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Activity assay of His-tagged *E. coli* DNA photolyase by RP-HPLC and SE-HPLC

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Abstract

Escherichia coli DNA photolyase was expressed as C-terminal 6× histidine-fused protein. Purification of His-tagged *E. coli* DNA photolyase was developed using immobilized metal affinity chromatography with Chelating Sepharose Fast Flow. By one-step affinity chromatography, approximate 4.6 mg DNA photolyase was obtained from 400 ml *E. coli* culture. The purified His-tagged enzyme was combined with two chromophors, FADH and MTHF. Using the oligonucleotide containing cyclobutane pyrimidine dimer as substrate, both reversed-phase high-performance liquid chromatography and size-exclusion high-performance liquid chromatography were developed to measure the enzyme activity. The enzyme was found to be able to repair the cyclobutane pyrimidine dimer with the turnover rate of 2.4 dimers/photolyase molecule/min.

Keywords: Photolyase; Chelating Sepharose Fast Flow; Chromophor; Oligonucleotide; Cyclobutane pyrimidine dimer

Abbreviations: CPD, cyclobutane pyrimidine dimer; FAD, flavin adenine dinucleotide; MTFH, methenyltetrahydrofolate; 8-HDF, 8-hydroxy-5-deazariboflavin; TEAA, triethylammonium acetate; ACN, acetonitrile; RP-HPLC, reversed-phase high-performance liquid chromatography; SE-HPLC, size-exclusion high-performance liquid chromatography.

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1. Introduction

DNA photolyase (DNA cyclobutane dipyrimidine photoyase, EC 4.1.99.3) is a specific enzyme which repairs DNA damage by utilizing near ultraviolet visible light (300–500 nm) as a cofactor. The enzyme binds to the thymine dimer in the dark with high affinity and specificity and then, upon absorbing a photon of near UV or visible light, repairs the damage, thereby restoring the integrity of the DNA [1]. Two types of DNA photolyase are known, one specific for cyclobutane pyrimidine dimer (CPD photolyase) [2], and the other specific for (6–4) photoproduct ((6–4) photolyase) [3]. DNA photolyase is a monomeric protein of M_r=55,000–65,000 Da and contains stoichiometric amounts of two chromophores [4]. One of the chromophores is flavin adenine dinucleotide (FAD) [5], and the other is either methenyl-tetrahydrofolate (MTHF) [6] or 8-hydroxy-5-deazariboflavin (8-HDF) [7].

Pure DNA photolyase can be obtained by a variety of purification methods. Malthorae et al. provided a common method for the purification of DNA photolyase, which included ammonium sulfate precipitation, Blue Sepharose column, and DNA-cellulose column [7]. This method has been employed to the purification of DNA photolyase from other species [8,9]. In addition, DNA photolyase, if combined with special fused tag, can be purified by affinity chromatography. Hitomi et al. reported that *Xenopus* (6–4) photolyase was expressed as glutathione *S*-transferase (GST)-fused protein and the fusion protein was purified by glutathione-Sepharose column [10]. Maltose-binding protein (MBP)-fused photolyase was also expressed, and purified by affinity chromatography [11–13].

In this article, *Escherichia coli* DNA photolyase was expressed as 6× histidine-fused protein. Then by one-step affinity chromatography, the enzyme was obtained with the purity of approximately 95%.

As to the activity assay of DNA photolyase, many methods were developed, which included gel retardation method [14], T4 endo V nicking assay [15], etc. Recently, reversed-phase high-performance liquid chromatography (RP-HPLC) method was employed to measure the activity of photolyase [16,17]. Based on their different hydrophobicity, the repaired DNA could be separated from the unrepaired DNA by reverse-phase column [18]. And by calculating the quantity of repaired DNA after various photoreactivation time, the photolyase activity could be measured. In our research, a much easier method of size-exclusion high-performance liquid chromatography (SE-HPLC) was developed to determine the activity of DNA photolyase.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strain BL21(DE3) was used as the host strain for the plasmid with *E. coli* phr gene. Plasmid pET-22 b(+) was used to construct pET-22 b(+)∷phr, which overproduced the *E. coli* DNA photolyase.

