URACIL–DNA GLYCOSYLASE FROM *BACILLUS STEAROTHERMOPHILUS*

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Received 24 July 1981

1. Introduction

Uracil residues are introduced into DNA as a result of the spontaneous cytosine deamination or occasional incorporation of the deoxyuridylate during DNA synthesis [1–3]. The first step of the base excision repair of uracil-contaminated DNA can be carried out by a DNA-glycosylase which catalyzes uracil–ribose bond hydrolysis. Such enzymes have been purified and studied in detail from bacteria and mammals [4–8]. Here, we describe the purification and general properties of an uracil–DNA glycosylase from thermophilic bacterium *Bacillus stearothermophilus*.

2. Materials and methods

The strain of *B. stearothermophilus* used was a gift from L. G. Loginova. The growth conditions were as in [9].

The substrate for determination of uracil–DNA glycosylase activity was prepared by copying activated salmon sperm DNA with *B. stearothermophilus* DNA polymerase I, using d[^{3}H]UTP (2000 cpm/pmol) as precursor [9]. Enzyme activity was measured in a reaction mixture (10 μl), containing 10 mM Tris–HCl (pH 8.0), 0.5 mM EDTA, 25 mM NaCl, 10 000 cpm d[^{3}H]UMP–DNA and various amounts of the enzyme. After incubation for 10 min at 60°C the mixture was chromatographed on Ecteola-cellulose paper in the system, water:ethylacetate:chloroform (7:2:1). Under these conditions the uracil and dUMP were separated in 10 min. The areas containing an uracil standard were cut out and the radioactivity counted in a toluene scintillator. One unit activity of uracil–DNA glycosylase was defined as the amount of enzyme that catalyzes the release of 1 nmol of free uracil/min under the described conditions.

Exonuclease activity was measured as in [9]. Endonuclease activities were determined by measurement of relaxation of the native, UV-irradiated or depurinated ColE1 DNA using an agarose gel electrophoresis technique [10].

Sodium dodecyl sulfate (SDS) slab gel electrophoresis was performed according to [11].

Protein concentration was determined either spectrophotometrically according to [12] or densitometrically by staining the protein after electrophoresis.

Table 1
Enzyme purification

Purification stage	Volume (ml)	Total protein (mg)	Spec. act. (units/mg)	Total act. (units)	Recovery (%)
Crude extract	100	3000	0.3	1000	100
$(NH_4)_2SO_4$ precip.	18	1600	0.6	960	96
DEAE-cellulose	60	600	1.2	750	75
Phosphocellulose	70	10	54	550	55
DNA–agarose	20	0.6	510	320	30
Sephadex G-75	20	0.2	700	140	14
Hydroxylapatite	6	0.06	1600	96	10

