Characterization of recombinant *Thermococcus kodakaraensis* (KOD) DNA polymerases produced using silkworm-baculovirus expression vector system

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Abstract

The KOD DNA polymerase from *Thermococcus kodakarensis* (Tkod-Pol) has been preferred for PCR due to its rapid elongation rate, extreme thermostability and outstanding fidelity. Here in this study, we utilized silkworm-baculovirus expression vector system (silkworm-BEVS) to express the recombinant Tkod-Pol (rKOD) with N- (rKOD-N) or C-terminal (rKOD-C) tandem fusion tags. By using BEVS we produced functional rKODs with satisfactory yields, about 1.1 mg/larva for rKOD-N and 0.25 mg/larva for rKOD-C, respectively. Interestingly, we found that rKOD-C shows higher thermostability at 95 °C than that of rKOD-N, while that rKOD-N is significantly unstable after exposing to long-period of heat-shock. We also assessed the polymerase activity as well as the fidelity of purified rKODs under various conditions. Compared with commercially available rKOD, which is expressed in *E. coli* expression system, rKOD-C exhibited almost the same PCR performance as the commercial rKOD did, while rKOD-N did lower performance. Taken together, our results suggested that silkworm-BEVS can be used to express and purify efficient rKOD in a commercial way.

Keywords: DNA polymerase, KOD, silkworm, baculovirus expression system

Introduction

Thermostable DNA polymerases (DNAPs) have been widely used for *in vitro* DNA manipulations, such as polymerase chain reaction (PCR), DNA sequencing and genotyping, etc. Generally, there are seven classified DNAP families (A, B, C, D, X, Y, and reverse transcriptase/RT) according to the sequence similarity of amino acids [1]. Family-B DNAPs were discovered in all archaeal lineages, and recently they have also been revealed as a co-operating member of integrase Cas1-dependent self-synthesizing mobile elements (termed casposons) in archaeal and bacterial [2, 3]. Among those Family-B DNAPs, several archaeal family-B DNAPs, such as *Pyrococcus furiosus* Pfu-Pol and *Thermococcus kodakarensis* Tkod-Pol, have been preferred to be used for PCR, especially for long fragment amplifications because of their extreme thermostability and outstanding fidelity along with integrated 3'-5' exonuclease activity (called proof-reading activity) [4–6], which *Thermus aquaticus* Taq-Pol from family-A DNAP lacks in its function. Like Taq-Pol, recombinant Pfu-Pol and KOD-Pol DNAPs have been successfully made as a commercial product by several biotech companies. To obtain marvelous DNAPs for a commercial application in terms of both quantity and quality, the remarkable and continuous efforts have been made including: 1) the improvements of expression and purification [7–11], 2) codon optimizations and mutagenesis [12–16], 3) the creation of chimeric enzymes with other DNAPs [17] and 4) the creation of fusion enzymes with a DNA-binding motif, such as Sso7d or helix-hairpin-helix (HhH) [18, 19]. To date, several protein expression systems derived from bacteria, yeast, and baculovirus/insect cells, have been utilized for efficient mass-production of active DNAPs [9, 20, 21]. As for production of family-B DNAPs, the native or site-mutated Pfu-Pols are produced in the *E. coli* [7, 13, 22] or *Spodoptera frugiperda* (Sf9)/baculovirus vector expression systems (Sf9-BEVS) [21], and their activities have been verified. Especially, Sf9-

BEVS produced ~100 mg/L active Pfu-Pol in the secretion form, indicating the possibility that the BEVS allows mass-production of DNAPs to be a commercial level [21].

It is reported that Tkod-Pol is less thermostable than Pfu-Pol, but that both two enzymes exhibit equivalent fidelity of ~0.4% mutation frequency [6, 17]. Moreover, Tkod-Pol demonstrates ~5-fold more rapid elongation rates (106-138 bases/s) and ~10-fold higher processivity (>300 bases/s) than those of Pfu-Pol in PCR [6]. It is later validated that KOD-Pol displays superior performance in normal and real time PCR [17]. To date, Tkod-Pol has been produced in *E. coli* [6] and *Pichia pastoris* [19] expression systems but not in BEVS.

It is known that the amount of recombinant proteins produced in silkworm-BEVS with silkworm larvae is often extremely higher than that in Sf9-BEVS with cultured cells [23]. In this study, to test whether silkworm-BEVS is suitable to produce recombinant Tkod-Pol (rKOD) in a commercial way, we expressed and purified his-tagged rKODs in N- (rKOD-N) or C-terminus (rKOD-C) and assessed the activities of purified rKODs under various conditions. The rKODs expressed in silkworm-BEVS are functional, and the production level of rKODs are satisfactory in a commercial way (about 1.1 mg/larva (rKOD-N) and 0.25 mg/larva (rKOD-C)). Compare to commercial *E. coli*-derived KOD-Pol, C-terminal his-tagged rKOD-C demonstrated equivalent processivity and is thermostable under continuous high-temperature toleration at 95 °C, while, N-terminal his-tagged rKOD-N is significantly unstable against continuous heat-shock. Besides, we noticed two unexpected rKOD protein isoforms in N-terminal, but not in C-terminal his-tagged constructs. It turns out that both short rKOD isoforms hold no DNA-Pol activity. Furthermore, we tested binding property of rKODs with DNA and RNA. Additionally, proofreading activity was exhibited from both purified rKODs. Taken together, our results of mass-production of rKODs from silkworm-BEVS would promote DNAP/PCR-based biological experiments to an economical

level in an accurate and time-saving manner and more importantly, may contribute to a better understanding of fundamental basics of DNAPs for further applications.