2.2. Cloning of E. coli phr gene

Using 5′ prime (5′-CG CAT ATG ACT ACC CAT CTG GTC TGG TTT CGC-3′) and 3′ prime (5′-GTG CTC GAG TTT CCC CTT CCG CGC CGC CTC A-3′), the *E. coli* phr gene was amplified from *E. coli* k12 genomic DNA. Then the phr gene was cloned into pET-22 b(+), and the reconstructed plasmid, pET-22 b(+)∷phr was obtained. Because the pET-22 b(+) vector carries an optional C-terminal 6× histidine-tag coding sequence, photolyase was expressed as His-tagged fusion protein.

2.3. Materials

Chelating Sepharose Fast Flow was purchased from Amersham Pharmacia Biotech. All restriction enzymes, T4 DNA ligase, dNTPs, Taq DNA polymerase and T4 polynucleotide kinase were from TaKaRa Biotech. Isopropyl β-D-thiogalactoside(IPTG) was bought from Sigma. The primes and the two types of oligonucleotide (Table 1) for preparing the substrates were synthesized by Sangon Corp. (Shanghai, China).

2.4. Purification of His-tagged E. coli photolyase

All purification steps were carried out at 4 °C and samples of all fractions were analyzed by SDS-PAGE. *E. coli* BL21(DE3)/pET-22 b(+)∷phr was grown in 400 ml Lauria broth to $A_{600\ nm}=0.8$ and then induced with IPTG (1 mM) for 3 h. The cells were harvested by centrifugation, resuspended in 12 ml lysis buffer (50 mM Tris, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, PH 7.4). The harvested cells were disrupted with French pressure cell (Thermo Spectronic, Rochester, NY) at 20,000 psi. Cell debris was removed by centrifugation at 18,000×*g* for 15 min. The supernatant (12 ml) was loaded onto a 3 ml Ni^{2+}-Chelating Sepharose Fast Flow column, mixed gently, and incubated for 20 min. Then the column was washed with 60 ml of washing buffer (50 mM Tris, 50 mM NaCl, 10 mM 2-mercaptoethanol, 100 mM imidazole, 10% glycerol, PH 7.4). The bound proteins were eluted with 6 ml elution buffer (50 mM Tris, 50 mM NaCl, 10 mM 2-mercaptoethanol, 500 mM imidazole, 10% glycerol, PH 7.4). Six-milliliter fractions were collected and dialysed against storage buffer (50 mM Tris, 50 mM NaCl, 10 mM DTT, 1 mM EDTA, 50% glycerol, pH 7.4).

Table 1
Two types of oligonucleotides for preparing substrates

15-mer	5′-AGA GCA GTT GAC ACG-3′
$d(pT)_{16}$	5′-TTT TTT TTT TTT TTT T-3′

2.5. Spectral measurements

The absorption spectra were obtained with a DU-640-nucleic acid protein analyser (Bechman, USA). The fluorescence measurements were made with a Spectronic Series2 Luminescence Spectrometer (Thermo Spectronic, Rochester, NY).

2.6. Preparation of 15-mer containing a thymine dimer and $d(pT)_{16}$ containing thymine dimers

15-mer containing a thymine dimer (which was called 15-mer(T<>T)) was prepared by irradiation of deoxygenated aqueous solution (100 μM 15-mer in 0.5 ml). The solution was sealed in a Pyrex (> 290 nm) tube, deaerated by high purity Argon (> 99.999% purity) bubbling for 15 min, and irradiated with a 300-W high pressure Hg lamp [19]. To improve the yield of 15-mer(T<>T), acetone was added as photo-sensitizer. The irradiated solution was concentrated on a Speedvac and the mixture was separated by reversed phase HPLC (Symmetry C_{18}; 3.9 mm × 150 mm, 300 Å, Waters) with a linear gradient of triethylammonium acetate (TEAA, 100 mM, pH 7.0), water and acetonitrile (ACN). The product collected was the purified oligomer containing a thymine dimer.

The preparation of $d(pT)_{16}$ containing thymine dimmers (which was noted as $d(pT)_{16}$(UV5 h)) was similar to that of 15-mer(T<>T) except that acetone was not used. Five hours of irradiation was used to for the preparation.

