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Cloning and high-level expression of *Thermus thermophilus* RecA in *E. coli*: purification and novel use in HBV diagnostics

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ABSTRACT

We studied the role of *Thermus thermophilus* RecA in enhancing the PCR signals of DNA viruses such as HBV. The *RecA* gene of a thermophilic eubacterial strain, *T. thermophilus*, was cloned and hyperexpressed in *Escherichia coli*. The recombinant RecA protein was purified using a single heat treatment step without the use of any chromatography steps, and the purified protein (>95%) was found to be active. The purified RecA could enhance the PCR signals of HBV and improve the detection limit of the HBV diagnosis by real time PCR. The yield of recombinant RecA was ~35 mg/L, the highest yield reported for a recombinant RecA to date. RecA can be successfully employed to enhance detection sensitivity for the diagnosis of DNA viruses such as HBV, and this methodology could be particularly useful for clinical samples with HBV viral loads of less than 10 IU/mL, which is interesting and novel.

Introduction

The recombinase A (RecA) protein (accession no. AAK15321.1) from the thermophilic bacterium *Thermus thermophilus* is a thermostable enzyme that plays important roles in homologous recombination and DNA repair.[1] DNA recombination in prokaryotes is achieved by catalyzing the pairing of homologous DNA sequences,[2,3] and DNA repair is achieved by protection of the damaged DNA ends.[4,5] This protein has the activities of single-stranded DNA dependent ATPase, DNA annealing, and exchanging of strands between two recombining DNA double helices, similar to *Escherichia coli* RecA (accession no. AFU91764.1) protein, but the optimal temperature for the strand exchange of RecA is between 50 and 60 °C.[6]

RecA-like proteins have been sequenced from over 60 different prokaryotic species[7,8] and it hydrolyzes ATP when bound to DNA, and its ATPase activity rate is proportional to the amount of RecA bound to the DNA[9–12] with the fact that the ATPase activity is used to measure its binding efficiency to DNA.[13] It has been shown that the RecA protein catalyzes in vitro DNA strand exchange reactions[14] with peak efficiency

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at approximately 60 °C,[6] a temperature that is fairly close to the Tm required for thermal denaturation of dsDNA.[15]

Numerous applications of RecA are reported. These include identification of different *Burkholderia cepacia* genomovars,[16] sequence-specific ligation of DNA using the *E. coli* RecA protein,[17] maintaining the integrity of chloroplast DNA molecules in *Arabidopsis*,[18] auto-cleavage of other serine proteases and repressors of the bacteriophage lytic cycle,[19–22] and its role in swarming motility in *E. coli*.[23]

Shigemori et al.[24] have used RecA in stimulating pairing between completely matched primers and target sequences and effectively eliminating non-specific PCR products. This specificity enabled them to amplify the sequences between any sites of interest without needing to consider the optimal priming sites for each PCR. Similarly, Fukui and Kuramitsu[25] have recently utilized MutS, a thermostable mismatch-recognizing protein from *T. thermophilus*, along with RecA to reduce non-specific amplifications in PCRs.

Hence, we realized the immense potential of RecA in PCR-based diagnostics and attempted to generate RecA protein by a simple purification method that would in turn make the PCR based detection of viruses more sensitive and cost-effective.

Material and methods

Reagents

All chemicals were of analytical grade. Oligonucleotides, used in this study, were from BioServe Technologies Pvt. Ltd, Hyderabad, India. Bradford protein assay reagents and IPTG were obtained from Amresco, USA. Standard RecA pure protein was procured from NEB, USA, while ATP was purchased from Thermo Fisher Scientific, USA. Protein molecular weight markers 11–180 kDa and 11–97 kDa were obtained from G-Biosciences, USA and Aristogene Life Sciences, Bangalore, India respectively.

