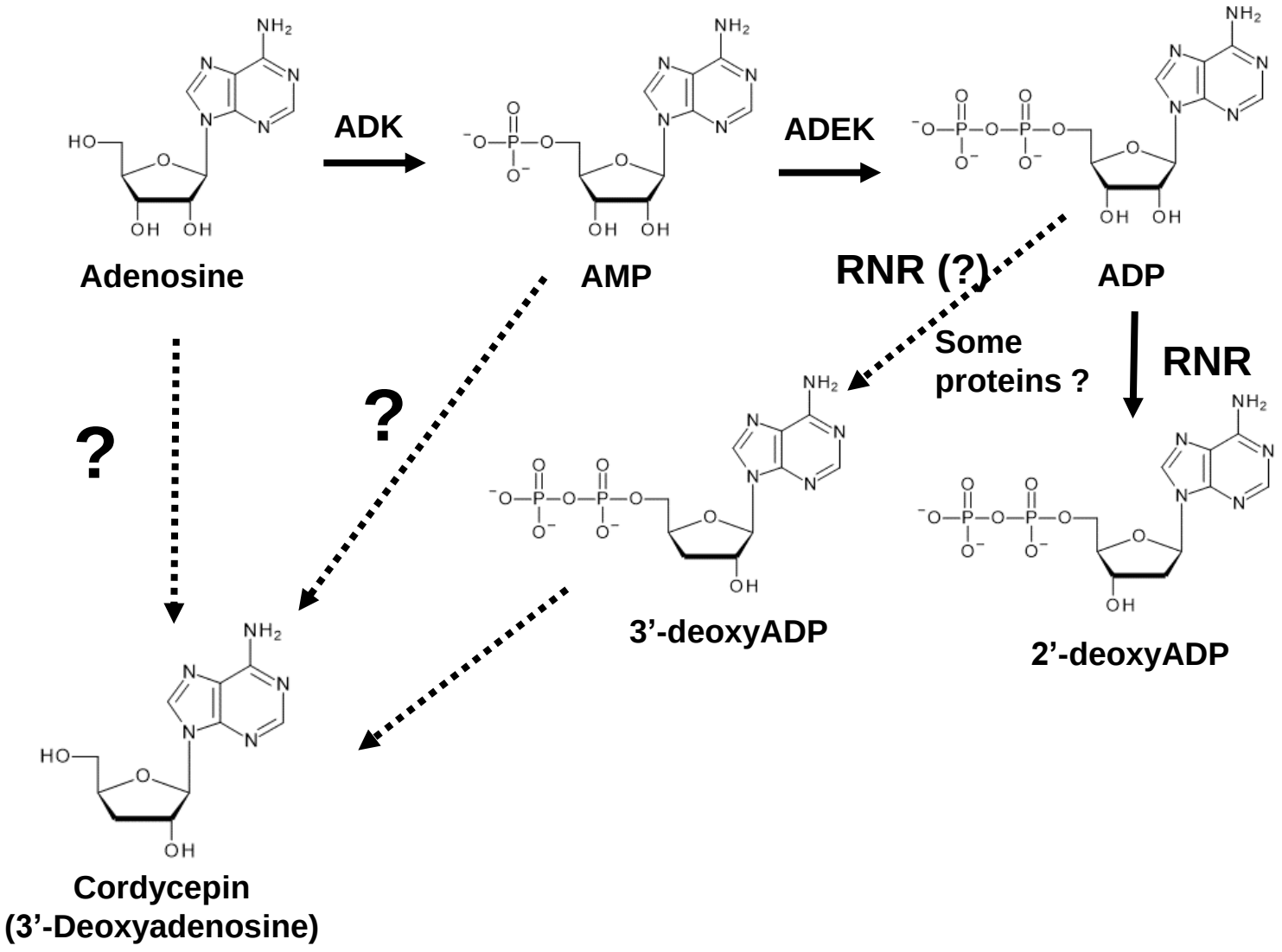


Fig. 1, Kato et al.