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

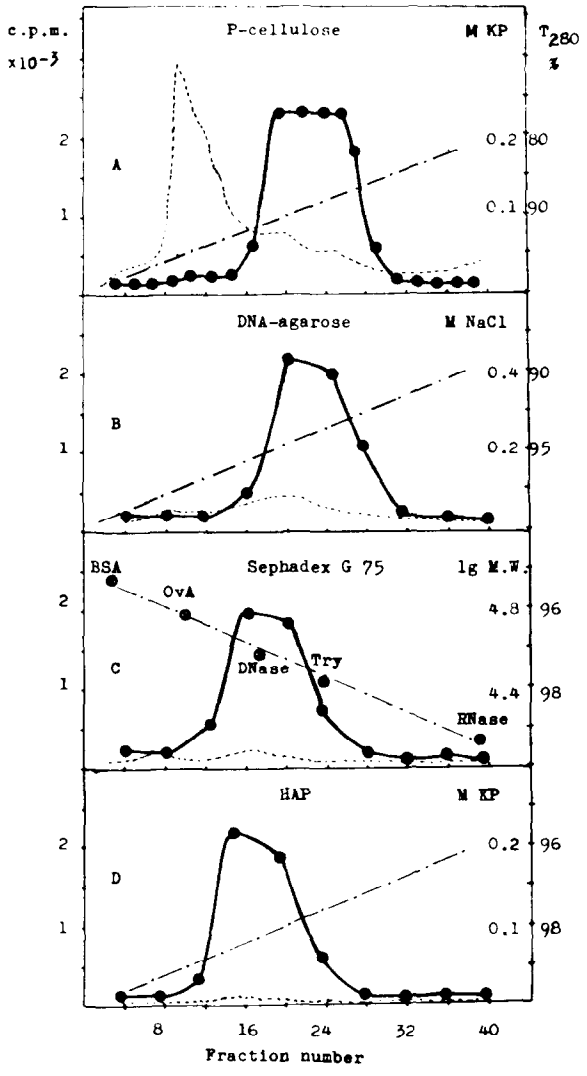

Fig.1. Purification profiles of the uracil–DNA glycosylase. Unadsorbed material from DEAE-cellulose was diluted 2-fold in B1 buffer and loaded onto a 50 ml phosphocellulose column, equilibrated with the same buffer. The proteins were eluted by a 400 ml linear gradient of 0.0–0.2 M KP in B1 buffer (A). Active fractions were diluted 3-fold in buffer B1 and layered on 10 ml DNA–agarose column. The elution was with a 100 ml linear gradient of 0.0–0.6 M NaCl in B1 buffer (B). The enzyme concentrated by ammonium sulfate was loaded on a 100 ml column of Sephadex G-75, equilibrated with B1 buffer, 0.5 M NaCl and eluted with the same buffer (C). Desalted enzyme was loaded on a 4 ml hydroxylapatite column, equilibrated with a buffer B1 and a linear gradient of 0.0–0.2 M KP in buffer B1 (20 ml) was applied (D).

3. Results

A summary of the purification procedure is given in the table 1. All operations were at 5°C, and centrifugations were for 30 min at 10 000 × *g*.

The bacterial cells (60 g) were stirred with 100 ml buffer B1 (10 mM potassium phosphate (KP) (pH 7.3), 0.5 mM EDTA, 1 mM NaN_3, 1 mM phenylmethyl sulfonylfluoride, 10% glycerol), containing 0.3 M NaCl, and disintegrated in a French press. The debris were removed by centrifugation at 40 000 × *g* for 60 min and fractionation by ammonium sulfate (35–70%) was performed. The protein was dissolved in 40 ml buffer B1. This suspension was desalted by Sephadex G-25 gel filtration in B1 buffer, containing 0.1 M NaCl and passed through a 200 ml DEAE-cellulose column, equilibrated with the same buffer. The enzyme was found in the unadsorbed material and purified by column chromatography on phosphocellulose, DNA-agarose, Sephadex G-75 and hydroxylapatite, as in fig.1.

When the purified uracil–DNA glycosylase was analyzed by discontinuous SDS–polyacrylamide slab gel electrophoresis, one main protein band was observed, representing ~70–80% of the stained material (fig.1E). Its position corresponds to an M_r of 28 000–30 000. A similar M_r-value was obtained by Sephadex G-75 gel filtration (fig.1C).

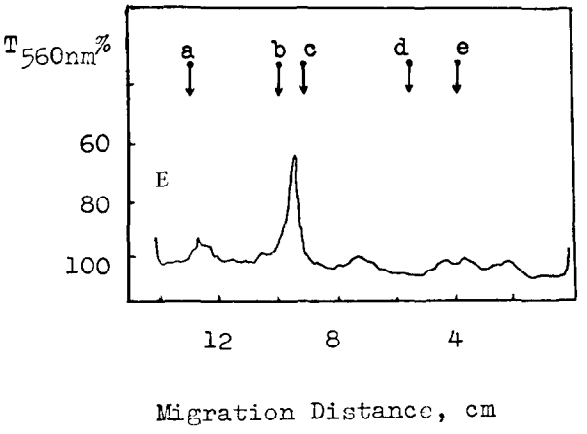

Fig.1E. Densitometric tracing of SDS–polyacrylamide gel electrophoresis of purified uracil–DNA glycosylase. The concentration of the gel was 12%. The gel was stained with Coomassie blue and scanned at 560 nm. About 5 μg enzyme was used. Protein standards (M_r): (a) bovine serum albumin (68 000); (b) ovalbumin (43 000); (c) pancreatic DNase (31 000); (d) trypsin (23 000; (e) pancreatic RNase (14 000).

