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Recombinant expression, purification, and characterization of XorKII: A restriction endonuclease from *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

An endonuclease from *Xanthomonas oryzae pathovar oryzae* (*Xoo*) KACC10331, XorKII, was recombinantly produced in *Escherichia coli* by applying the stationary state induction method, which was necessary to prevent the unwanted lysis of *E. coli* cells. XorKII was purified by immobilized metal affinity chromatography on an FPLC system. The yield was 3.5 mg of XorKII per liter of LB medium. The purified recombinant XorKII showed that it recognized and cleaved to the same site as PstI. It behaved as a dimer as evidenced by the size exclusion chromatography. The specific activity of the purified XorKII was determined to be 31,300 U/mg. The enzyme activity was monitored by cleaving lambda DNA or YEp24 plasmid as substrates. The enzyme was the most active at 10 mM Tris–HCl pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol at 37 °C. XorKII was easily inactivated by heating at 65 °C for 5 min, but retained most of the original activity after incubation at 37 °C for 24 h.

Introduction

Restriction endonucleases play an important role in defending bacteria against invading viruses. They cooperate with methyl transferases so that unmethylated foreign DNA at N4 or C5 at cytosine or N6 at adenine within the recognition sequence gets cleaved while the properly methylated host DNA is protected [1]. These restriction endonucleases can be classified into four different types: Type I, II, III, and IV [2]. Among these four types, Type II restriction endonucleases are known to be homodimeric or tetrameric, cleave DNA within or close to the specific recognition sites, and require divalent ions such as Mg^{2+} for catalysis.

Xanthomonas oryzae pv. *oryzae* (*Xoo*)[1] is a member of the γ-subdivision of the Proteobacteria and causes bacterial blight on rice [3]. Bacterial blight is a vascular disease resulting in white lesions along the leaf veins. Severe infestation can cause yield losses as high as 50%. With a goal to better understand the pathogen and thus minimize the possible losses by this pathogen, the *Xoo* genome project was completed [4]. However, the restriction/modification system of the KACC10331 strain was highly active, and genetic analysis of this strain has failed. To overcome this predicament, it was thought to be essential to first understand the restriction/modification system.

In this work, we focused on XorKII, one of the two restriction endonucleases from *Xoo*. The other endonuclease, XorKI, was reported elsewhere [7,8]. XorKII was known to be an isoschizomer of PstI [5]. However, in the previous report [5], XorKII was directly produced from *Xoo* and purified only to a minor degree, so a detailed analysis was impossible. The previous result was reported in 1980, and there has been little further work done except some on methyl transferases [10]. This was rather curious, and is likely due to the fact the production of necessary amount of pure enzyme was very difficult. Here we report the optimized production and purification of recombinant XorKII and its biophysical and biochemical properties.

Materials and methods

Plasmid construction

The genomic DNA of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) KACC10331 was prepared as described in [4]. The gene coding for XorKII was amplified by PCR. The primers were designed based on the *Xoo* genome sequence (GenBank Accession No. NC_006834). The forward primer was 5′-GGG CCC GGA TTC GTG AGC TTG CCT CCC TAC GTC-3′, and the reverse primer, 5′-GGG CCC CTC GAG TTA AAC GCC GTG CAT CAA CGT-3′. The PCR product was purified, cut by BamHI and XhoI, purified again, and ligated with the pET-28a vector (Novagen, Madison, WI, USA), which was previously digested with the same enzymes. The resulting plasmid was named pET-28a/XorKII. The plasmid was brought into Rosetta2(DE3)/

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[1] *Abbreviations used: Xoo*, *Xanthomonas oryzae* pv. *oryzae*; ORFs, open reading frames.

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pLysS (Novagen, Madison, WI, USA) for recombinant expression. This strain did not have the cognate methyl transferase gene for protection against XorKII.

Small scale expression test

A single colony was used to inoculate a 3 ml LB medium supplemented with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. When fully-grown, 10 μl of the culture was used as an inoculum to 3 ml each of the following media: 50%, 60%, 70%, 80%, 90%, and 100% LB. The cultures were grown overnight at 37 °C in a shaking incubator. The next morning, a proper volume of 10× LB medium was added to the culture so that the final concentration of the nutrient in the culture became equal to LB medium. IPTG was also added at the same time to the final concentration of 0.5 mM to induce protein production. The culture was further grown for another 3 h, and 50 μl were taken from each culture and harvested by centrifugation. The cells were resuspended in 50 μl of 10 mM Tris–HCl pH 7.5 containing 8 M urea and mixed with 50 μl of 2× SDS sample buffer for analysis.