2.7. The activity assay by RP-HPLC

A 100-μl photoreactivation solution, which contained 1 μM DNA photolyase and 30 μM 15-mer(T<>T) in reaction buffer (50 mM Tris, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% gylcerol, PH 7.4), was added to a cuvette. The mixture were purged with argon for 15 min and photoirradiated with a UVA lamp (predominantly 365 nm; the distance between the cuvette and the lamp was 2 cm) for different time. Then, the solution was boiled for 5 min and centrifugated at 16,000×*g* for 10 min. A 20-μl portion was subjected to RP-HPLC. The RP-HPLC conditions were as following: mobile phase, 4% ACN in 100 mM TEAA (PH 7.0) for 0–5 min; 4–8% ACN in 100 mM TEAA (PH 7.0) for 5–25 min; flow rate, 0.75 ml/min; column, Xterra MS C_{18} (4.6 mm × 50 mm, Waters); column temperature, 80 °C; detection wavelength, 260 nm. *E. coli* photolyase activity was evaluated from the peak area of repaired DNA in the chromatogram.

2.8. The activity assay by SE-HPLC

A 100-μl photoreactivation solution containing 1 μM DNA photolyase and 5μM $d(pT)_{16}$(UV5 h) (containing 28 μM dimer) in reaction buffer was added to a cuvette. The photoreactivation process was the same to the above. A 20-μl portion was subjected to SE-HPLC. The SE-HPLC conditions were as following: mobile phase, 25 mM Tris–HAc (PH 8.7); flow rate, 0.75 ml/min; column, Protein-Pak 300SW (7.8 mm × 300 mm, Waters); column temperature, 25 °C; detection wavelength, 260 nm. *E. coli* photolyase activity was evaluated from the increment of the peak area.

3. Results

3.1. Overproducion and purification of E. coli photolyase

To obtain pure *E. coli* DNA photolyase for in vitro characterization, an overproducing plasmid, pET-22 b(+)::phr was constructed. pET-22 b(+)::phr was obtained by inserting the NdeI-XhoI fragment of *E. coli* phr gene into the site between the T7 promoter and T7 terminator of the expression vector pET-22 b(+). Upon induction of BL21(DE3)/pET-22 b(+)::phr with IPTG, a 50,000-Da protein was overproduced to 20% of total cellular proteins (Fig. 1). The mass of the overproduced protein was similar in size to what was predicted for the phr gene product from the nucleotide sequence (M_r=53,994 [20]). After one-step purification with Chelating Sepharose Fast Flow, about 4.6 mg His-tagged photolyase was obtained (with the purity of approximately 95%) from 400 ml induced *E. coli* culture.

3.2. Spectral properties of DNA photolyase

The purified His-tagged enzyme has a blue color with visible absorption peaks at 384 nm and at 580 nm, which are attributable to MTHF and FADH, respectively (Fig. 2A) [21]. When the excitation wavelength is 390 nm, the enzyme had an emission maximum at 470 nm due to MTHF and a shoulder around 505 nm due to $FADH_2$ (Fig. 2B) [22]. The presence of $FADH_2$ was the result of the reducing ability of DTT, which was contained in the sample buffer. The spectral properties showed that the purified enzyme was the blue form E-MTHF-$FADH^{\cdot}$, which was reported to have high activity [23].

3.3. Preparation of 15-mer(T<>T)

To improve the yield of 15-mer(T<>T), acetone was added as photo-sensitizer during its preparation. Different acetone concentration and different irradiated time were used, and then the products were analyzed by RP-HPLC for the determination of the quantity of

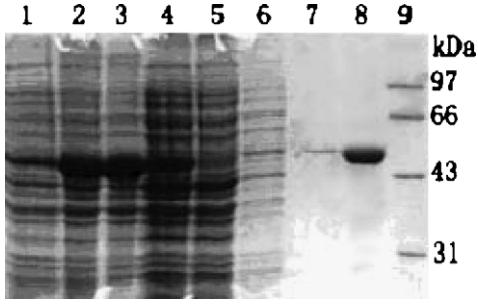

Fig. 1. Coomassie blue staining of proteins separated on 12% SDS-PAGE during purification of photolyase from BL21(DE3)/pET-22b(+)::phr. Lane 1, BL21(DE3)/phr(N+X) uninduced; lane 2, cells induced with IPTG; lane 3, cell debris; lane 4, supernatant ready for purification; lane 5–7, collected solution with washing buffer during purification; lane 8, the purified photolyase; lane 9, protein marker. The numbers at the right margin indicated mass (in kDa) of the markers.