Materials and Methods

Cells and silkworms

The cultured silkworm cell line, NIAS-Bm-oyanagi2 (BmO2) was cultured in insect IPL-41 medium (Sigma, St. Louis, MO) supplemented with 10 % fetal bovine serum (FBS, Gibco, Grand Island, NY). The silkworm strains (n70) were provided by the silkworm stock center of Kyushu University supported by the National BioResource Project (NBRP, <http://www.shigen.nig.ac.jp/silkwormbase/index.jsp>). The larvae were regularly reared on mulberry leaves at 25-27 °C.

Construction of recombinant baculoviruses for rKOD

Thermococcus kodakarensis KOD DNA polymerase (Tkod-Pol, mature type, NCBI accession No. 29671) gene was chemically synthesized (Genscript, Picataway, USA) and cloned into pUC57 vector. The Tkod-Pol gene was amplified with KOD-Plus-Neo DNA polymerase (TOYOBO, Tokyo, Japan) using gene-specific primers (listed in Table 1) and further inserted into the modified pENTR11 vectors containing an N-terminal or C-terminal 8His-Strep-6His-TEV, TEV-8His-Strep-6His tag, respectively (Figure 1A). The resulting constructs were termed pENTR11L21-dHisStrepTEV-Tkod-Pol (N) and pENTR11L21-Tkod-Pol-TEVStrepdHis (C), respectively. The transfer plasmids for generating recombinant baculovirus were subsequently constructed using gateway LR reaction between each entry vector and pDEST8 vector (Invitrogen, Carlsbad, California) following the manufacturer's protocol (Fig. 1A). Recombinant baculoviruses were further generated using BmNPV/T3

116 bacmid system as described previously [24]. Concisely, 1×10^5 BmO2 cells in 24-well plates
117 were transfected with 200 ng purified bacmid DNA using FuGENE HD transfection kit
118 (Promega, Madison, WI). Then 4 days after transfection, the culture medium was gathered
119 and centrifuged at 1,000 g for 10 min at 4 °C. The resulting supernatants with recombinant
120 P1 viruses were used for several subsequent infections to obtain high titer virus according to
121 the protocols described in the manufacturer's manual (Invitrogen). Instead, cell pellets re-
122 suspended in ice-cold PBS were subjected to Western blotting analysis for the recombinant
123 Tkod-Pol polymerases, rKOD-N or rKOD-C, respectively. The P3 viral stocks were kept in
124 the dark at 4 °C until use for infecting silkworm cells or larvae. The viral titer was determined
125 by the end-point dilution method [25].

127 **Pilot evaluation of rKOD polymerases from infected cultured silkworm cells and larvae**

128 BmO2 cells (1×10^5 cells /well) or n70 silkworm larvae (fifth instar larvae at day 3) were
129 infected with the recombinant viruses at a multiplicity of infection (MOI) of 1 or 1×10^5
130 plaque-forming unit (PFU) per larva, respectively. At 4 days post-infection (dpi), the infected
131 cells or fat body (1 mg) were recovered by centrifugation at 1,000 g (7780II Kubota, Tokyo,
132 Japan) for 10 min. The intracellular proteins were prepared from fat body tissue cells or cell
133 pellet in 1 ml modified lysis buffer (PBS, pH7.5, complemented with 0.05% NP-40 and PIC
134 (Protease Inhibitor Cocktail, Roche)) and rotated (2 rpm, RT-5 Taitec, Tokyo, Japan) for 4
135 hrs at 4 °C. The samples containing rKOD were heat-shocked at 75 °C for 10 min and then
136 centrifuged at 15,000 g for 30 min at 4 °C. As for cell protein fractions, the supernatant
137 containing soluble rKOD was further concentrated to a final 25 µl by ultrafiltration using
138 Amicon 30 K filters according to the manufacturer's instructions (Millipore). Subsequently, 5
139 µl of each lysate was utilized for SDS-PAGE gel loading or directly applied for PCR to

amplify the attL1-L2 fragment from the 10 ng pENTR11 (Invitrogen) plasmid DNA template using attL1 and attL2 primers (listed in Table 1).

Purification of rKODs produced in silkworm larvae

As for the large-scale production of rKODs, fat bodies were collected from 10 infected n70 larvae at 4 dpi and dissolved in buffer A (20 mM Tris-HCl pH 7.4, 0.5 M NaCl, 0.1% CHAPS, PIC (1 tablet/50ml, Roche)) and rotated (2 rpm, RT-5 Taitec, Tokyo, Japan) at 4 °C overnight. Then the lysates were centrifuged at 52, 500 g for 30 min at 4 °C. The supernatant was filtered using a 0.45-µm filter (Millipore) and subjected to the purification process.