Protein production

A single colony was used to inoculate a 1000 ml medium containing 7 g of bactotrypton (BD, Franklin Lakes, NJ, USA), 3.5 g of yeast extract (BD, Franklin Lakes, NJ, USA), and 7 g of NaCl, supplemented with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. The culture was grown overnight at 37 °C in a shaking incubator. The next morning, 3 g of bactotrypton, 1.5 g of yeast extract and 3 g of NaCl were directly mixed with the fully-grown culture. After the nutrients were completely dissolved, IPTG was added to the final concentration of 0.5 mM to induce protein production. The culture was further grown for another 3 h, and harvested by centrifugation at 5000 rpm for 10 min at 4 °C. The harvested cells were resuspended in 50 ml of 10 mM Tris–HCl pH 8.0 and frozen at −20 °C.

Protein purification

The cells were lysed by freeze-and-thaw, and the DNA was fragmented by ultrasonication. The soluble fraction was retained after centrifugation at 15,000 rpm for 20 min at 4 °C and then loaded onto the HiPrep Chelate column (5 ml) charged with Ni^{2+} (GE Healthcare, Piscataway, NJ, USA). Imidazole gradient of 10 mM to 300 mM was applied to the column on ÄKTA Basic system (GE Healthcare, Piscataway, NJ, USA). The fractions containing XorKII were pooled, concentrated, and buffer exchanged with 100 mM Tris–HCl pH 8.0 containing 200 mM NaCl, 2 mM DTT, 0.2 mM EDTA, and 0.1% Triton X-100 by Amicon Ultra (Millipore, Billerica, MA, USA). The resulting concentrated XorKII (4 ml) was mixed with the same volume of 100% glycerol and kept at −20 °C.

Size exclusion chromatography

Size exclusion chromatography was performed with a Superdex 200 10/30 column (GE Healthcare, Piscataway, NJ, USA), which was pre-equilibrated with 10 mM sodium phosphate buffer pH 7.4 containing 1 M NaCl on ÄKTA Basic system (GE Healthcare, Piscataway, NJ, USA). The proteins used for setting up a standard curve were BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Endonuclease activity test

The YEp24 vector (GenBank Accession No. L09156) with three PstI sites was used as a substrate. Enzyme digestion was performed at 37 °C. Buffers used to find the optimal cleavage condition were as follows: Buffer 1, 10 mM Tris–HCl pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol; Buffer 2, 50 mM NaCl, 10 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol; Buffer 3, 100 mM NaCl, 50 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol; Buffer 4, 50 mM potassium acetate, 20 mM Tris-acetate pH 7.9, and 10 mM Magnesium Acetate, 1 mM dithiothreitol. For the inactivation test, XorKII was preincubated at 65 °C for 5, 10, 15, 20, 25, 30 min before mixing with the substrate. For the stability test, XorKII was preincubated at 37 °C for 4, 8, 12, 18, and 24 h before mixing with the substrate.

Specific activity determination

The concentration of XorKII was determined by using Protein Assay kit (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used to set up the standard curve. One unit was defined as the amount of XorKII to cleave 1 μg of lambda DNA at least once in an hour. The reaction mixtures contained 5 μl of 10× reaction buffer, 1 μg of DNA substrate, and the total volume was set to 50 μl. A serial dilution of the purified XorKII was made, and 1 μl from each dilution was added to the reaction mixture. The mixture was incubated at 37 °C for an hour, and the reaction was stopped by incubation at 80 °C for 15 min. The degree of cleavage was visualized by agarose gel electrophoresis. The highest dilution showing at least one cleavage was used to determine 1 U of XorKII.

Results and discussion

XorKII gene cloning and plasmid construction

Since the genome sequences of *Xoo* was reported, we analyzed the open reading frames (ORFs) to search for the gene coding for Type II restriction endonucleases. A candidate gene was located at sequences between 4219262 and 4218168 on the complementary strand although it was not annotated yet. This ORF had a 93% sequence identity with R.Xph I (GenBank Accession No. AF042157). From the fact that RM system is mobile, and the gene coding for endonuclease and its cognate methylase are located close to each other on the chromosome [6], we expected to find a methylase gene nearby. Fortunately, a putative methylase gene was found right next to the XorKII gene in the *Xoo* sequence, which increased the probability that this candidate was actually the XorKII we were searching for. The XorKII gene was amplified from the *Xoo* genome and inserted into pET-28a between BamHI and XhoI sites. As a consequence of using these cloning sites, the resulting protein would have 34 additional amino acid residues to the N-terminus of the wild-type protein. As will be discussed below, this stretch of additional residue did not cause any detrimental effects on the endonuclease activity.

Protein production

The cell growth and protein induction were performed in a rather non-typical way. This was because the cells died when forced to produce XorKII at 37 °C. It was suspected that the produced endonuclease molecules were too toxic to cells in their mid-log phase. The previous version of the stationary phase induction method [9] was attempted, but the cell lysis could not be lessened. Instead of diluting the fully-grown culture as the previous method described [9], we tried growing cells in a less nutrient medium and then adding back the omitted amount of nutrients for further growth. Upon adding the nutrients, IPTG was added for the induction of protein production. As seen in Fig. 1, the best result could be obtained if 70% LB medium had been used in its ini-