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Purification and characterization of Qβ replicase with a His-tag

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Abstract

A His-tag was added to the C-terminal of Qβ replicase, an RNA-dependent RNA polymerase of RNA coliphage Qβ, to facilitate enzyme purification. The purified His-tagged enzyme assumed almost the same template specificity as the wild type purified by a conventional method when MDV-poly(+) RNA or Qβ RNA was used as the template. Here, we showed the efficiency of the approach surmounts the present available ones. The availability of Qβ replicase of quality affords its implementation for the synthesis and amplification of RNA molecules as well as further elucidation on the molecular mechanism of the enzyme reaction. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Qβ replicase, an RNA-dependent RNA polymerase of RNA coliphage Qβ, consists of four subunits of which three are host-derived proteins (ribosomal protein S1, and protein elongation factors, EF-Tu and EF-Ts) [1]. Only the β-subunit, which is composed of 588 amino acid residues, is encoded by the phage RNA. The Qβ-replicase can specifically transcribe in vitro the Qβ RNA as well as RNA from closely related phage [1]. S1 and another host protein, HF-I [2] are required for the recognition of only Qβ viral plus-strand RNA as a template. In contrast, the enzyme lacking S1 and HF-I can transcribes poly(rC), MDV-1 RNA, and Qβ minus-strand RNA, as does the holoenzyme of Qβ replicase [1]. The complexity behind the replication machinery with Qβ replicase as the catalyst, hence, still demands more intensive research works toward further elucidation of the molecular mechanisms of RNA replication.

Besides being an attractive enzyme, Qβ replicase showed potential for in vitro synthesis of RNA molecules [3,4] and gene detection by exponent amplification of target RNAs [5,6]. Furthermore, the enzyme can be a significant constituent of an in vitro self-replication system, which has been constructed using a DNA polymerase as a minimum model of life [7]. How-

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ever, although several purification methods have already been developed [8–10], convenient way of purification and sufficient supply of pure Qβ replicase yet pose difficulties for the rapid progress on works involving this particular enzyme. To overcome the problem, we developed a method whereby a His-tag was added to the C-terminal of the β-subunit to improve the efficiency of purification than those available [8–10]. The approach not only accelerated purification but also yielded purified His-tagged Qβ replicase with similar template specificity as the wild-type enzyme.

2. Experimental

2.1. Bacterial strains and plasmids

The bacterial strains used were *Escherichia coli* 594/F′ *lacI*^{q} (*sup*$^+$ *gal* Str^r *recA44*/F′ *lacI*^{q} *lac*$^+$ *proAB*) [11] and BL21 (DE3) (Novagen). Plasmid pRQ1 contains the wild-type β-subunit gene of Qβ replicase [11]. PET-QRH, a hybrid plasmid containing the gene of Qβ replicase β-subunit with a C-terminal His_6 tag in the multicloning site of pET21a(+) (Novagen), was prepared in this study. Plasmid pUC-MDV-LR carrying the segment corresponding to MDV-poly(+) RNA was described previously [12].

2.2. DNA manipulation

Preparation of plasmid DNA, enzyme reactions, and transformation of *E. coli* cells were carried out as described by Maniatis et al. [13].