Purification of the rKODs was modified from our previously published protocol [26]. Concisely, a two-step purification protocol was executed based on the terminal tandem His and STREP tags. Firstly, lysates containing rKODs were purified by nickel affinity chromatography with HisTrap excel column (GE Healthcare Bioscience, Piscataway, NJ), and eluted by increasing concentrations of imidazole solution buffers (100-500 mM, see supplemental Fig. 1A and 1B). Then concentrated (Amicon 30 K filter, Millipore) elution was buffer-changed in binding buffer B (100 mM Tris-HCl pH 8.4, 150 mM NaCl, 1 mM EDTA, PIC (1 tablet/50ml, Roche)). A final 25 ml diluted solution was applied to StrepTrap HP column (GE Healthcare), followed by elution with the buffer B containing 2.5 mM desthiobiotin. All fractions were separated through 10% SDS-PAGE electrophoresis and visualized with Coomassie Brilliant Blue (CBB) R-250. The purified rKODs were subsequently dialyzed in a storage buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 0.1% Tween-20, 0.1% Nonidet P-40, 50% Glycerol). Finally, the purified rKOD proteins were quantified via ImageJ software using bovine serum albumin (BSA) as standard (Supplemental Fig. 1C).

Western blot analysis

All protein samples were electrophoresed on a 10% SDS-PAGE under the reducing condition and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Milford, MA), and further blocked in 5% skim milk (Wako, Tokyo, Japan), followed by incubation with HisProbe-HRP (1: 20000; Thermo Scientific, Rockford, IL) at room temperature. The protein bands were imagined using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

MALDI-TOF MS

MALDI-TOF MS was performed following our previous protocols [27]. In brief, four distinct rKOD protein bands (N, S1, S2, C) were excised from CBB-R250 stained gels, respectively, washed twice with Milli-Q water to remove any excess liquid. Then gels were destained with 300 μ L of 1:1 (v/v) solution of 50 mM ammonium bicarbonate and 100% acetonitrile (ACN) for 30 min at 37 °C. The supernatant was discarded and repeat the above step until the gels are clear. Subsequently, gel pieces were dehydrated with 100% ACN for 5 min at 37 °C, then reduced the protein in 50 μ L of 10 mM DTT for 20 min at 37 °C. The proteins were in-gel digested with 10 μ g/mL of trypsin (Sequencing Grade, Promega, Madison, WI, USA) at 37 °C for 12 h, followed by sonication treatment. Finally, the resulting peptides were collected and further mixed with an equal volume of saturated α -Cyano-4-hydroxycinnamic acid (CHCA, Sigma) in 0.1% TFA and 50% ACN. The desalted and enriched mixture by ZIC-HILIC microtips (Protea, Morgantown, WV, USA) was further analyzed with MALDI-TOF mass spectrometer (AXIMA-CFR Plus, Shimadzu, Kyoto, Japan) and the spectra were collected in the reflectron mode according to manufacturer's instructions. Finally, the resulting spectra were used to analyzed the possible deletions of S1 and S2 forms of N-terminal rKODs.

Electrophoretic mobility shift assay (EMSA)

200 ng of 800 bp double-stranded DNA (dsDNA) or 200 ng total RNA isolated from cultured silkworm BmN4 cells was mixed with indicated amount of purified rKODs in a binding buffer containing (10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1 mM DTT). To verify the single-stranded DNA (ssDNA) binding activity of the rKODs, 800 bp PCR product was first denatured at 100 °C for 10 min followed by cooling on ice rapidly to acquire ssDNA. Protein K (50 µg/ml) treatment was used as a negative control. After a 1-hour incubation at 37 °C, samples were loaded on 1% agarose gel running at 10 V/cm in TAE buffer, followed by staining with ethidium bromide (EtBr, 0.5 µg/ml). Subsequently, the fraction bound was quantified using ImageJ software to estimate the binding parameter, apparent K_d (non-linear curve fitted, Hill equation).

DNA polymerase activity assay

A simplified PCR-based assay was performed to determine the relative DNA polymerase activities by comparing the band intensities as described previously [7, 21]. In brief, same amount of purified rKODs, which are equal to 1U of purchased standard KOD (Toyobo, Japan), were used for PCR to amplify the attL1-L2 fragment from the 10 ng pENTR11 (Invitrogen) plasmid DNA template using specific primers (listed in Table 1). The PCR was initiated at 95 °C for 2 min followed by 30 cycles of 95 °C for 15 s, 58 °C for 15 s, 68 °C for 10 s, and a final hold of 68 °C for 10 min. Subsequently, EtBr-stained DNA bands were quantified and density compared relatively in ImageJ. To test the usability against various templates, we directly utilized silkworm genomic DNA, silkworm RNA reverse-transcribed cDNA, plasmid DNA (pENTR11), pENTR11 transformed *E. coli* (DB3.1, Invitrogen)

colony, baculovirus-infected silkworm hemolymph, and silkworm fat body tissues in PCR to amplify target DNA fragments using gene-specific primers (Table 1).