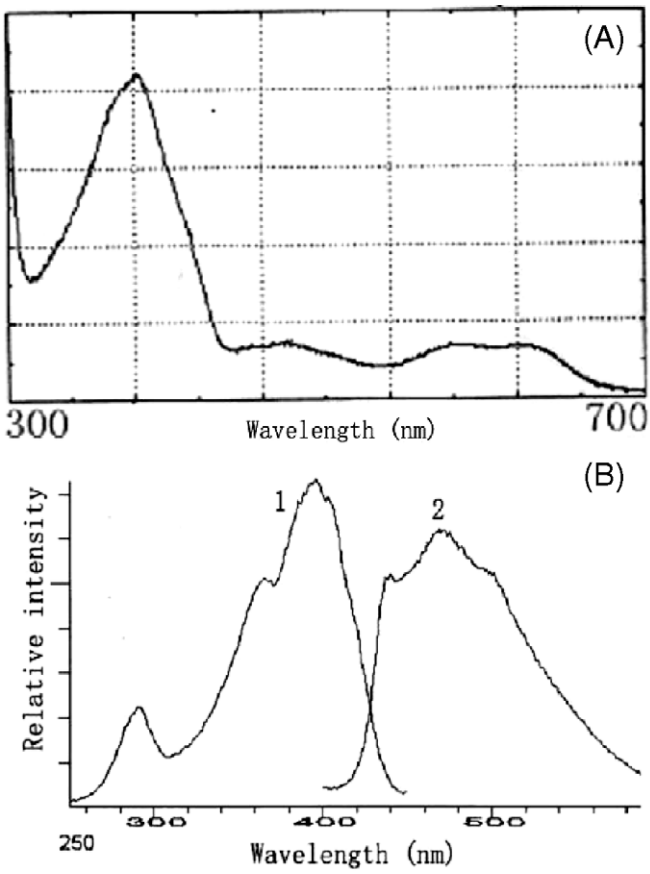

Fig. 2. Spectroscopic analyses of His-tagged *E. coli* photolyase. (A) absorption spectra of His-tagged *E. coli* photolyase. The purified photolyase is blue in color with absorption maxima at 384 and 580 nm. (B) exciting and emission spectrum of the enzyme (curve 1 and curve 2, respectively). The excitation spectrum was taken at an emission wavelength of 470 nm; the emission spectrum was taken at an exciting wavelength of 390 nm.

15-mer(T<>T). As shown in Fig. 3 and Table 1, acetone greatly contributed to the formation of cyclobutane thymine dimmers. The 15-mer(T<>T) was produced with high yield (approximately 30%) when 20% acetone and 30 min of irradiation were employed. Long-time irradiation, however, decreased the yield of 15-mer(T<>T).

3.4. Activity assay by RP-HPLC

Photoreactivation solution containing DNA photolyase and 15-mer(T<>T) in reaction buffer was irradiated for various time. Then RP-HPLC was used to detect the quantity of 15-mer, which represented the activity of the enzyme. As shown in Fig. 4, DNA photolyase catalyzed 15-mer(T<>T) into 15-mer during photoreactivation, and the repaired DNA increased in the manner of photoreactivation time. The eluting peak at 12.1 min and 19.4 min showed eluting peak of 15-mer(T<>T) and that of 15-mer. Peak