2.3. Purification of wild-type Qβ replicase

E. coli 594/F′ *lacI*^{q} cells harboring pRQ1 were grown to a cell density of OD_{660} 0.25–0.30 at 37°C for about 12–16 h on standing in LB medium [13] (40 × 1.2 l) containing 50 μg/ml ampicillin, after which isopropyl-β-D-thio-galactopyranoside (final 2 mM) was added and the cultivation continued with shaking for 2 h. The bacterial cells suspended in 350 ml of buffer I (50 mM Tris/HCl, pH 7.6, containing 5 mM $MgCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20% glycerol) were disrupted with a French pressure cell. To the supernatant (380 ml) obtained by centrifugation, 20% dextran solution (95 ml) and 30% poly(ethylene glycol) 6000 (117 ml) solution were added slowly. After stirring for 30 min at 4°C, the phases were separated by centrifugation. The upper phase (420 ml) was discarded and to the lower phase was added in the order of 303 ml of buffer II (50 mM Tris/HCl, pH 7.6, containing 5 mM $MgCl_2$, 1 mM EDTA, and 5 mM 2-mercaptoethanol), 21 ml of 1 M $MgCl_2$, 117 ml of 30% poly(ethylene glycol) solution, and solid NaCl (final 5 M). The mixture was stirred for 1 h at 4°C before the phases were separated by centrifugation. The upper phase was dialyzed overnight against buffer I at 4°C, and the precipitate formed was removed by centrifugation. To the supernatant (490 ml) was added solid $(NH_4)_2SO_4$ to 35% saturation. After stirring for 20 min at 4°C, the phases were again separated by centrifugation. Solid $(NH_4)_2SO_4$ was added to the lower phase (420 ml) until 60% saturation and the mixture was stirred for 20 min at 4°C. The precipitate collected by centrifugation was dissolved in 40 ml of buffer I. The solution was dialyzed against buffer II for about one day with change of buffer once, and applied to a DEAE-cellulose column (3 × 20 cm) equilibrated with the same buffer. The column was washed with 200 ml of the same buffer, and the enzyme was eluted with a NaCl gradient of 0–0.4 M (total 500 ml). Active fractions were collected, dialyzed against 50 mM Tris/HCl (pH 7.6) containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20% glycerol for about one day with change of buffer once, and applied to a phospho-cellulose column (3 × 20 cm) equilibrated with the same buffer. The column was washed with 200 ml of the same buffer, and the enzyme was eluted with a NaCl gradient of 0–1.0 M (total 500 ml). Active fractions were pooled, dialyzed

against buffer II for about 1 day with change of buffer once, and applied to a DEAE-Sepharose CL-6B column (3 × 10 cm) equilibrated with the same buffer. The column was washed with 50 ml of the same buffer, and the enzyme was eluted with a NaCl gradient of 0–0.4 M (total 200 ml). Active fractions were collected, dialyzed against buffer I, and used as the purified wild-type enzyme.

2.4. Purification of His-tagged Qβ replicase

E. coli BL21(DE3) cells harboring pET-QRH were grown to a cell density of OD_{660} 0.25–0.30 at 37°C for about 12–16 h on standing in LB medium (14 × 1.4 l) containing 50 μg/ml ampicillin before the addition of isopropyl-β-D-thiogalactopyranoside (final 2 mM) and the culture was further cultivated for 2 h with shaking. The purification procedures were essentially the same as above until the step of DEAE-cellulose column chromatography. Active eluates were collected, dialyzed against 0.1 M potassium phosphate (pH 8.0) containing 5 mM 2-mercaptoethanol for about one day with change of buffer once to remove the NaCl and Tris, and applied to a Ni-NTA Superflow (Qiagen) column (3 ml) equilibrated with the same buffer. The column was washed with 100 ml of 0.1 M potassium phosphate (pH 8.0) containing 5 mM 2-mercaptoethanol and 10 mM imidazole, and the enzyme was eluted with 5 ml of 0.1 M potassium phosphate (pH 8.0) containing 5 mM 2-mercaptoethanol and 0.5 M imidazole. Active fractions were collected, dialyzed against 50 mM potassium phosphate (pH 8.0) containing 5 mM 2-mercaptoethanol for about one day with change of buffer once, and used as the purified His-tagged enzyme.

2.5. Enzyme assay

RNA polymerase activity assay was carried out at 35°C using MDV-poly(+) RNA or Qβ RNA as a template. MDV-poly(+) RNA was prepared by in vitro transcription of pUC-MDV-LR DNA with T7 RNA polymerase as described in Ref. [12]. At each purification step, the replicase activity was constantly monitored by gel electrophoresis of the enzyme reaction product as follows: The reaction mixture (50 μl) contained 0.8 mM each of ATP, CTP, GTP, and UTP, 20 μg/ml MDV-poly(+) RNA, 125 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM 2-mercaptoethanol, 5 mM phosphoenol pyruvate, 10 μg/ml pyruvate kinase, 74 units/ml DNase I, 10 μg/ml rifampicin, and 5 μl of enzyme sample. The reaction was carried out for 30 min and the RNA was extracted with phenol/chloroform, precipitated with ethanol, dissolved in 10 μl water, and analyzed by agarose-gel electrophoresis.