A protocol described previously was applied for rKODs to determine the optimal reaction conditions by varying individual parameters, pH, extension temperature, and MgSO_4 and KCl concentration. Various cations (Ca^{2+} , Cd^{2+} , Co^{2+} , Cu^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Sr^{2+} , Zn^{2+}) were screened for the possible activations of rKOD at a final concentration of 1.5 mM. Mn^{2+} and Co^{2+} cations were further optimized against increasing final concentrations (0~3 mM). To compare the relative activities, mean values for each experimental condition were further plotted against highest (100%) intensity. As for efficiency verification of long PCR, several PCR primer pairs (listed in Table 1) were designed for 2 kbp, 5 kbp and 8 kbp amplicon, respectively. The standard KOD (Toyobo, Japan) was used as positive control.

As for the activity identifications of rKOD-N products (rKOD-N, rKOD-S1, and rKOD-S2), four distinct rKOD protein bands (N, S1, S2, C) were excised from SDS-PAGE gels (CBB-unstained; CBB-stained gel was used as reference during excision), respectively, washed twice with Milli-Q water to remove any excess liquid. Then sliced gels were equilibrated in 500 ml PBS, frozen-thawing for three times using liquid nitrogen, and further subjected to vortex for 3 hrs at 4 °C. The extracted rKODs were subsequently dialyzed in a storage buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 0.1% Tween-20, 0.1% Nonidet P-40, 50% Glycerol), concentrated through ultrafiltration (Amicon 30 K filter, Millipore) and detected in SDS-PAGE. Same PCR parameters were followed as described above for activity identifications of each purified isoform. The rKOD-C and KOD-T were used as controls.

Thermostability Assay

2 µg of each purified rKOD (N or C) was tested for stability at 37 °C or 95 °C by incubating in 1× reaction buffer (10 mM Tris-HCl, pH 8.8, 1 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.01% Triton-100, and 0.1 mg/ml BSA) for different time indicated (0-12 h) and then verifying relative polymerase activities by PCR as described above. Differences in density of EtBr-stained bands were quantified using ImageJ.

Fidelity assays

Mutation frequency of DNA polymerase was measured according to the method reported elsewhere [4, 6]. An entire lacZ gene containing plasmid (Agilent) was inverse PCR amplified with indicated KOD by Blue-F and Blue-R primers (Table 1) holding 20 bp homologous sequences. Subsequently, the PCR products were digested with *DpnI* to eliminate background plasmid contaminations and further purified with FastGene Gel/PCR Extraction Kit (Nippon genetics, Japan). Then an equal amount of DNA was used for *E. coli* (Top10, Invitrogen) transformation. The transformed *E. coli* was incubated for 30 min at 37°C and plated on LB plates containing 1 mg/ml X-gal (Sigma). When calculating mutation frequencies, light blue and white plaques were considered as mutants.

Proofreading assay

Proofreading activities of rKODs were measured as previously reported method [4], except that 60-mer long template oligomer DNA (Temp) and 31-mer complementary primers to the template with or without 3' mismatches (WT, M1, M2, M3, see details in Fig. 5A) at the *EcoRV* site are used for extension. See detailed sequence information in figure 5A. The elongation reaction was performed at 72 °C for 10 min in a 20 µl mixture of 1× reaction buffer (10 mM Tris-HCl, pH 8.8, 1 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.01% Triton-100, and 0.1 mg/ml BSA) supplemented with 0.2 mM of each dNTP, 60-mer template

(500 ng), 31-mer primer (250 ng), and indicated rKOD-pol. The purified resulting products were digested with *EcoRV* enzyme (Takara, Japan) and then resolved on a 12 % polyacrylamide gel (run in Tris/borate/EDTA buffer) and visualized by EtBr (0.5 µg/ml).

Results

Active rKODs can be expressed in silkworm cells

To test whether an active rKOD can be expressed in silkworm, we designed two constructs with N-terminal (rKOD-N) or C-terminal (rKOD-C) tandem His-Strep-His tags (Fig. 1A). Using these two rKOD constructs, we successfully generated corresponding BmNPVs in cultured silkworm cells as described in "Materials and Methods". Subsequently, the expressions of rKOD-N and rKOD-C were detected in virus-infected BmO2 cells at 3 dpi by Western blot. As shown in figure 1B, correct expression bands at approximately 94.5 kDa were detected in all culture media, as well as soluble and precipitate portions of cell lysates, from both rKOD-N and rKOD-C samples. It is also noticed that the expression level of rKOD-N is higher than that of rKOD-C. Moreover, unlike the clear single band from rKOD-C, it is noteworthy to discover other two smaller bands in the soluble portion of rKOD-N cell lysates, indicating that the different expression patterns exist between rKOD-N and rKOD-C in silkworm cells.

To tell if the expressed rKODs are functional enough to perform a PCR, we achieved pilot experiments to see their activity. As described in "Materials and Methods", we prepared the soluble crude lysates with heat-shocked (75 °C for 10 mins) from the cultured cells or from the fat bodies of silkworm larvae, then we directly added them to PCR reaction. As demonstrated in figure 1C, except the mock-infected samples, both rKOD-N and rKOD-C crude lysates, from either cultured cells or fat bodies, resulted in successful amplification of

target bands. These results indicate that silkworm cells produce efficient rKODs for gene PCR manipulation.