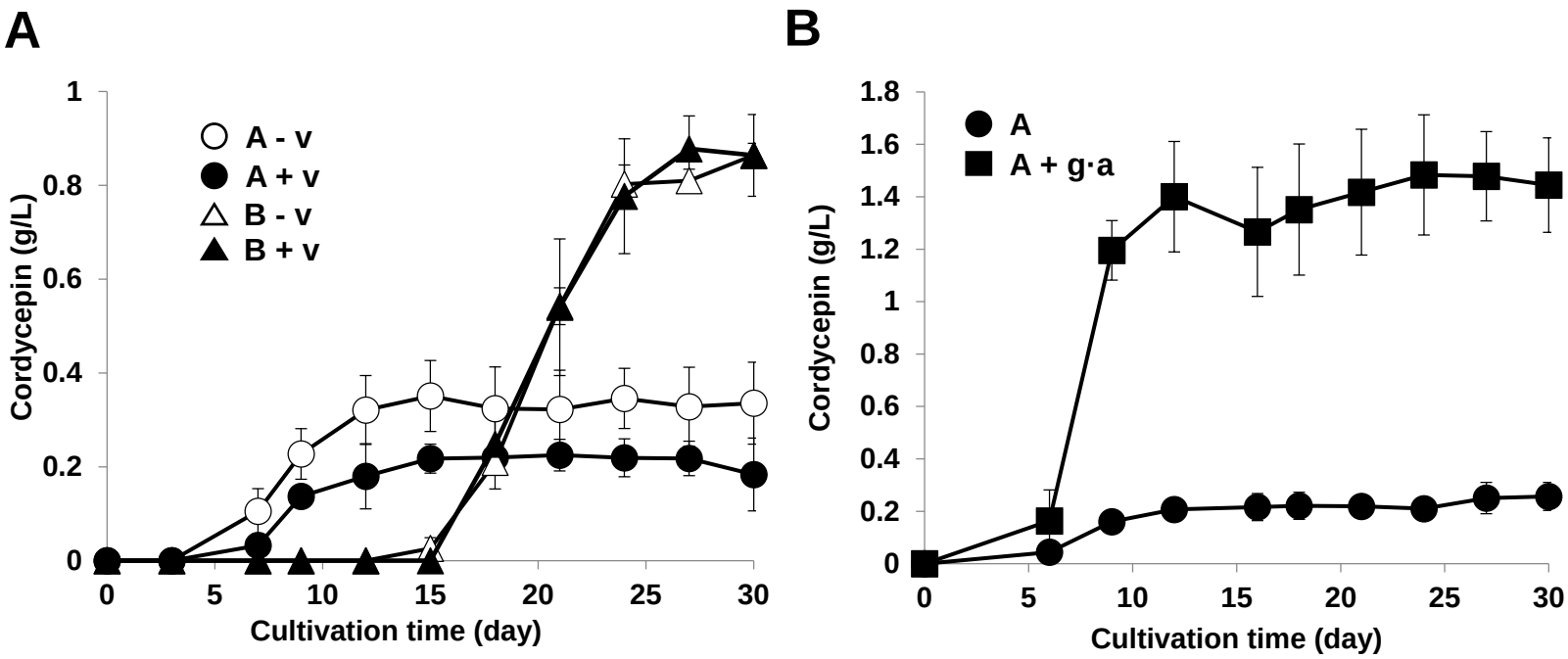
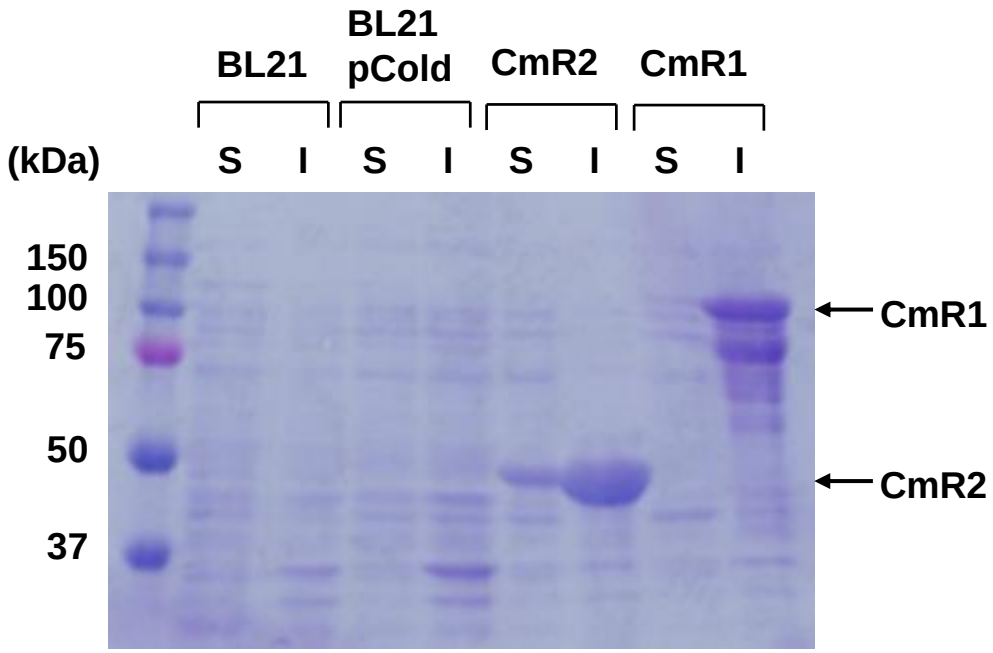


