Heat-labile uracil-DNA glycosylase: purification and characterization

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Abstract A uracil-DNA glycosylase (UNG) from a psychrophilic marine bacterium (BMTU 3346) has been purified to apparent homogeneity. The enzyme has a molecular weight of 23400 Da. It is stable in complex buffers (containing glycerol/BSA), whereas it is heat-labile in dilute buffers (free of stabilizers) with a half-life of 2 min at 40°C. Due to the thermolability, uracil-DNA glycosylase is suitable for application in the carryover prevention technique showing less residual activity and/or a slower reactivation rate than the usually applied UNG from *Escherichia coli*.

Key words: Uracil-DNA glycosylase; Carryover prevention; PCR; Amplification

1. Introduction

Uracil-DNA glycosylases (uracil DNA *N*-glycosylase, UNG; EC 3.2.2.3) are a highly conserved and specific class of DNA repair enzymes ([1] and references therein). The biological function of uracil-DNA glycosylases is the specific removal from DNA of the normal RNA base uracil, liberating free uracil and generating apyrimidinic sites [2]. Uracil-DNA glycosylases have been identified in a variety of prokaryotic and eukaryotic organisms and in different families of viruses [1]. Originally, the enzyme was described in *Escherichia coli* [3,4]. From bacterial origins, UNG has also been characterized from *Bacillus subtilis* [5], *B. stearothermophilus* [6], *Micrococcus luteus* [7], *Mycoplasma lactucae* [8] and *Thermothrix thiopara* [9]. Uracil arises in DNA by deamination of cytosine and via misincorporation of deoxyuridine triphosphate during DNA synthesis generating a promutagenic U:G mismatch, which if not corrected leads to a C→T transition mutation in the next round of DNA synthesis [2].

The amplification of DNA by polymerase chain reaction (PCR) can be affected by contamination leading to false positive results. Possible sources of contamination are (1) cross-contamination between samples, (2) DNA contamination from the laboratory, and (3) carryover contamination of amplification products and primers from previous PCRs [10]. Carryover contamination is concerned as the major source of false positive results [11]. To overcome this problem an effective method has been developed to avoid carryover contamination in PCR. The carryover prevention technique consists of two steps: (1) incorporation of dUTP in all PCR products (substituting dUTP for dTTP or incorporating dUTP during synthesis of the primers); and (2) treatment of subsequent PCR mixtures with uracil-DNA glycosylase, followed by thermal inactivation of UNG prior to the actual PCR [10].

UNG degrades contaminating uracil-containing DNA leaving the natural (thymine-containing) target DNA unaffected. For this application usually the uracil-DNA glycosylase from *E. coli* is used. However, it has been reported that the enzyme is not completely inactivated by heat denaturation, leading to degradation of the PCR product affecting its integrity and yield. Furthermore, reactivation of UNG after PCR has been discussed [12].

In the present paper, the purification and characterization of a heat-labile uracil-DNA glycosylase is described. The enzyme has low thermal stability in buffers usually used in PCR technology. Data are presented showing that this thermolabile UNG is inactivated more rapidly and shows less residual activity and/or a slower reactivation rate than the corresponding enzyme isolated from *E. coli*.

2. Materials and methods

2.1. Materials

Phenyl-Sepharose fast flow, Q-Sepharose fast flow high load, Superose 12 (HR 10/30), Sephadex G-50 and polyacrylamide gels (8–25%) were obtained from Pharmacia (Uppsala, Sweden). Coomassie brilliant blue G-250 was from Serva (Heidelberg, Germany). [^{3}H]dUTP (15.3 Ci/mmol) was purchased from Amersham (Braunschweig, Germany). Standard 1 medium was purchased from Merck (Darmstadt, Germany). Hydroxyapatite was obtained from IBF Biotechnics (Villeneuve-la-Garenne, France). The DIG-Taq DNA sequencing kit, DIG-luminescent detection kit, UNG from *E. coli* and all other reagents were from Boehringer Mannheim GmbH (Mannheim, Germany).

2.2. Bacterial strain

The strain BMTU 3346 was originally isolated from a marine sample (taxonomic characterization in progress). Cells were grown at 20°C in nutrient broth medium (Standard 1 medium, Merck).

2.3. Purification

All steps were performed at 4°C.

2.3.1. Crude extract. 120 g frozen cells were suspended in 400 ml of buffer 1 (10 mM potassium phosphate, pH 7.5, 1 mM β-mercaptoethanol). 100 mg lysozyme were added and the suspension was stirred for 30 min. Cells were disrupted at 8000 psi using an APV-Gaulin press.