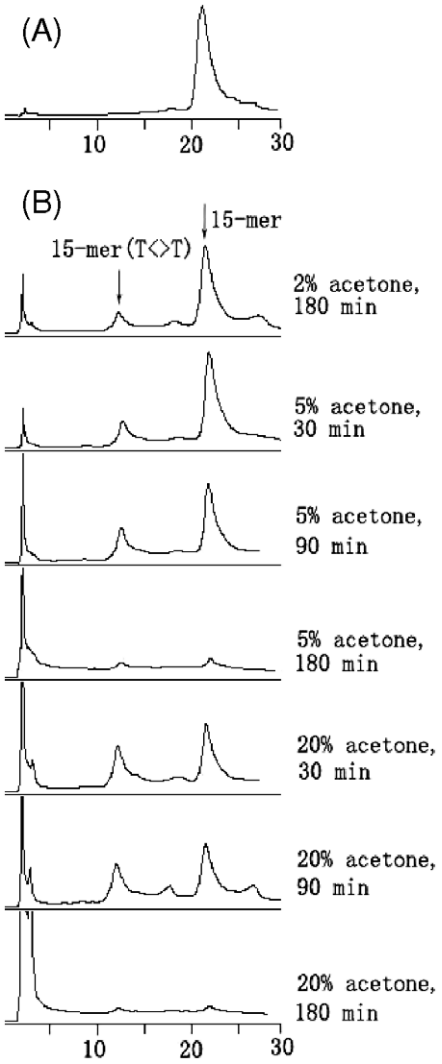

Fig. 3. Analysis by RP-HPLC on a C_{18} column (3.9 mm × 150 mm, 300 Å, Waters) of various preparation products during preparation of 15-mer(T<>T). The RP-HPLC conditions were as following: mobile phase, 6% ACN in 100 mM TEAA (pH 7.0) for 0–5 min and 6–8% ACN in 100 mM TEAA (pH 7.0) for 5–30 min; flow rate, 0.75 ml/min; column temperature, 60 °C; detection wavelength, 260 nm. (A) RP-HPLC trace of standard sample of 15-mer without CPD. (B) series of RP-HPLC traces of the synthesis of 15-mer(T<>T). The arrow peak at 12.5 and 22.4 min showed eluting peak of 15-mer(T<>T) and that of 15-mer without CPD. The numbers at the right indicated acetone concentration and irradiated time for corresponding sample.

area was calculated by Millennium32 software (Waters). The photoreactivation curve using 15-mer(T<>T) as substrate was shown in Fig. 5. Based on the data, the turnover rate was 2.4 dimers/photolyase molecule/min, which is similar to others' result [24]. After photoreaction for 60 min, the turnover proportion is 82%.

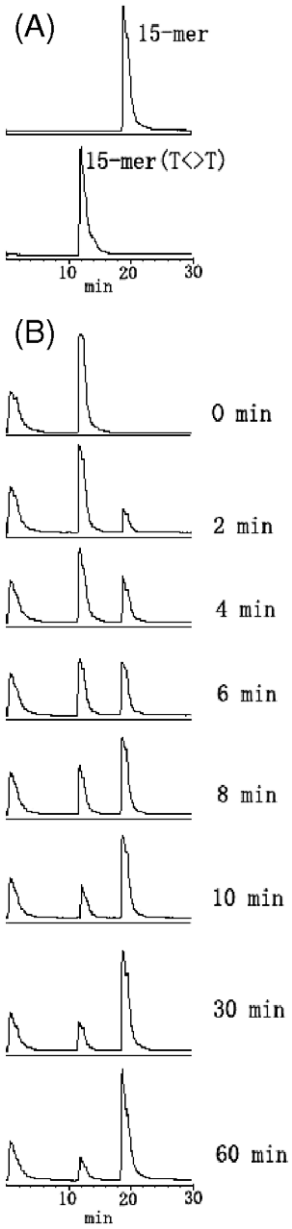

Fig. 4. RP-HPLC traces of the aliquots taken during the photoreactivation with 15-mer(T<>T). The RP-HPLC conditions were as following: mobile phase, 4% ACN in 100 mM TEAA (pH 7.0) for 0–5 min; 4–8% ACN in 100 mM TEAA (pH 7.0) for 5–25 min; flow rate, 0.75 ml/min; column, Xterra MS C_{18} (4.6 mm × 50 mm, Waters); column temperature, 80 °C; detection wavelength, 260 nm. (A) RP-HPLC traces of standard samples of 15-mer and 15-mer(T<>T). (B) series of RP-HPLC traces of the 15-mer(T<>T) after different time of photoreactivation by DNA photolyase. The peak at 12.1 and 19.4 min showed eluting peak of 15-mer(T<>T) and that of 15-mer repaired. The numbers at the right indicated the corresponding photoreactivation time.