Silkworm-BEVS with a silkworm larva can scale up the amount of rKOD protein products

Given that the silkworm larvae can be used to scale-up the resulting yield of expressed proteins [23], we expressed rKODs in silkworm larvae, N70 strain, by infecting with recombinant viruses and purified rKODs from silkworm larvae through a two-step purification procedure (see Material and Methods for purification details). As shown in supplemental figure 1A and 1B, clear bands were detected in fractions eluted both at 100 and 500 mM imidazole concentration from His purification column. Similar to the Western blot results of cultured cells (Fig. 1B), a single main band was found in the rKOD-C elution fractions, whereas unexpected smaller two bands—rKOD-S1 and rKOD-S2—were seen in rKOD-N fractions even after the second purification through Strep-column (Fig. 2A and 2C). We also estimated the yields of purified rKODs expressed in silkworm larvae (Supplemental Fig. 1C). The estimated yields of purified rKODs were 1.1 mg (rKOD-N) and 0.25 mg (rKOD-C) per infected silkworm larva. Combined with the results from our pilot activity experiments, we confirmed that silkworm-BEVS with silkworm larvae can provide satisfactory amounts of active rKODs.

Only full length of rKOD-N retains its activity

To characterize the unexpected expression products of rKOD-N, we separated rKOD-N, rKOD-S1 and rKOD-S2 seen in figure 2C by gel filtration column (Fig. 2D). Then we added each protein to PCR reaction to check their activity. As shown in Figure 2E, only the entire length of rKOD-N exhibited DNA polymerase activity. Therefore, we sent rKOD-S1 and

rKOD-S2 to MALDI-TOF MS to check their amino acid sequences and analyzed the resulting spectra. From the spectra distribution of trypsin-digested peptides, we found that rKOD-S1 and rKOD-S2 lacks some peptides in DNA polymerase domain, causing both truncated proteins to be inactive in DNA polymerase functions.

rKODs hold preferred DNA binding property

KOD-Pol is known to bind to dNTPs and DNA chain as KOD/primer/template duplex [28]. To test whether rKODs that produced by silkworm-BEVS hold the instinctive DNA-binding property, we performed electrophoresis mobility shift assay (EMSA) by using different substrates, dsDNA, ssDNA, and mRNA, to assess the possible binding activity of purified rKODs. First, in figure 3A, we validated that our purified rKODs have no nuclease activity to dsDNAs or mRNAs. Then we incubated rKODs with dsDNA or mRNA, the results of which showed dsDNA mixture was shifted but not mRNA (Fig. 3B). Subsequently, detailed binding activities of rKODs were tested towards the ssDNA substrate, which was demonstrated in figure 3C. From the differences in density of the DNA fraction bound, we estimated the binding parameters for ssDNA-rKOD-N or -rKOD-C, respectively. The results indicated that KOD exhibited specific DNA binding property at roughly apparent K^d of 150 nM.

rKODs produced in silkworm-BEVS can be used for PCR with dirty samples

It is known that a successful PCR amplification relies on DNA template quality. We then first assessed the usability of rKODs for various PCR applications, especially, to amplify template DNAs in harsh samples. In this study, we employed samples: pENTR11 plasmid DNA, silkworm genomic DNA, silkworm cDNA, pENTR11 in *E. coli* colony, baculovirus-infected silkworm hemolymph, and silkworm fat body. As demonstrated in Fig. 4, like the

commercial KOD-T, rKOD-N and rKOD-C were equally active to amplify correct fragments of interest with all samples (Fig. 4A). We also validated that rKODs purified from silkworm larvae are free from any silkworm genomic contamination (Fig. 4A, 2nd panel). These results indicate that rKODs produced in silkworm-BEVS are active and clean enough for the routine DNA manipulations such as colony PCR and microbial contamination.

Terminal tags are influential for thermostability and function of silkworm-BEVS rKOD

To further characterize rKODs produced in silkworm-BEVS, we first examine the thermostability of rKODs by heating rKODs at 95 °C for a long duration (see Material and Methods). Upper panel in figure 4B shows control experiment, where we incubated rKODs at 37 °C for up to 12 hrs. Both the rKOD-N and rKOD-C are stable without apparent degradation by proteases. Then we incubated rKOD-N and rKOD-C at 95 °C, finding that rKOD-N, surprisingly, was denatured rapidly when it was incubated at 95 °C (Fig 4B, middle panel). In line with a protein denaturation, the loss of protein activity of rKOD-N was seen when these heat-shocked rKOD-N proteins were subjected to PCR reaction (Fig. 4B, down panel, Fig. 4C). On the other hand, rKOD-C demonstrated similar thermostability to KOD-T and even retained ~70% of original activity up to 8 hr incubation at 95 °C, which is significantly more active than KOD-T did (Fig 4C). These results indicate that N-terminal tag affects the thermostability of rKOD produced in silkworm-BEVS but that C-terminal tag does not interfere with the performance of rKOD. Figure 4D shows mutation frequency of rKODs in PCR reaction. rKOD-N and rKOD-C have a comparable mutation frequency to KOD-T (about 0.081%, 0.086%, and 0.111%, respectively). We also varied the amount of template DNA from 1 ng to 30 ng in PCR reaction. As the template DNAs increased, the resulting PCR products by KOD-N and KOD-C rise steadily, but KOD-N saturated with lower amount

of template DNAs than KOD-C did. This result is consistent with the previous finding that N-terminal tag affected the rKOD polymerase activity produced in silkworm-BEVS (Fig. 4E).