The replicase activity of the purified enzymes was measured as the increase in the fluorescence emission at 509 nm (excitation at 488 nm) of the enzyme reaction mixture using a Hitachi F-2000 fluorescence spectrophotometer. The reaction mixture (0.2 ml) contained 2.5 mM each of ATP, CTP, GTP, and UTP, 4.5 ng/ml MDV-poly(+) RNA or 77.8 ng/ml Qβ RNA, 138 mM Tris/HCl (pH 7.8), 22 mM $MgCl_2$, 0.5 mM EDTA, 10,000-times diluted SYBR Green II (Molecular Probes), and 20 μl of enzyme sample. The amount of RNA synthesized was estimated using the standard curves drawn for the two RNA templates (see Fig. 2). Enzyme concentration was calculated based on the values of absorbances at 280 and 260 nm using the equation [14], $(1552\,A_{280} - 757.3\,A_{260})$ μg/ml, to correct the effect of possible contaminating RNA.

3. Results and discussion

3.1. Enzyme purification

Three steps of column chromatographies are required to yield about 1 mg of purified wild-type Qβ replicase from a 48-l culture, while for the His-tagged enzyme, it took only two steps of chromatographies to yield about 0.6 mg of

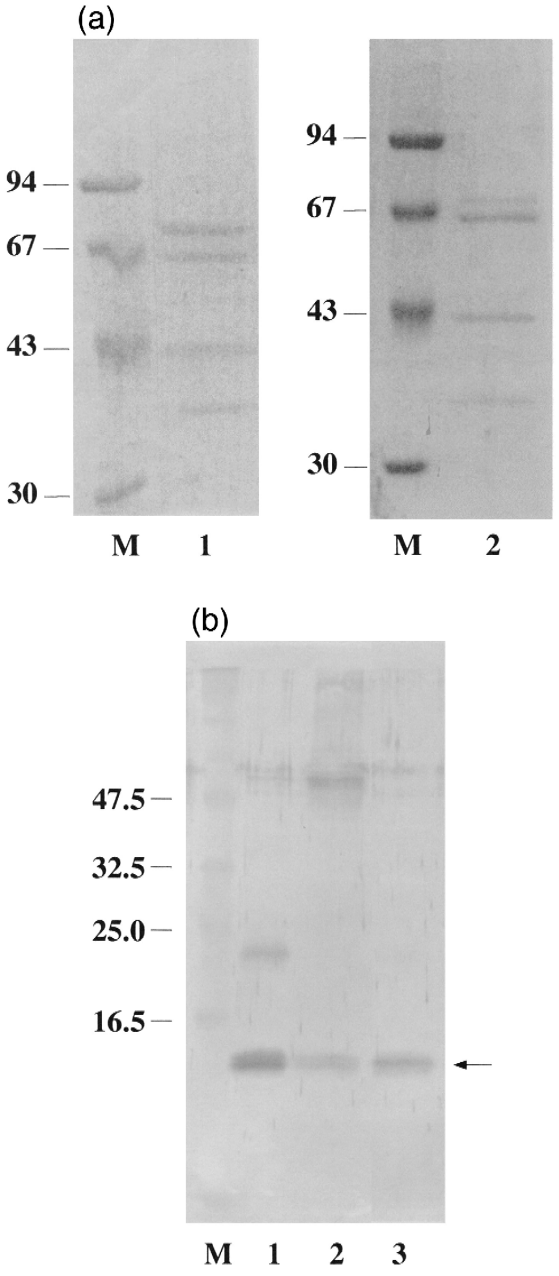

Fig. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the purified enzymes. (a) 10%-gel electrophoresis of the wild-type Qβ replicase (1.9 μg, lane 1), His-tagged Qβ replicase (1.5 μg, lane 2), and marker proteins with the indicated size in kDa on the left (lane M). Protein bands were visualized by Coomasie brilliant blue R-250. Electrophoresis was carried out according to Laemmli [15]. (b) Western blot analysis using anti-HF-I antibody after 15%-gel electrophoresis of the purified HF-I (0.1 μg, lane 1), wild-type enzyme (1.2 μg, lane 2), His-tagged enzyme (1.4 μg, lane 3), and marker proteins (lane M). The arrow indicates the position of HF-I monomer. HF-I sometimes aggregates and forms oligomers.

