ENDONUCLEASES FOR APURINIC SITES IN PLANTS

Lise THIBODEAU and Walter G. VERLY

Département de Biochimie, Université de Montréal, Canada, et Biochimie, Faculté des Sciences, Université de Liège, Belgium

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1. Introduction

Many chemical or physical agents damaging DNA result in a loss of purines or pyrimidines; moreover this loss occurs spontaneously at a slow rate [1,2]. To keep its genetic information intact, a cell must be able to repair depurinated or depyrimidinated DNA. An endonuclease specific for apurinic sites was first discovered in *Escherichia coli* by Verly and Paquette [3]; the enzyme was used for the successful repair of depurinated DNA in vitro [4] and it has been completely purified [5]. Ljungquist et al. [6] found in *Escherichia coli* a second endonuclease for apurinic sites, responsible for about 10% of the total activity, which they named endonuclease IV. Endonucleases for apurinic sites were found in other bacteria and also in animal tissues [7,8]; the present paper shows that enzymes with the same specificity exist in higher plants, fungi and algae.

2. Materials and methods

Seed embryos of *Phasaeolus multiflorus* were taken after a 4-day germination in the dark. Roots and primordial leaves came from young plants raised for 10 days after germination with a natural cycle of light and dark, then for 24 h in darkness. The fruiting bodies of the fungus *Agaricus campester* were bought from the market. The photosynthetic blue alga *Anacystis nudulans* (Indiana Culture Collection 625) was cultured in a synthetic medium [9] under fluorescent tubes with 2% CO_2 in air bubbling; after one week, the cells were collected by centrifugation.

Tissues were ground with a Polytron in buffer A (0.05 M Tris–HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 8). The homogenates, after filtration through several layers of cheesecloth, were centrifuged at 100 000 × *g* for 90 min, the supernatants were used for the enzyme assay. The cells of *Anacystis nidulans* were ground with the help of alumina in buffer B (0.05 M Hepes, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 8); the suspension was centrifuged at 5000 × *g* for 15 min to discard the alumina and the cellular debris. A 100 000 × *g* supernatant was then prepared and dialyzed against buffer A.

To prepare nuclei, *Phasaeolus multiflorus* embryos were ground in Honda's buffer (2.5% ficoll, 5% dextran, 0.1% bovine serum albumin, 0.25 M sucrose, 25 mM Tris–HCl, 4 mM 2-mercaptoethanol, 1 mM $MgCl_2$, pH 8.5). After filtration through several layers of cheesecloth, the homogenate was centrifuged at 4000 × *g* for 15 min. The sediment, dispersed in Honda's buffer, was layered on top of a discontinuous gradient of sucrose (1.0 M and 1.7 M) in Honda's buffer and centrifuged at 53 000 × *g* for 60 min. The purified nuclei of the sediment, after sonication, were used to prepare chromatin, and the chromatin was dissociated into DNA, histones and non-histone proteins according to the methods of Gilmour and Paul [10] and Stein et al. [11].

To isolate the chloroplasts, the *Phasaeolus multiflorus* leaves were ground in Honda's buffer. The homogenate, after filtration through several layers of cheesecloth, was centrifuged at 4000 × *g* for 15 min, and the supernatant submitted to another centrifugation at 18 000 × *g* for 15 min. The sediment, dispersed in Honda's buffer, was placed on a four-layer discontinuous gradient of sucrose (1.0 M, 1.5 M, 2.0 M and 2.5 M) in Honda's buffer, and centrifuged at 53 000 × *g* for 50 min. The chloro-

plasts were collected between the 1.5 M and 2.0 M sucrose layers, dispersed in Honda's buffer, and spun down at 18 000 × *g* for 15 min. The chloroplasts of the sediment were lysed in distilled water and the preparation was dialyzed against buffer A.

Aliquots of 300 μl of the various crude extracts in buffer A were incubated with 300 μl of SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 8) containing 6 μg of ^{3}H-labelled DNA, either normal, or alkylated (with methyl methanesulfonate; 550 methylated sites per 10^6 daltons), or alkylated-depurinated (160 apurinic sites and 390 methylated sites per 10^6 daltons); the $MgCl_2$ concentration was 10 mM. The mixtures were incubated at 37°C and aliquots were taken after various periods of time; carrier DNA and perchloric acid (to have a 5% final concentration) were successively added and, after centrifugation, the radioactivity of the supernatant was determined. The results are corrected for controls without enzyme; they are expressed as fractions of the substrate total radioactivity.