Optimal conditions are determined for rKODs

To determine an optimal condition for PCR reaction by rKODs, we varied individual parameters in the reaction buffer and determined optimal values. Figure 4F shows rKOD-N and rKOD-C activities under different pH, temperature, KCl, and MgSO₄ concentration. The optimal condition for PCR by rKOD is: 8.5 (pH), 75 °C (temperature at extension), 50 mM (K⁺) and 1.5-2 mM (Mg²⁺). Furthermore, besides Mg²⁺, we screened other divalent metal ions to find a possible rKOD cofactor by using rKOD-C, which showed more polymerase activity than rKOD-N. The results from figure 4G exhibited that Co²⁺ and Mn²⁺ can also activate rKOD-C. rKOD-C increased its activity by ~1.8-fold with Mn²⁺ at a final concentration of 1.0 mM than that with Mg²⁺ at a final concentration of 1.5 mM, while rKOD-C with Co²⁺ shows less activity by 0.6-fold at a final concentration of 1.5 mM.

As for the efficiency verification of long PCR, several PCR primer pairs (listed in Table 1) were used for 2 kbp, 5 kbp and 8 kbp amplicons from bacmid DNA (Fig. 4H). It is reported that KOD-Pol could provide a high elongation rate of about 106-138 bases/s [6]. Compared with positive control of KOD-T, high extension rate (106-138 bases/s; 6) was observed in both rKOD-N and rKOD-C from silkworm-BEVS, except that rKOD-N is relatively low productive as judged from the DNA band intensity. This result also agrees with the previous finding that the N-terminal tag of rKODs leads to less polymerase activity.

Finally, to analyze 3'-to-5' exonuclease activity of the rKODs from silkworm-BESV, an 80-mer long DNA template and four 31-mer primers containing 0 (WT), 1 (M1), 2 (M2), and 3 (M3) mismatches at the 3' terminus of *EcoRV* site were used as a substrate for proofreading assay (Fig. 5A). After primer extension reaction by rKODs, we examined the

extended products by digesting with *EcoRV* enzyme whether mismatches at the 3' terminus of *EcoRV* site are properly removed or not. As demonstrated in Fig. 5B, all rKOD-T, rKOD-N, and rKOD-C could extend WT primer to an 80-bp (lane1, 3, 4), and the resulting extended products could be digested by *EcoRV*, leading that two bands—51-bp and 29-bp—were seen (lane2, 4, 6). Then we used substrates containing M1, M2, and M3 mismatched primers. Both rKOD-N and rKOD-C extended all mismatched substrates, and resulting products could be digested by *EcoRV*, suggesting that 1 nt, 2 nt, and even 3 nt mismatch nucleotides at the 3' terminus of primer were successfully removed by exonulcease activity of rKODs. Moreover, as shown in lanes 5, 11, 17 and 23, rKOD-C provided a better performance in resulting products, which again verified the usability of this enzyme in this study.

Discussion

In this report, we could achieve about 1.1 mg and 0.25 mg of pure rKOD-N and rKOD-C per a virus-infected silkworm larva by silkworm-BEVS. Nowadays, BEVS has been preferred for mass-production of higher eukaryotic recombinant proteins, partly because lepidopteran insects or cultured cells hold similar post-translational modifications (PTMs)—phosphorylation [29] and glycosylation [23, 25]—to higher eukaryote, while *E. coli* expression system lacks most of the eukaryotic PTMs. Additionally, by incorporating human glycosyltransferases, lepidopteran insects or cultured cells could be also engineered to produce human-like glycoproteins with similar or identical glycol-branches, which is expected to apply for further pharmacological analysis and clinical uses [30, 31]. More promisingly, versatile baculovirus multigene expression vectors enable us to produce and study heterologous multiprotein complexes *in vivo* and *in vitro* [32, 33]. Recently in our group, various proteins have been mass-produced by BEVS with either cultured silkworm cells or silkworm larvae. It is significant that silkworm larvae could easily scale-up the yields

without any additional equipment and favorably ensure the original activity, stability, and solubility of produced proteins of interest avoiding inclusion bodies formation from *E. coli* expression system [34–39]. Moreover, silkworm-BEVS with silkworm larvae is a time-saving method as compared to the cultured cell-based BEVS [29].

Wang et al. [19] reported that the secreted form of a rKOD-Sso7d expressed from yeast *P. pastoris* resulted in an amazing ~250 mg/L product before purification. A useful platform has been established in our laboratory to produce proteins of interest in a secretion form as native protein or protein with silkworm endogenous signal peptide (SP) [35, 36, 38]. We showed that proteins expressed as a secretion form sometimes lead to redundant post-translational modifications, glycosylation, for example, which causes partial loss of functions in some cases [38]. Therefore, technically, it is better to, but not always, choose to express a protein of interest in its native form, secreted or intracellular, to avoid losing its original biological activity. Interestingly, the secreted form of glycosylated rKOD-Sso7d demonstrated higher thermostability (100 °C, up to 11 hrs) than *E. coli* produced rKOD-Sso7d [19]. We confirmed that rKOD-C expressed by silkworm-BEVS with silkworm larvae retained ~70 % of original activity after 95 °C incubation for 8 hrs (Fig. 3B, 3C), which is better than commercial KOD-T expressed in *E. coli*. Further engineering of KOD protein, such as alternation of expression fashion into secreted form with desired modifications and/or site mutation of certain amino acid, should be considered for thermostability improvement.