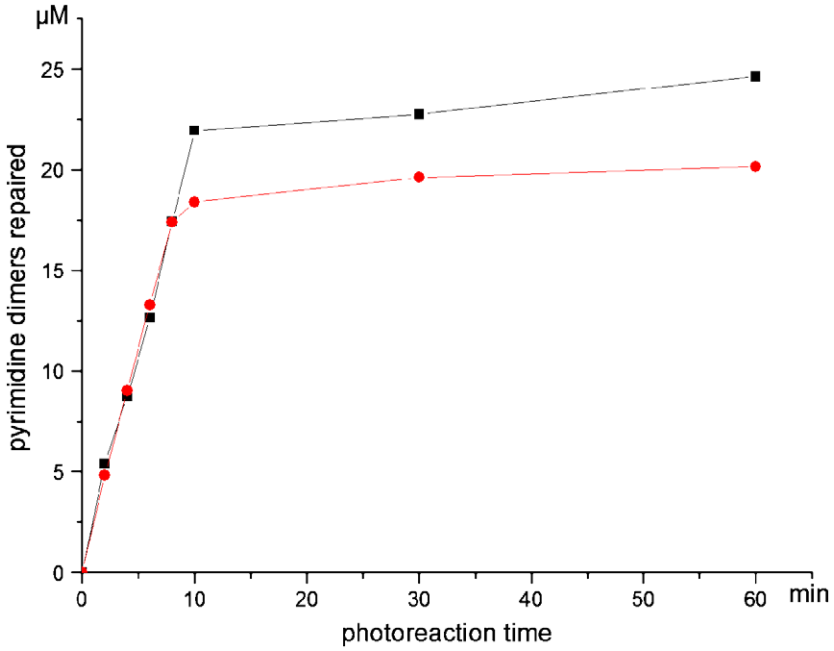

Fig. 5. Catalytic activity of *E. coli* DNA photolyase. Black and red lines represented the photoreactivation curve of the enzyme using 15-mer(T<>T) and $p(dT)_{16}$(UV5 h) as substrates.

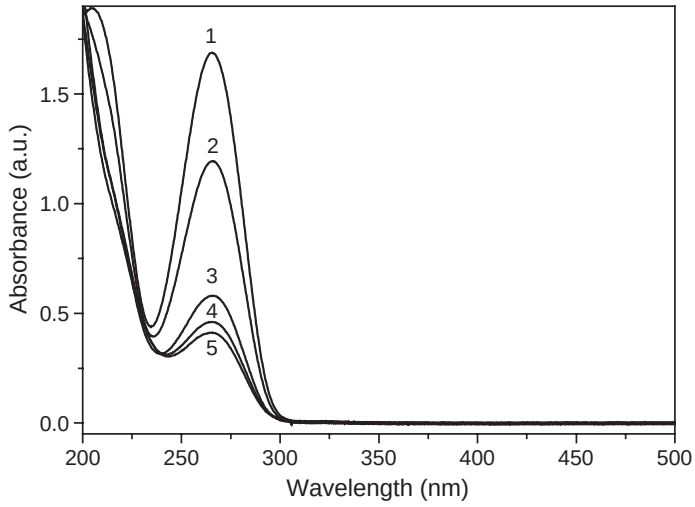

Fig. 6. UV–VIS spectra of $d(pT)_{16}$ after irradiation of 300 W high-pressure Hg lamp. Curve 1 was the spectrum of 10 μM $d(pT)_{16}$ before irradiation. Curves 2–5 were recorded 0.5, 1, 3, 5 h, respectively, after irradiation at 25 °C.

3.5. Preparation of $d(pT)_{16}$(UV5 h)

The oligonucleotide of $d(pT)_{16}$ was irradiated for various time, and the products were detected by UV–VIS scanning. As shown in Fig. 6, the thymine dimerization approached steady after 5 h of irradiation, and thus the time was used for the preparation of $d(pT)_{16}$(UV5 h). The degree of thymine dimerization for $d(pT)_{16}$(UV5 h) was approximately 70%, calculated by SE-HPLC (Fig. 7A).