purified enzyme from a 20-l culture. Clearly, addition of a His-tag to the C-terminal of the β-subunit of Qβ replicase facilitates purification and gives a higher yield of the enzyme.

The homogeneity of the enzymes was confirmed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis [15] (Fig. 1a). Both enzymes migrate as four protein bands corresponding to S1, β-subunit, EF-Tu, and EF-Ts from the top. In addition, Western blot analysis [16] confirmed that both enzyme preparations contained the host factor protein, HF-I (Fig. 1b). To analyze the amount of contaminating RNA in an enzyme preparation, the value of A_{280}/A_{260} was calculated [14]. The A_{280}/A_{260} values of the purified wild-type and His-tagged enzymes were 0.55 and 1.03, respectively, indicating a much lesser amount of RNA in the His-tagged enzyme preparation. The accurate value of the RNA content can be calculated after knowing the absorption coefficient of the enzyme though. A lower level of RNA contamination was attained probably due to the extensive

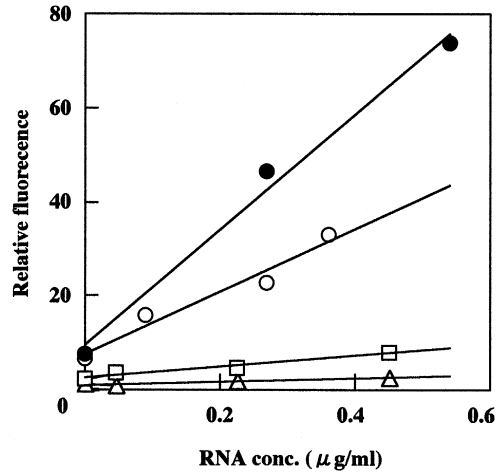

Fig. 2. Sensitivity of SYBER Green II in detecting RNA. Fluorescence of SYBER Green II was measured at 509 nm (excitation at 488 nm) in 138 mM Tris/HCl (pH 7.8) containing 22 mM $MgCl_2$, 0.5 mM EDTA, 2.5 mM each of ATP, CTP, GTP, and UTP, and the indicated concentration of MDV-poly(+) RNA (closed symbols) or Qβ RNA (open circles). Dilution factors of SYBER Green II from the commercial preparation (Molecular Probes) were 10,000 (● and ○), 20,000 (□), and 50,000 (△).