Cloning and construction of RecA expression construct

The synthetic gene of *T. thermophilus RecA* was custom synthesized from Integrated DNA Technologies, Inc. (IDT), USA, and cloned into the pUC57 vector. The nucleotide sequence of the *T. thermophilus RecA* gene was adjusted for codon bias without altering any of the amino acids. The *RecA* gene was excised from the pUC57-*RecA* plasmid as a NdeI/HindIII fragment and cloned into the pET26b vector into similar restriction sites. The recombinants were screened by PCR using T7 promoter specific primers, forward 5′ TAATACGACTCACTATAGGG 3′ and reverse 5′ GCTAGTTATTGCTCAGCGG 3′, and the positive clones were further confirmed by restriction analysis and DNA sequencing. The pET26b-*RecA* plasmid was designated as pCAN008.

The plasmid DNA of pCAN008 was introduced into ER2566 competent cells (NEB, USA) for expression of recombinant RecA. The protocol was essentially as described by Paul et al.[26] Briefly, the induction of RecA was effected by the addition of 1 mM IPTG when cells in LB medium (containing 20 μg/mL kanamycin) reached an OD_{600} value between 0.5 and 0.8. The induction proceeded for 4 h by incubating the culture at 37 °C, and then the induced cells were pelleted at 7670 × *g* for 10 min. The harvested cells were resuspended in 25 mM Tris. Cl, pH 7.5 and sonicated in a sonicator (Omni Sonic Ruptor 400, GA, USA) at 50% amplitude for one min on/off for 10 min in cold. The sonicated lysate was spun at 9000 × *g* for 20 min at 4 °C and the supernatant obtained was used for purification of the RecA expressed protein.

RecA purification by heat treatment

RecA is known to be thermostable,[27] and we utilized this property to purify the recombinant RecA protein. Different volumes of crude lysate of RecA were heated at 75 °C for 30 min, after which the samples were centrifuged at 30,670 × *g* for 10 min, and the supernatants were analyzed on 12% SDS–PAGE for RecA protein followed by silver staining as described by Nesterenko et al.[28] Protein concentrations were determined by Bradford's method[29] to quantify the total yield of RecA per liter of induced culture under the described experimental conditions.

ATP hydrolysis assay

ATPase activity was determined using a QuantiChrom ATPase/GTPase Assay Kit DATG-200 (EnzyChrom, USA). Phosphate standards were made by taking varying concentrations of phosphate (0–50 μM) in nuclease free water as per manufacturer's instructions. Equal amounts of proteins of in-house purified RecA and commercial RecA (New England Biolabs, UK) were used for detecting the ATPase activity. After incubating the RecA proteins with ATP, 200 μL of the supplied reagent was added, and contents were incubated for 30 min at room temperature. The OD of the samples, reflecting free phosphate release due to ATP hydrolysis by RecA, was read at $OD_{620\,nm}$ in an ELISA plate reader (Tecan Group Ltd, Männedorf, Switzerland).

Human leucocyte antigen (HLA) PCR

Human gDNA is the source of the template for the amplification of HLA genes.[30] The different protocols for HLA typing reported require use of gDNA between 5 and 30 ng/μL[31] and DNA concentrations lower than 2 ng/μL have been found to compromise genotyping results severely.[32] Since RecA is known to prevent false priming, we hypothesized that samples with lower concentrations of genomic DNA might display higher gene amplifications in presence of RecA. To validate this hypothesis, we carried out PCRs of HLA genes from human gDNA in presence and absence of RecA. The primers for amplification of HLA class I genes, namely, HLA-A-exon 2 and exon 3, HLA-B-exon 2 and exon 3 and HLA-C-exon 2 and 3, were as described by Itoh et al.,[33] while the primers for HLA class II genes such as DPB1, DQB1 and DRB1, targeting exon 2, were according to Lange et al.[30] The PCR reaction was carried out in 25 μL reaction volume and the PCR mixture comprised 1× ammonium sulfate buffer, pH 8.3; 2.5 mM magnesium chloride; 0.2 mM dNTPs; 5% DMSO; 0.2% Tween-20; 0.04% BSA; 0.4 μM HLA primers; and 1 unit of Taq DNA polymerase (ThermoFisher Scientific, USA) with 40 ng of gDNA. PCR conditions were as follows: Initial denaturation

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