DNA polymerases have always been extensively studied for its great biological and economic significance. In the current study, we chose and mass-produced a Pol B KOD-pol from Archaea *T. kodakaraensis*, which was identified as a thermostable and efficient polymerase with high-fidelity [6]. A tandem His-strep-His tag at the N- or C-terminal of rKOD (Fig. 1A) was designed to facilitate protein purification processes and to improve the purity of the resulting proteins of interest. As indicated in our results of purification (Fig. 2A,

2B, 2C and Supplemental Fig 1A, 1B), silkworm-BEVS expressed rKODs demonstrated high binding affinity to both His and Strep columns. As a result, we successfully obtained highly purified rKOD-C through simple two step purifications. (Fig. 2A, 2B). As for the purification methodology, Takagi et al. [6] used a heparin column to purify a stable mature KOD without any fusion tags from the *E. coli* expression system. We also identified in this study that purified rKOD-N and rKOD-C possessed DNA-binding activity (Fig. 3B, 3C). Since low thermostability was discovered from N-terminal but not C-terminal tag-fused rKOD, it also would be worthy to express and purify native KOD in silkworm-BEVS by using heparin column to avoid potential negative effects from terminal fusion tags.

It is shown that purified rKOD-C but not rKOD-N showed comparable polymerase activity and fidelity with the commercial KOD (Fig. 4), even though rKOD-N can also be used in PCR to amplify target DNA fragments (Figs. 1C, 4A) and exhibited proofreading activity (Fig. 5B). Significant loss-of-function was detected when rKOD-N was used for long PCR assay (Fig. 4H). As shown by MALDI-TOF MS analysis, we found that the truncated products—rKOD-S1 and rKOD-S2—missed some peptides in the polymerase domain. Because the same smaller expression products were observed from yeast [19] and *E. coli* expression system (data not shown), it is possible that N-terminal tags changed the mRNA structure and further affected the resulting proteins, or that N-terminal tags contribute negatively to the overall thermostability of KOD and therefore reduce its activity as a DNA polymerase, or that some specific endogenous protease of *E. coli* cleaves KOD only when N-terminal tag was fused. It demonstrated that three cations, Co^{2+} , Mn^{2+} , and Mg^{2+} could activate polymerase activity of KOD (Fig. 4H, left panel). Interestingly, Mn^{2+} exhibits ~1.6-fold more activity than Mg^{2+} does (Fig. 4H, right panel). It is reported that both the accuracy of base selection and the specificity of exonuclease activity are altered by the substitution of Mn^{2+} for Mg^{2+} [40]. These results could be further applied to accelerate colony PCR for

amplifying long DNA fragments under a time-saving model or be used for introducing random mutations with a Mn^{2+} containing reaction buffer.

Besides, rKODs displayed efficient proofreading activities as shown in figure 5B. According to previous reports that mutations at H147 or I142 in the unique loop of the exonuclease domain away from the Thumb-2 subdomain of KOD resulted in alternations of polymerase activities, 3'-5' exonuclease activities, PCR fidelities and/or elongation rates [41, 42]. With these supportive results, we could produce various rKOD in silkworm-BEVS with distinct purposes, such as introducing random mutations into a gene by using I142R KOD in an error-prone PCR.

In this study, we produced intracellular forms of rKODs utilizing silkworm-BEVS and verified their characterizations along with the commercial rKOD, which was expressed in *E. coli* expression system. As a result, we successfully obtained active rKODs with satisfactory yields from silkworm larvae. Additionally, the rKODs exhibited preferable PCR performance, and especially, rKOD-C has almost the same performance compared to the commercial rKOD. Taken together, our results suggested that silkworm-BEVS can be used as an alternative method for producing active rKOD in a commercial way.

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Conflict of interest The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1. (A) Schematic representation of constructed recombinant Tkod-Pol (rKOD) with N- or C-terminal His-Strep-His tandem fusion tags. Bacmid DNAs and BmNPV P3 viruses were obtained using the methods as described in Materials and methods (B) Expression verifications of rKODs in virus-infected BmO2 cells at 3 dpi by Western blot using His-Probe. *M*, 100 kDa protein standard marker; *Medium*, culture medium from infected cells; *Soluble*, soluble portion of cell lysates; *Precipitate*, insoluble portion of cell lysates. # represented two unexpected products from N-rKOD. (C) Pilot function evaluation for rKOD expressed from virus-infected BmO2 cells or silkworm fat body, respectively. Crude proteins from indicated samples were loaded to SDS-PAGE gel or utilized directly for PCR to amplify attL1-L2 fragment from the 10 ng pENTR11 (Invitrogen) plasmid DNA template using attL1 and attL2 primers (listed in Table 1). *M*, DNA or protein standard marker KOD-T, commercial KOD purchased from Toyobo Company *Mock*, without viral infection; N: N-terminal tag fused rKOD; C: C-terminal tag fused rKOD. Detailed sample preparations were described in Materials and methods.