Fig. 2, Kato et al.



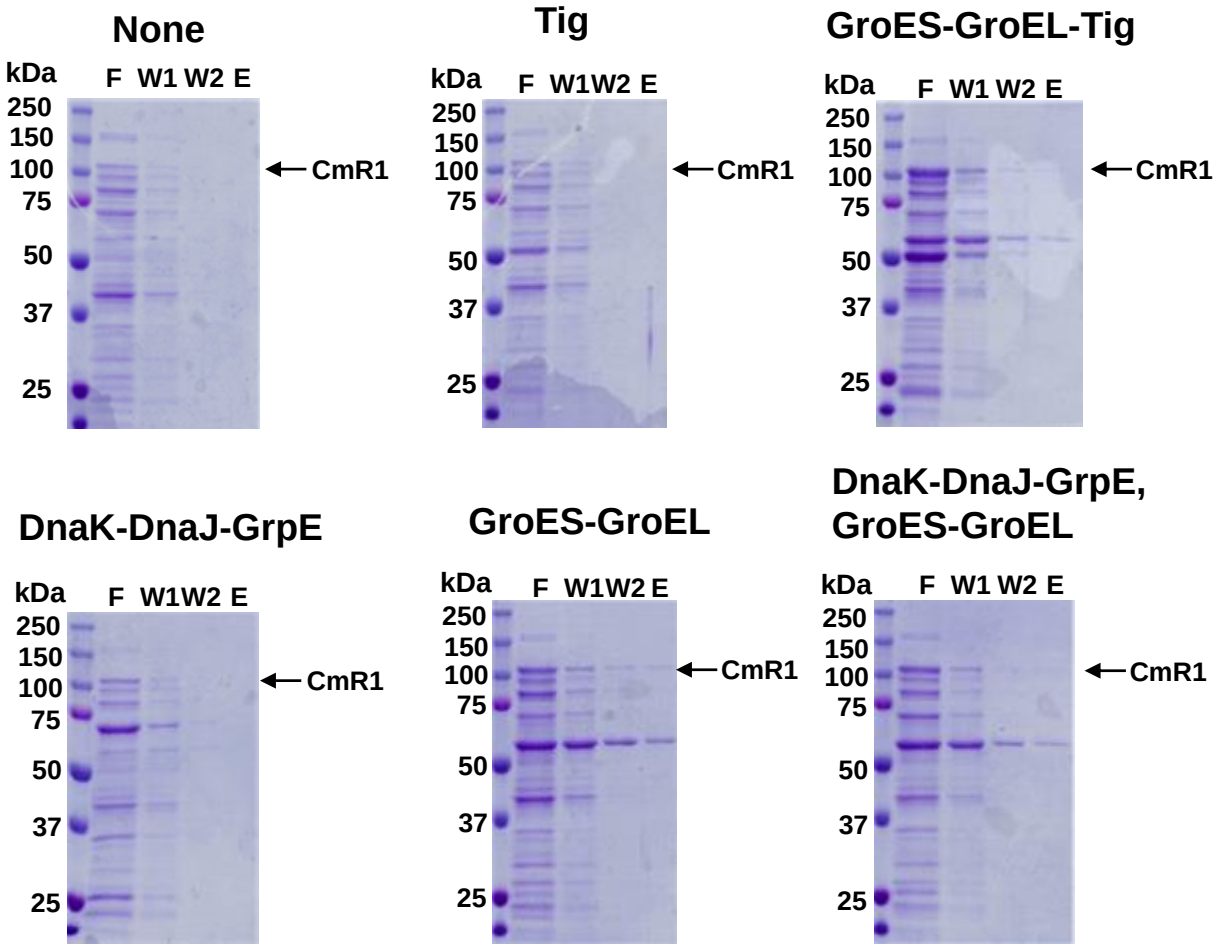

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Fig. 4, Kato et al.