2.3.2. Polymin precipitation. To the crude extract 24 ml of polymin P (10%) were slowly added and stirred for 30 min. The precipitate was centrifuged at 13000×*g* for 30 min. The resulting supernatant was dialyzed against buffer 1.

2.3.3. Hydroxyapatite chromatography. The dialyzed supernatant was applied to a hydroxyapatite column (2.6 cm×10 cm) equilibrated with buffer 1. The column was washed with 500 ml of buffer 1 and the enzyme was eluted using a linear gradient of potassium phosphate (10 mM–1 M), pH 7.5 in a volume of 1500 ml. The flow rate was 5 ml/min and 10-ml fractions were collected. The uracil-DNA glycosylase was eluted between 50 and 150 mM potassium phosphate. Active fractions were pooled and dialyzed against buffer 2 (10 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol).

2.3.4. Chromatography on Q-Sepharose fast flow high load. The dialyzed pool was applied to a Q-Sepharose fast flow high load col-

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Abbreviations: UNG, uracil-DNA glycosylase; BSA, bovine serum albumin

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umn (2.6 cm×10 cm), equilibrated with buffer 2. The column was washed with buffer 2 and the enzyme was eluted with a linear NaCl gradient (0–1 M) in 1.5 l of buffer 2. At a flow rate of 10 ml/min 10-ml fractions were collected. The enzyme eluted at about 250 mM NaCl. Active fractions were pooled and solid ammonium sulfate was added to a final concentration of 1.3 M.

2.3.5. Chromatography on Phenyl-Sepharose fast flow. The enzyme solution was applied to a Phenyl-Sepharose fast flow column (1.6 cm×10 cm), equilibrated with buffer 3 (100 mM potassium phosphate, pH 6.0, 1 mM β-mercaptoethanol, 1 M ammonium sulfate). The column was washed with 200 ml of buffer 3 and the enzyme was eluted using a linear gradient of 50 ml of buffer 3 and 50 ml of buffer 4 (100 mM potassium phosphate, pH 6.0, 1 mM ß-mercaptoethanol, 10% glycerol). Fraction size was 4 ml at a flow rate of 2.5 ml/min. The active fractions were pooled and dialyzed against storage buffer (50 mM HEPES-KOH, pH 8.0, 1 mM EDTA, 1 mM DTT, 300 mM NaCl, 50% glycerol).

2.4. SDS gel electrophoresis

SDS gel electrophoresis was performed using 8–25% gradient gels on a Pharmacia phast system. The molecular weight of the uracil-DNA glycosylase was determined using a molecular weight standard consisting of lysozyme (14300 Da), trypsin inhibitor (20100 Da), triosephosphate isomerase (26600 Da), aldolase (39200 Da), glutamate dehydrogenase (55500 Da), and fructose-6-phosphate kinase (85200 Da). Gels were stained with Coomassie brilliant blue G250.

2.5. Enzyme assays

2.5.1. Standard assay. The standard reaction mixture (50 µl) consisted of 1 µg M13mp11 U-amber ss-DNA in 60 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA. Enzyme was added in a suitable dilution. After incubation for 60 min at 37°C, 16.5 µl of 0.6 M NaOH were added and the mixture was incubated for 5 min at 37°C. After addition of 16.5 µl of 0.6 M HCl the samples (20 µl) were applied to agarose gel electrophoresis (1%). 1 U of uracil-DNA glycosylase is defined as that amount of enzyme that degrades 1 µg of M13mp11 U-amber ss-DNA in 60 min at 37°C.

2.5.2. Radioactivity assay. Preparation of [^{3}H]uracil-labeled DNA substrate was performed by random primer labeling [13]. The reaction mixture (5 ml) contained 2.5 mg calf thymus DNA, 5000 U Klenow fragment, 1 ml hexanucleotide mixture (3.125 mg/ml) in 0.1 M Tris-HCl, pH 7.2, 20 mM $MgCl_2$, 0.2 mM DTE, 0.4 mg/ml BSA, 0.5 µM of dCTP, dGTP, dATP each, 23 nM dUTP and 2.6 nM [^{3}H]dUTP. After incubation for 1 h at 37°C non-incorporated nucleotides were removed by gel filtration on a column of Sephadex G-50 (2.5×10 cm), equilibrated in 10 mM Tris-HCl, pH 8.0. Fractions containing [^{3}H]dUMP-DNA were pooled and concentrated in vacuo.