Fig. 2. Purification and expression pattern analysis of rKODs from silkworm fat body. Fat bodies were collected from each 10 N (A) or C (B) rKOD virus-infected n70 larvae at 4 dpi and dissolved in the lysis buffer. Then a two-step purification protocol was executed based on the terminal tandem His and STREP tags (see Materials and methods for detailed procedures). M, molecular markers; FT, flow-through; Lanes 2–5, nickel affinity chromatography; lanes 6–8, Strep-Tactin affinity chromatography; lane 4, integrated elution fraction by 100 and 500 mM imidazole; lane 8, elution fraction by 2.5 mM desthiobiotin. All samples were resolved in 10% SDS-PAGE and visualized by CBB staining. (C) The purified rKODs, N and C, were compared with commercial Tkod-Pol together in a single SDS-PAGE gel. T, commercial KOD purchased from Toyobo. The arrows indicate the low molecular bands of rKOD-N termed S1 and S2, respectively. All products from rKOD-N were further isolated from gel (D) and utilized directly in PCR to amplify target fragment indicated by an arrow, where rKOD-C and KOD-T were used as control (E). S1 and S2 protein were subjected to MALDI-TOF MS analysis and the spectra were collected in the reflectron mode according to manufacturer's instructions. Finally, the resulting spectra distributions were plotted in (F, upper panel) and used to determine the possible deletions of S1 and S2 forms of N-terminal rKODs (f, lower panel).

Fig. 3. Purified rKODs hold preferred DNA binding property (A) Purified rKODs were tested for possible nuclease contaminations. 200 ng of 800 bp double-stranded DNA (dsDNA) or 200 ng total RNA from cultured silkworm BmN4 cells was mixed with 0.164 µg purified rKODs in a binding buffer with or without DNase or RNase at 37 °C for 3 h and protein K (50 µg/ml) treated samples were loaded on 1% agarose gel. As for test of binding ability towards indicated substrates, protein K (50 µg/ml) was not added and the resulting mixture was directed applied for electrophoresis (B). To verify ssDNA binding activity of the rKODs,

200 ng ssDNA were incubated with increasing amount of BSA or rKOD (C). After a 1-hour incubation at 37 °C, samples were separated on 1% agarose gel (c). Protein K (50 µg/ml) treatment was used as a negative control. Subsequently, the fraction bound was quantified using ImageJ software to estimate the binding parameter, apparent K_d (non-linear curve fitted, Hill equation).

Fig. 4. Evaluation of purified rKODs. (A) Each purified rKOD of equal amount of commercial Tkod-Pol (0.164µg, 1U) was used for PCR to amplify target fragment from various indicated templates. As for detection of possible silkworm genomic contamination, a 50-cycle programed PCR was employed to amplify silkworm endogenous GAPDH gene. (B) Stability and thermostability of the purified rKODs were verified by incubation at 37 °C or 95 °C for 0-12 h. After incubation, the resulting protein was displayed in SDS-PAGE or assayed its activity in PCR, respectively. It is observed that a rapid degradation in N-terminal tag fused rKOD but not in rKOD-C. Detailed comparisons were demonstrated in (C), in which KOD-T was utilized as control. Subsequently, mutation frequency was confirmed by method described in “Fidelity assays” and further summarized in (D). When using increasing template DNA, the resulting PCR products rise steadily and could be positively productive even at a low concentration of template DNA (E). Then, some essential parameters, pH, extension temperature, KCl and MgSO₄ concentration were examined in (F). Furthermore, total nine cations were screened for their possible activation of KOD polymerase which were shown in (G). Detailed analyses demonstrated that and Mn²⁺ triggers ~1.8-fold of activity at a final concentration of 1.0 mM than Mg²⁺ (1.5 mM), while Co²⁺ is a weak metal ion activator for the KOD polymerase. (H) Long PCR performance was evaluated by employing bacmid DNA template to amplify 2 kbp, 5 kbp or 8 kbp fragment, respectively.

686 **Fig. 5.** Proofreading assay of rKODs. (A) Schematic representation of used oligo primers.
687 Mutated sites and *EcoRV* cleavage sites were indicated by arrows. (B) The elongation
688 reaction was performed at 72 °C for 10 min in a 20 ul mixture of the reaction buffer
689 supplemented with 0.2 mM of each dNTP, 60-mer template (500 ng), 31-mer primer (250
690 ng), and indicated rKOD-pol. The purified resulting products were digested with *EcoRV*
691 enzyme and resolved on a 12 % polyacrylamide and visualized by EtBr (0.5 µg/ml).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

SuppFigure 1

Supplemental Fig. S1 Detailed first purification of N-terminal (**A**) and C-terminal (**B**) tag-fused rKODs by nickel affinity chromatography. M, molecular markers; FT, flow-through; Lanes 5–7, elution fraction by 100 mM imidazole; Lanes 8–13, elution fraction by 500 mM imidazole. All samples were resolved in 10% SDS-PAGE and visualized by CBB staining. (**C**) The purified rKODs were quantified by ImageJ software using BSA as a standard. Lanes 1–5, BSA; Lanes 6–8, rKOD-N; Lanes 9–11, rKOD-C.