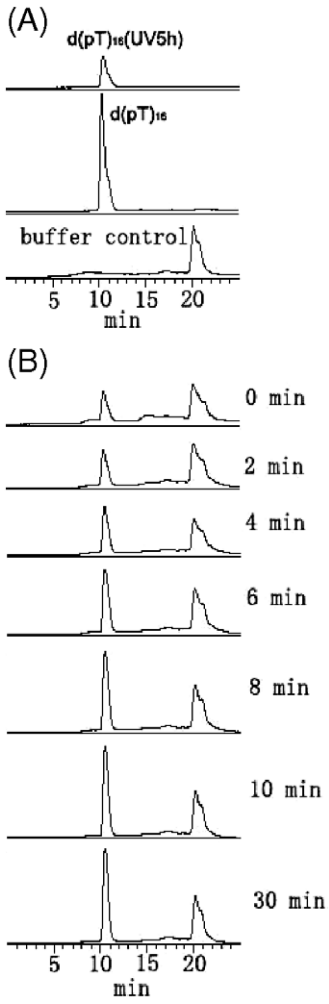

Fig. 7. SE-HPLC traces of the aliquots taken during the photoreactivation using $d(pT)_{16}$(UV5 h) as the substrate. The SE-HPLC conditions were as following: mobile phase, 25mM Tris–HAc; flow rate, 0.75 ml/min; column, Protein-Pak 300 SW (7.8 mm × 300 mm, Waters); column temperature, 25 °C; detection wavelength, 260 nm. (A) SE-HPLC traces of standard samples. (B) series of RP-HPLC traces of the $d(pT)_{16}$(UV5 h) after different time of photoreactivation by DNA photolyase. The peak at 10.6 showed eluting peak of all oligonucleotides. The numbers at the right indicated the corresponding photoreactivation time.

3.6. Activity assay by SE-HPLC

The activity of DNA photolyase was also determined by SE-HPLC. Because CPD DNA, the substrate of the enzyme has the same molecular weight to the repaired DNA, CPD DNA and the repaired DNA were eluted at the same time while using the method of SE-HPLC. Also because CPD has no absorbance at 260 nm but restores the ability while repaired, the enzyme activity could be measured by calculating the increment of absorbance at 260 nm.

Photoreactivation solution containing DNA photolyase and $d(pT)_{16}$(UV5 h) in reaction buffer was irradiated for various time, and then subjected to SE-HPLC. As shown in Fig. 7B, all oligonucleotides, whether repaired or not, shared one eluting peak at 10.6 min. Because CPD has no absorbance at 260 nm, the peak area at 10.6 min increased when the CPDs in $d(pT)_{16}$(UV5 h) were repaired. Enzyme activity was evaluated from the increment of oligonucleotides' peak area in the chromatogram. Using the $d(pT)_{16}$(UV5 h) as substrate, the turnover rate was 2.4 dimers/photolyase molecule/min. After photoreaction for 60 min, the turnover percentage is 72%, which is lower than the value when 15-mer(T<>T) was used as substrate (Fig 5). The possible reason was that there were other photoproducts in $d(pT)_{16}$(UV5 h) (such as (6–4)photoproduct), which can not be repaired by CPD photolyase.

4. Discussion

Attachment of a hexa-His tag is a common strategy in recombinant protein production, and the use of such a tag greatly simplifies the process of purification [25]. In our research, *E. coli* DNA photolyase was expressed as His-tagged fusion protein. And by one-step purification employing immobilized metal affinity chromatography with Chelating Sepharose Fast Flow, DNA photolyase was obtained in high purity (approximately 95%). This purification method not only shortened operation time, but also elevated the quantity of active photolyase. Approximately 4.6 mg enzyme was obtained from 400 ml IPTG-induced culture. The output is much high compared with other purification methods.

To measure the activity of photolyase, its substrate, CPD DNA is needed. In our study, two types of substrates, $d(pT)_{16}$(UV5 h) and 15-mer(T<>T) were prepared. The dimerization of thymine in 15-mer, which has one pair of thymine, is more difficult than that in $d(pT)_{16}$. Approximately 70% thymines in $d(pT)_{16}$ formed dimers after 300 W Hg lamp irradiation for 5 h, but thymine dimers rarely appeared in 15-mer under the same conditions (data no shown). The possible reason is that only one pair of thymines in 15-mer can be dimerized, while the probability of dimerization in $d(pT)_{16}$ is much higher.