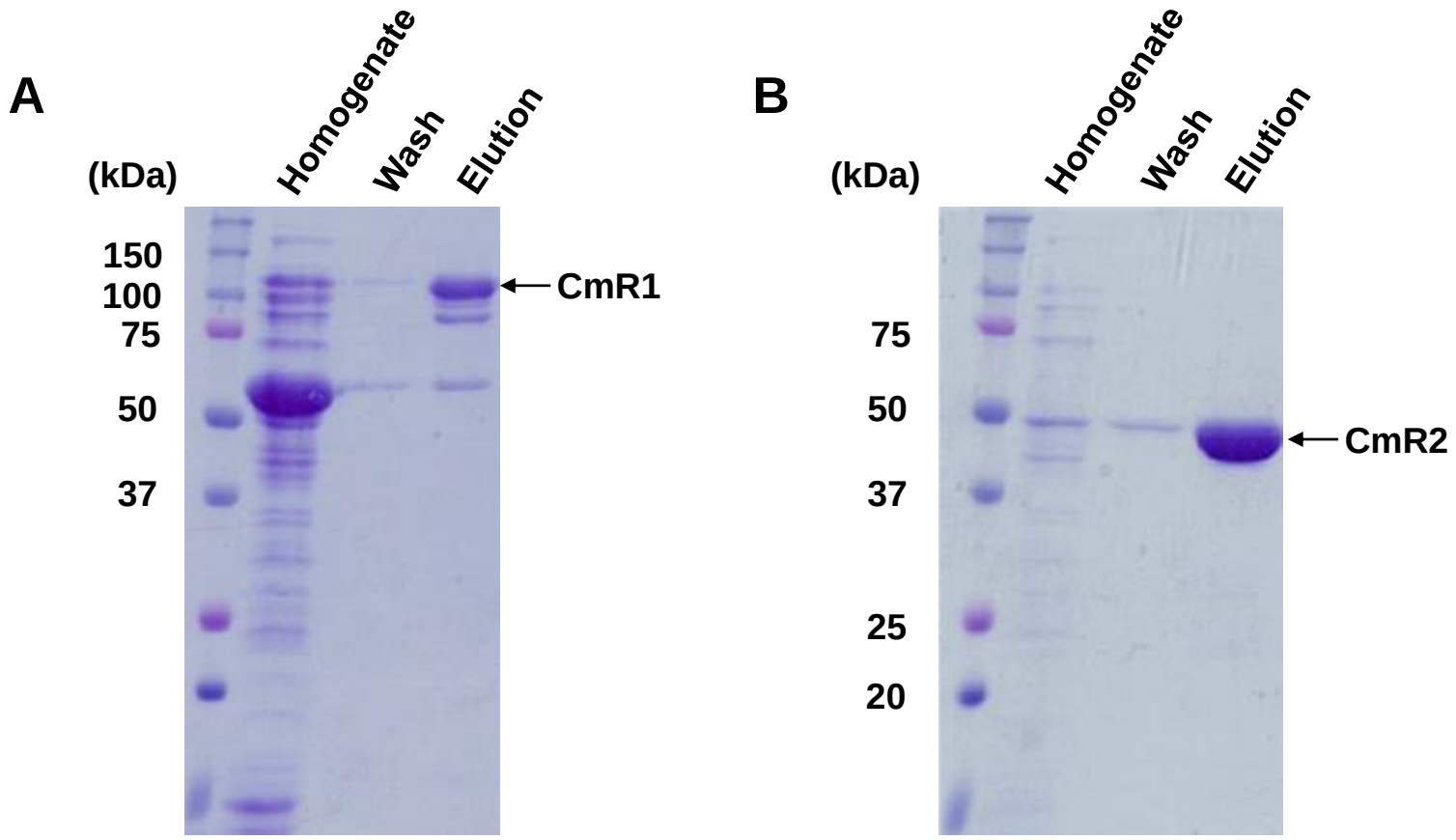

Fig. 5, Kato et al.