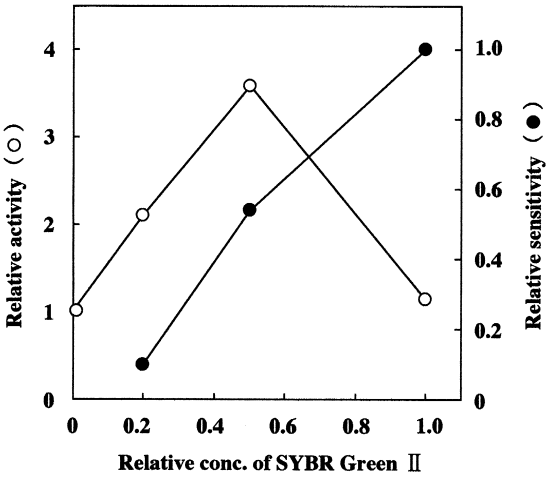

Fig. 3. Effect of SYBR Green II concentration on the activity of the His-tagged Qβ replicase (○) and sensitivity of the assay system (●). The assay conditions were as described in the Experimental section except for the concentration of MDV-poly(+) RNA and SYBR Green II. The relative dye concentration of 1 corresponds to 10,000-times diluted commercial preparation. The purified His-tagged enzyme and 45 ng/ml MDV-poly(+) RNA were used for the assay. The relative activity at zero dye concentration was estimated as follows: sampling was done at 1-min interval from precisely the same enzyme reaction mixture but without the dye. The reaction was stopped immediately by boiling upon sampling. To each of the sampled solution was added the same amount of diluted dye and the fluorescence emitted in each solution was measured. The relative activity of 1 corresponds to 0.41 ng RNA/s. The sensitivity of the assay system was calculated from the sensitivity of RNA detection and activation factor, and was expressed relative to the value obtained at the relative dye concentration of 1.

washing prior to the elution of the enzyme from the Ni-NTA column.

3.2. Sensitivity of the replicase activity measurement

The commonly used radiochemical assay for RNA polymerases, although highly sensitive, requires extensive manipulation and time. Here, we measured the polymerase reactions in real time by the increase in the fluorescence of a dye in accord to the methods developed by Burg et al. [17] with some modifications. The real time assay is essential to kinetic and mechanistic studies [18]. The fluorescent intercalating dye SYBR Green II was used instead of propidium iodide. Fig. 2 shows that the sensitivity of SYBER Green II in detecting MDV-poly(+) RNA increases with the increase in the dye concentration. This suggests that the RNA molecules have several kinds of dye-binding sites with different affinities. However, too high a concentration may interfere with the enzyme reaction. As shown in Fig. 3, at relative concentration of 1, which corresponds to 1 in 10,000 dilution in Fig. 2, SYBR Green II dye has only a small effect on the enzyme activity (relative activity is 1.14) relative to that without the dye. Furthermore, the dye is shown to enhance the activity at lower concentrations. The assay system overall sensitivity is determined by the sentivity of RNA detection and activation factor as shown in Fig. 3. Hence, the relative concentration of 1 was used in the measurement of replicase activity. Under these conditions, the sensitivity of the assay system is about 160 times that of the system [17] using 1 μg/ml of propidium iodide, which enhanced the activity twice (data not shown).

The sensitivity of SYBER Green II varies with the type of RNA such that detecting Qβ RNA is about 1.6 times of that for MDV-poly(+) RNA at dye concentration of 1 in 10,000 dilution (Fig. 2). For quantifying the Qβ replicase activity, the curves drawn from the data obtained with MDV-poly(+) RNA and Qβ RNA at dye concentration of 1 in 10,000 dilution in Fig. 2 were used as the standards for the two kinds of RNA templates, respectively.

Table 1
Template specificity of Qβ liplicases

Enzyme	Specific activity (μg RNA/s/mg enzyme)		Template specificity (A)/(B)
	MDV-poly(+)RNA (A)	QβRNA (B)	
Wild-type	0.61	0.083	7.4
His-tagged	0.32	0.045	7.0

3.3. Enzymatic properties

The specific activities of the wild-type and His-tagged Qβ replicase were measured using MDV-poly(+) RNA or Qβ RNA as a template. The His-tagged enzyme has a bit lower specific activity for both templates, while the template specificity of the two enzymes was nearly the same (Table 1). Decrease in activity can be an effect of the added His-tag and/or the decrease in the relative amount of the S1 subunit in the preparation of the His-tagged enzyme (Fig. 1a). Thorough examination on the role of each subunit on the enzyme reaction using the His-tagged enzyme may provide additional insight to the molecular mechanisms of RNA replication.

The approach presented allows pure Qβ replicase to come in handy and be a convenient tool for RNA synthesis and amplification and for the construction of an in vitro self-replication system.

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