The traditional methods for the preparation of 15-mer(T<>T) employed high irradiation energy and long time of UV exposure [17,19]. In our research, acetone was added as photo-sensitizer in the preparation of 15-mer(T<>T), and it was found that acetone increased the reaction rate greatly. When 20% (v/v) acetone was added, the output was raised to approximately 30% after only 30 min of irradiation by 300 W Hg lamp. Acetone has high energy in the triplet state (79–82 kcal/mol), which makes the photosensitization process possible [26,27]. After acetone is excited by UV light, a collision between the

Table 2
Preparation of 15-mer(T<>T) under various conditions

Acetone concentration (v/v)	2%	5%	5%	5%	20%	20%	20%
Irradiation time (min)	180	30	90	180	30	90	180
15-mer undimerized (%)	73.40	81.04	61.85	2.48	49.76	43.92	1.20
15-mer (T<>T) (%)	10.97	15.85	21.00	2.19	29.56	29.16	1.70
Other product (%)	15.63	3.11	17.15	95.33	20.68	26.92	97.10

triplet acetone and the thymine will accomplish an energy transfer, which results in thymine dimer. Thus high irradiation is unnecessary when acetone is used in the preparation of 15-mer(T<>T).

In the presence of acetone, however, long-time irradiation reduced the CPD output in 15-mer. When more than 5% acetone was used, the ratio of 15-mer(T<>T) was less than 3% after 180 min of irradiation (Table 2). The possible reason is that long-time irradiation could bring destruction to oligonucleotide when acetone is used.

A variety of methods have been developed to detect the activity of DNA photolyase. Recently, RP-HPLC was used to measure the activity of *Aspergillus nidulus* photolyase [16] and of *E. coli* DNA photolyase [17]. In this paper, both RP-HPLC and SE-HPLC were developed for the activity assay of photolyase. When 15-mer(T<>T) or $d(pT)_{16}$(UV5 h) was used as substrate, the percentage of repaired DNA was 82% and 72% respectively after 60 min of photoreactivation. The 15-mer(T<>T) used was purified and all contained pyrimidine cylobutane dimers, while the $d(pT)_{16}$(UV5 h) was the mixture of different photoproducts and part of them could not be repaired by CPD photolyase. So less $d(pT)_{16}$(UV5 h) could be repaired than 15-mer(T<>T). Both methods, however, gave out the same value of the turnover rate, which was 2.4 dimers/photolyase molecule/min in the first 8 min.

Compared to RP-HPLC, SE-HPLC had more virtues when used for the activity assay of *E. coli* DNA photolyase. The manipulation of SE-HPLC is much easier than that of RP-HPLC. And in SE-HPLC, the purification of the substrates, CPD oligonucleotides was omitted.

In summary, *E. coli* DNA photolyase was expressed as His-tagged fusion protein, and was purified by one-step affinity chromatography. The substrates of photolyase, 15-mer(T<>T) and $d(pT)_{16}$(UV5 h), were prepared; and it was found acetone greatly increased the output of 15-mer(T<>T). Both RP-HPLC and SE-HPLC were developed for the activity assay of *E. coli* DNA photolyase, and both methods resulted in the turnover rate of 2.4 dimers/photolyase molecule/min.

5. Simple description of the method and its application

E. coli DNA photolyase was expressed as C-terminal 6× histidine-fused protein. Purification of His-tagged *E. coli* DNA photolyase was developed using immobilized metal affinity chromatography with Chelating Sepharose Fast Flow.

RP-HPLC method was recently developed to measure the activity of DNA photolyase. In our research, some progress was developed about this method, which was showed in the

process of the preparation of DNA substrate containing one thymine dimmer. It was that acetone was added as photo-sensitizer in the preparation of 15-mer(T<>T) and increased the reaction rate greatly.

In addition, the new method, SE-HPLC was also developed to determine the activity of DNA photolyase. Photoreactivation solution containing DNA photolyase and $d(pT)_{16}$(UV5 h) in reaction buffer was irradiated for various time, and then subjected to SE-HPLC. The activity of DNA photolyase was evaluated from the increment of oligonucleotides' peak area with 260 nm absorbance in the chromatogram.

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