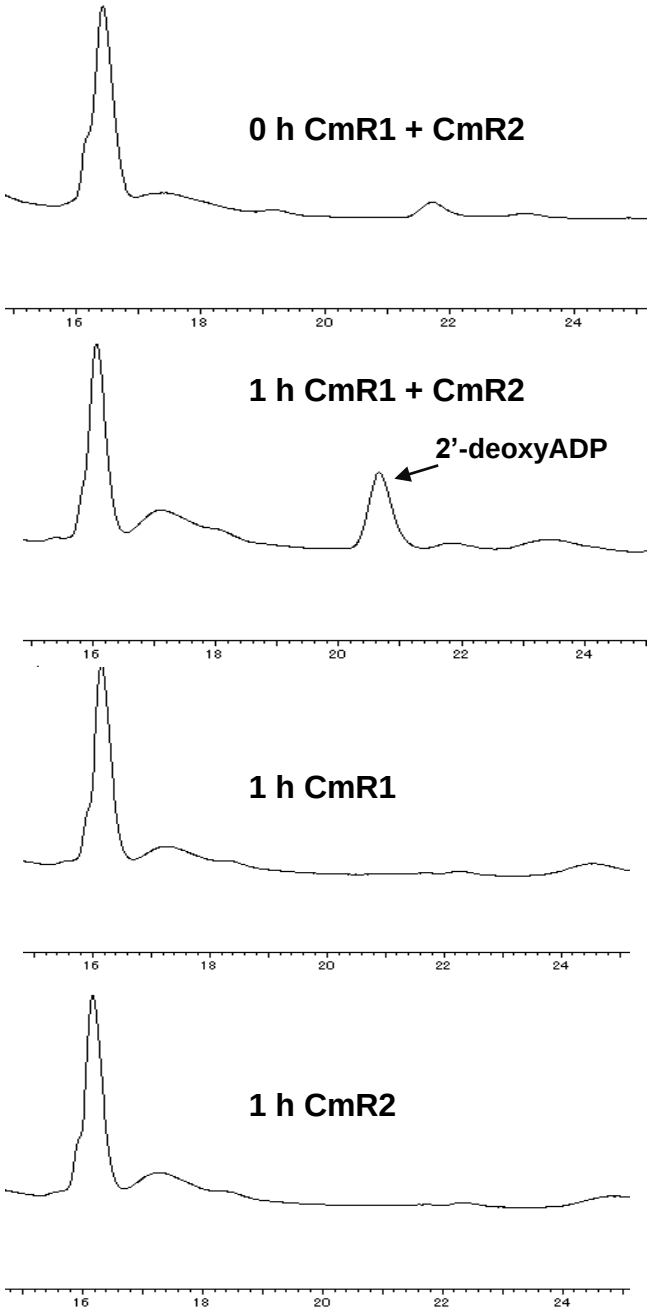

Fig. 6, Kato et al.