Table 2
General properties of uracil–DNA glycosylase

Incubation condition	Activity (%)
Complete system	100
– enzyme	1
+ ATP (1 mM)	110
+ EDTA (20 mM)	86
+ $MgCl_2$ (10 mM)	86
+ $MnCl_2$ (2 mM)	81
+ $CoCl_2$ (2 mM)	35
+ *p*-Chloromercuribenzoate (1 mM)	74
+ *N*-Ethylmaleimide (10 mM)	100
+ Dithiothreitol (10 mM)	100
+ Uracil (1 mM)	70
+ Thymine (1 mM)	102
+ 5-Bromo uracil (1 mM)	96
+ NaCl (0.1 M)	70
+ NaCl (0.2 M)	60
+ NaCl (0.3 M)	15
+ SDS (0.1%)	12
+ Triton X-100 (1%)	100
+ Urea (1 M)	100
+ Spermidine (1 mM)	100
+ Daunomycin (1 μg/ml)	23
+ Actinomycin D (1 μg/ml)	30
+ Ethidium bromide (1 μg/ml)	88

The general properties of the enzyme are summarized in table 2.

Uracil–DNA glycosylase was free from exonuclease and endonuclease activities. Successive treatment of native or UV-irradiated (120 J/m^2) ColE1 DNA by uracil–DNA glycosylase and apurinic endonuclease (AP-endonuclease was purified from the same strain of bacteria) did not induce chain breaks.

The enzymic reaction remained linear during 30 min at 60°C and its velocity is proportional to the amount of active protein in the assay mixture up to 0.5 μg/ml.

As estimated from a double reciprocal plot, the K_m of uracil–DNA glycosylase for dUMP is 0.4 μM.

The enzyme exhibited extreme thermostability. It could be heated at 60°C (growth temperature) for 60 h without any loss of activity at all steps of purification.

A linear Arrhenius plot gives an energy of activation ~13 kcal/mol at 20–44°C and ~4 kcal/mol at 44–60°C (fig.2).

If the cells were grown for ⩾12 h and ammonium sulfate precipitation during purification was omitted, at least 5 chromatographically distinct forms of uracil–DNA glycosylase were observed. They can be distinguished by their M_r-value and thermostability (not shown). This finding is probably a result of protein modification in the process of sporulation. Only one main activity was observed if bacteria were harvested in the logarithmic growth phase.

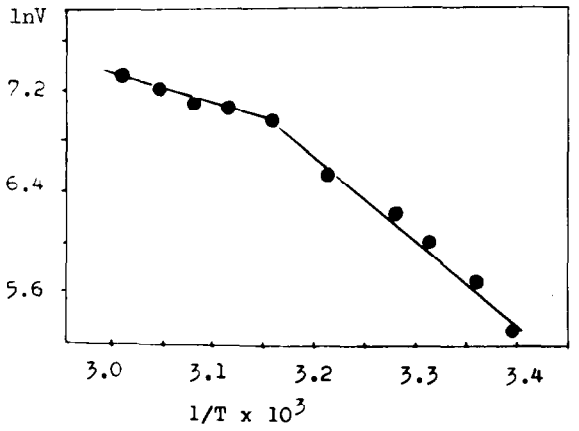

Fig.2. Arrhenius plot, ln V vs $1/T$. Time of incubation of the reaction mixture was 10 min at indicated temperature. Concentrations of the substrate and the enzyme were 500 nM and 0.2 nM, respectively.

3. Discussion

The specific activity of uracil–DNA glycosylase in *B. stearothermophilus* extract is not higher than that from mesophilic organisms. Probably, the contamination of the uracil residues into DNA does not play a significant role in spontaneous damaging of DNA of the thermophile. Furthermore, a purified enzyme resembles the mammalian uracil–DNA glycosylase more closely than those of *Escherichia coli* or *Micrococcus luteus* [1–4]. It has an M_r of 30 000 K_m of 0.4 μM, turnover no. of ~100, whereas the corresponding values for *E. coli* enzyme are 24 000, 50 nM and 800 [4].

Uracil–DNA glycosylase from *B. stearothermophilus* appears to be thermostable at up to 0.2 μg protein/ml. The addition of albumin does not provide any protecting effect, suggesting that intrinsic thermoresistance of the enzyme is the result of its primary structure.

Fig.2 illustrates the effect of the temperature on the rate of the glycosylase reaction. The Arrhenius plot is a biphasic curve with transition temperature at 44°C. This transition probably, reflects a confor-

mational change of the protein. Other thermophilic enzymes have the same feature [13].

In many other properties (see table 2) uracil–DNA glycosylase from *B. stearothermophilus* is similar to its bacterial and mammalian counterparts [4–8].

Acknowledgement

We thank V. I. Makhno for providing a part of the cell extract.

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