The radioactive test mixture (50 µl) consisted of 5 µl of labeled calf thymus DNA (10000 cpm = 0.82 pMol [^{3}H]uracil) in 60 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml BSA. The reaction was initiated by adding the enzyme in a suitable dilution. After incubation at 37°C for 10 min the reaction was chilled on ice followed by the addition of 100 µl of herring sperm DNA (1 mg/ml) and 300 µl of trichloracetic acid (10%). After incubation for 10 min at 4°C, the samples were centrifuged at 5000×*g*. 400 µl of the supernatant were removed and the radioactivity was determined in a liquid scintillation counter.

2.6. Tests for contaminating activities

The absence of contaminating activities was investigated in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTE. Enzyme fractions (20 µl) were incubated with the respective nucleic acids. Nicking activity was assayed by incubating 1 µg pBR322 for 16 h at 37°C. Single and double strand nucleases were tested using M13mp9-ss DNA and λ/*Eco*RI, *Hin*dII, respectively; incubation time was 16 h at 37°C. The absence of RNases was tested by incubating samples with 5 µg MS 2 RNA for 1 h at 37°C. For the test of exonucleases the samples were incubated with 1 µg ^{3}H-labeled DNA for 4 h at 37°C and the release of ^{3}H-labeled nucleotides was assayed.

2.7. Protein determination

Protein concentrations were determined by the method of Lowry et al. [14].

2.8. Carryover prevention

Thermolabile UNG was tested for application in carryover prevention. As artificial contamination, dU-containing DNA (generated by PCR) was treated with UNG followed by PCR. The target of the amplification was a 1.1 kb fragment of collagen gene. Reactions contained 2.2 ng–2.2 fg dU-containing target, 1.6 µM of oligonucleotide primers (5′-TAA AGG GTC ACC GTG GCT TC-3′ and 5′-CGA ACC ACA TTG GCA TCA TC-3′) 2 units of UNG, 2.5 units of Taq DNA polymerase in 100 µl of 10 mM Tris-HCl, pH 8.9, 50 mM KCl, 1.5 mM $MgCl_2$; dATP, dCTP, dGTP each at 200 µM and 600 µM dUTP. After incubation for 10 min at 20°C, UNG was inactivated by incubation at 95°C for 10 min. PCR consisted of 30 cycles of 10 s at 94°C, 30 s at 60°C, 60 s at 72°C. Aliquots of the reactions were electrophoresed on 1% agarose gels and stained with ethidium bromide [15].

2.9. Heat inactivation

Heat inactivation was performed in a buffer consisting of 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KCl. 2 units of uracil-DNA glycosylase were diluted in 100 µl buffer and incubated at different temperatures. At different periods samples were removed, chilled on ice and the remaining activity was determined in the radioactive test system.

2.10. Characterization of residual activity

Residual activity of UNG following heat inactivation and PCR was characterized by monitoring the degradation of a DIG-labeled dU-PCR product. The PCR product was a 103 bp fragment of pUC18 generated by using the pUC18 sequencing forward primer (5′-DIG-GTA AAA CGA CGG CCA GT-3′) and the pUC18 sequencing reverse primer (5′-CAG GAA ACA GCT ATG AC-3′) flanking the multiple cloning site of pUC18. The amplification reaction (100 µl) contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, dATP, dCTP, dGTP each at 200 µM, 600 µM dUTP, 2.5 units Taq DNA polymerase, 1 ng pUC18/*Pst*I, primers (see above) each at 1 µM and 2 units of UNG. UNG was inactivated by treatment at 95°C for 2–10 min prior to amplification. PCR was performed as follows: 25 cycles of 94°C, 1 min; 50°C, 1 min; 72°C, 3 min; followed by a soak file at 4°C. During the soak time samples (20 µl) were removed at different periods, treated with 5 µl of 0.6 M NaOH for 5 min at 37°C and 5 µl of 0.6 HCl. Finally 4 µl of formamide dyes were added, the samples were heated to 95°C for 5 min and subjected to a sequencing gel [15]. Blotting and detection of DIG-labeled (intact and degraded) PCR products were performed as described in the manual of the DIG-Taq DNA sequencing kit and the DIG-luminescent detection kit.

The influence of the buffer on the amount of remaining activity was assessed under different conditions. For the PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$) the pH value was varied (pH 8.3, 8.6, 8.9 and 9.2). Additionally the effect of DMSO (1% and 3%) and ammonium sulfate (10 mM and 30 mM) was studied using PCR buffer, pH 8.3.

3. Results

3.1. Purification and characterization

Uracil-DNA glycosylase from BMTU 3346 was purified to apparent homogeneity with a specific activity of 4×10^5 U/mg. Under the conditions described above, no contaminating activities were detectable in the final fraction of UNG. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis the enzyme showed a single band. From the mobility of molecular weight markers the molecular weight of the uracil-DNA glycosylase was determined to be 23400 Da (± 1000). Preliminary data of gel filtration experiments on Superose 12 show that the UNG coelutes with chymotrypsinogen A (molecular weight 25000 Da) indicating a monomeric structure in solution.

3.2. Stability

In storage buffer the uracil-DNA glycosylase is stable at −20°C for months. Under reaction conditions at 37°C the

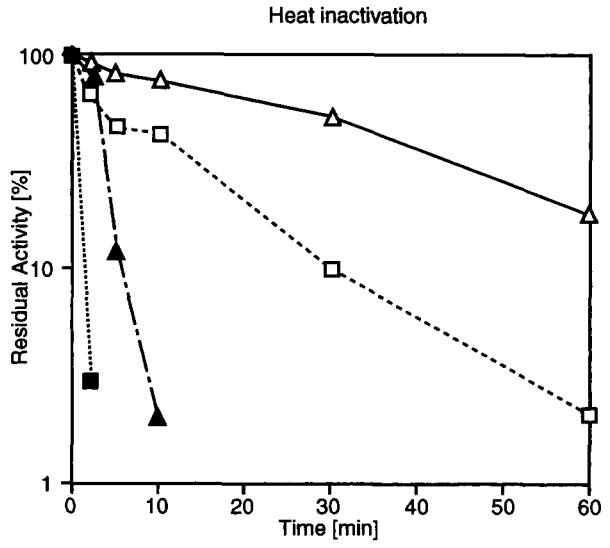

Fig. 1. Thermostabilty of UNGs from BMTU 3346 (closed symbols) and *E. coli* (open symbols). 2 units of UNG were incubated in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ at 40°C (triangles) and 45°C (squares). At different times sample were removed and the residual activity was determined.

enzyme shows a linear kinetics for at 1 h. In 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ the enzyme is rapidly inactivated by elevated temperatures. At 40°C (45°C) the half-life was determined to be 2 min (0.5 min) (Fig. 1). At pH 8.9 similar data were obtained. For the UNG from E. coli the half-life at pH 8.3 were determined to be 27 min at 40°C and 8 min at 45°C respectively (Fig. 1).

3.3. Residual activity of heat-labile UNG after application in carryover prevention

UNG from BMTU 3346 was applied in the carryover prevention technique [10]. Incubation of 2 units of enzyme for 10 min at 20°C with dU-containing template (2.2 ng–2.2 fg) prevented amplification of this target by PCR. In control reactions the UNG did not affect the amplification of dT-containing template (data not shown).

The degradation of an amplified 103 bp DIG-labeled dU-PCR product was followed to detect residual activity of UNG after heat inactivation. Removal of any of the dUs in the PCR product caused chain scission upon alkali treatment and appearance of oligomer degradation products. Fig. 2 examines the fate of dU-PCR products (after inactivation for 2 min at 95°C followed by PCR) during incubation at 4°C. For the heat-labile UNG no significant amounts of degraded dU-PCR products were observed between t=0 and 4 h (Fig. 2A, lanes 1–3). After 16 h of incubation degradation is observed (Fig. 2A, lane 4). The UNG from *E. coli* showed stronger degradation of dU-PCR products starting at t=0 (Fig. 2B, lanes 1–4). Without UNG no degradation is observed indicating the stability of the dU-PCR products during the experimental conditions. Addition of UNGs after the last PCR cycle results in total degradation.

In experiments to minimize the amount of remaining activity by application of different buffer and salt conditions in the heat inactivation step no conditions for further minimization of the remaining activity were found.

Extension of the inactivation at 95°C to 10 min resulted in similar degradation patterns, indicating that the inactivation was not significantly improved by prolongation of the inactivation time. For the UNG from BMTU 3346 detailed analysis of the degradation kinetics revealed the appearance of degraded dU-PCR products at t = 10 h (data not shown).

4. Discussion

The uracil-DNA glycosylase from the psychrophilic marine strain BMTU 3346 has been purified. With respect to the monomeric character and the molecular weight the enzyme is similar to the uracil-DNA glycosylases from other prokaryotic organisms [3–9]. The molecular weight of 23400 Da closely resembles those of the enzymes from *B. subtilis* and *E. coli* having a molecular weight of 24000 and 24500 Da, respectively [5,4]. Like other UNGs the enzyme described excises uracil from single and double stranded DNA.

The uracil-DNA glycosylase is stable in complex buffers as used under storage and reaction conditions. Preliminary data indicate that glycerol and BSA have the most stabilizing effect. However, in dilute buffers as usually applied in PCR technology the enzyme is extremely heat-labile. For the uracil-DNA glycosylase from *E. coli* several heat-labile mutants

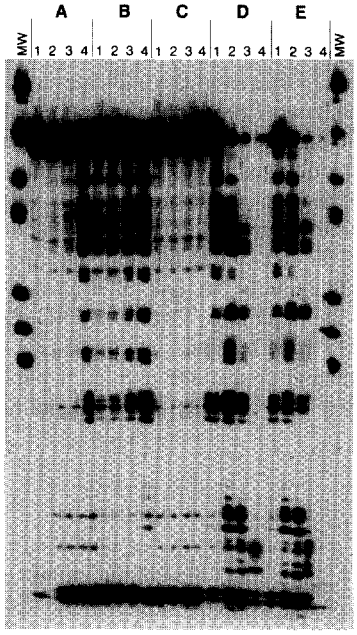

Fig. 2. Characterization of residual UNG activity. The degradation of a 103 bp DIG-labeled dU-PCR product was monitored. 100 µl amplification reactions containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, 600 µM dUTP, 2.5 units of Taq DNA polymerase, 1 ng pUC18/*Pst*I and 1 µM of each primer (DIG-labeled pUC18 sequencing forward primer, pUC18 sequencing reverse primer) were prepared. Samples were treated at 95°C for 2 min followed by PCR (25 cycles of 94°C, 1 min; 50°C, 1 min; 72°C, 3 min; followed by incubation at 4°C). Samples (20 µl) were removed at different times, treated with 5 µl of 0.6 M NaOH for 5 min at 37°C and 5 µl of 0.6 M HCl. After addition of formamide dye (4 µl) aliquots of each were analyzed by gel electrophoresis on a sequencing gel, blotting and chemoluminescence detection. The mixtures contained (A) 2 units of UNG from BMTU 3346, (B) 2 units of UNG from *E. coli*; (C) negative control without UNG. As positive controls UNGs were added after the last cycle, (D) 2 units of UNG from BMTU 3346, (E) 2 units of UNG from *E. coli*. Samples were removed at t = 0 (lane 1), t = 1 h (lane 2), t = 4 h (lane 3) and t = 16 h (lane 4).

have been described. However, attempts to purify one of the mutant enzymes were unsuccessful due to the instability of the mutant enzyme [16]. Because of its characteristics the UNG from BMTU 3346 is suitable for application in an improved carryover prevention technique. For the UNG from *E. coli* it has been reported sufficient UNG activity survives the heat-inactivation and the PCR to degrade uracil-containing DNA during extended incubation at 4 or 25°C [12]. Therefore, the performance of the UNG from BMTU 3346 in carryover prevention technique was compared to that of the enzyme from E. coli. Both enzymes are suitable to prevent amplification of an artificial DNA contamination. Attempts to precisely characterize the residual UNG activity after heat inactivation and PCR were unsuccessful due to insufficient sensitivity of the radioactive test system used [12]. Therefore, a DIG-labeled 103 bp uracil-containing PCR product was used to detect residual UNG activity by monitoring the appearance of degradation products. After inactivation for 2 min at 95°C followed by PCR, degraded PCR products were detectable immediately after the last PCR cycle in the reaction mixture containing UNG from *E. coli*. This strongly indicates that an amount of UNG from *E. coli* survives the inactivation enough to degrade the PCR product. After prolonged incubation at 4°C the amount of degraded PCR product increases.

The PCR mixture containing UNG from BMTU 3346 showed no degraded PCR products after the last PCR cycle. Within the limits of detection this indicates a complete inactivation of the enzyme. The reaction containing the heat-labile UNG shows an significant amount of degraded dU-PCR product only after prolonged (> 10 h) incubation. On incubation at 25°C, degradation proceeds more rapidly with both UNGs (data not shown). Therefore, PCR samples should be kept at 4°C to decrease the risk of degradation. The application of the heat-labile UNG reduces the risk of degradation within the first hours of the soak file. For the UNG from *E. coli* a reactivation after PCR has been discussed [12]. However, under the experimental conditions used it is impossible to discriminate whether the accumulation of degradition products is the result of residual enzyme activity over time or/and is influenced by regain of activity by reactivation.

The results clearly demonstrate that the UNG from BMTU 3346 is more heat-labile than the enzyme from *E. coli*. It shows less residual activity (or regain of activity) than the enzyme from *E. coli* and is more suitable to avoid the degradation of dU-PCR product at least within the first hours of the soak file. Therefore, the application of the UNG described here provides an improvement of the carryover prevention technique.

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