For the determination of the enzyme activity, 20 μl of SSC containing 0.4 μg of alkylated-depurinated [^{3}H]DNA were incubated with 20 μl of the enzyme preparation in buffer A for 15 min at 37°C before measuring the acid-soluble radioactivity. The enzyme preparation was always sufficiently diluted to have an excess of substrate and work near V_{max} (treatment of the alkylated-depurinated DNA with NaOH yielded an acid-soluble fraction of 0.35). The enzyme unit is the activity necessary to give an acid-soluble fraction of 0.1 in the assay.

3. Results and discussion

Seed embryos, leaves or roots of *Phasaeolus multiflorus* were ground in buffer A and 100 000 × *g* supernatants were prepared. Aliquots from these supernatants were tested on normal, alkylated, and alkylated-depurinated DNAs. The data for the embryos are presented in fig.1a: the supernatant has little action on normal DNA, a slightly greater action on alkylated DNA (a few apurinic sites appear during incubation), but this latter DNA becomes a much better substrate when the alkylated sites are replaced by apurinic sites. Similar results (not shown) were obtained with the 100 000 × *g* supernatants from leaves and roots, proving that an endonuclease for apurinic sites is present in all the tissues of this higher plant.

A 100 000 × *g* supernatant of *Anacystis nidulans* cells was prepared as described in Materials and methods. Slices of caps and stalks from *Agaricus campester* fruiting bodies were ground in buffer A; because of its very high enzyme activity, the 100 000

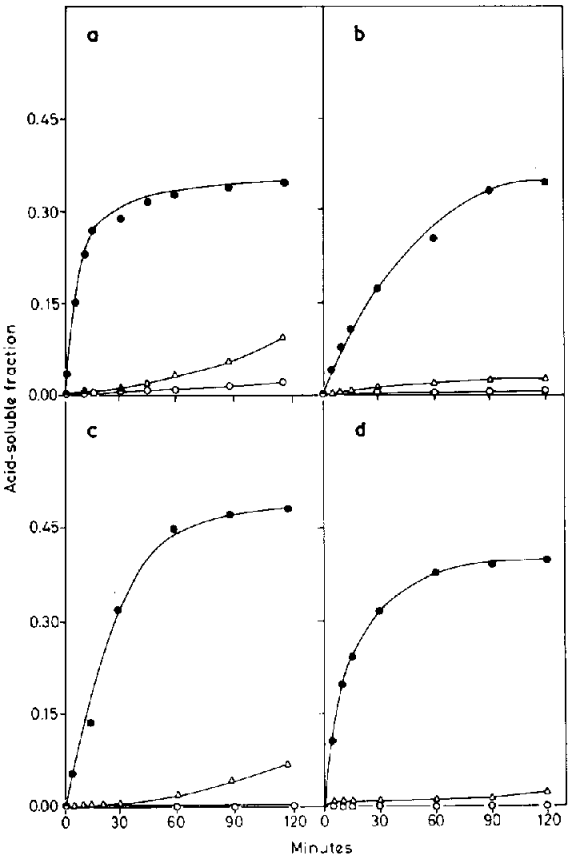

Fig.1. Endonucleases for apurinic sites in *Phasaeolus multiflorus, Anacystis nidulans,* and *Agaricus campester.* To 300 μl of crude extract in buffer A, are added 300 μl of SSC containing 6 μg of ^{3}H-labelled DNA which is either normal (○), alkylated (△) or alkylated-depurinated (●); the mixture is incubated at 37°C and 20 μl aliquots are taken at various times (minutes) to measure the acid-soluble radioactivity (acid-soluble fraction). (a) Nucleo-cytoplasmic extract of *Phasaeolus multiflorus* embryos; (b) extract of *Phasaeolus multiflorus* chloroplasts; (c) extract of *Anacystis nidulans* cells; (d) 60-fold diluted extract of *Agaricus campester* fruiting bodies. In every case, the sensitivity of DNA to nuclease action mostly depends on the presence of apurinic sites.

X *g* supernatant was diluted 60-fold with buffer A. Figure 1 (c and d) shows the effect of these supernatants on normal, alkylated and alkylated-depurinated DNAs; the same picture always emerges indicating the presence of endonucleases for apurinic sites.

The endonuclease for apurinic sites was purified from the 100 000 X *g* supernatant in buffer A of *Phasaeolus multiflorus* embryos. A complete description of this purification will appear elsewhere. After removal of the nucleic acids with streptomycin sulfate and fractional precipitation with ammonium sulfate, the enzyme was chromatographed on DEAE-cellulose and DNA-cellulose. It was then found practically pure on polyacrylamide gel electrophoresis in the native form or denatured in sodium dodecyl-sulfate; its molecular weight, estimated by filtration through a Sephadex G-75 column, is around 40 000. The purified enzyme has no action on normal DNA strands or on alkylated sites; it is strictly specific for apurinic sites. Its action on apurinic sites is greater in native than in denatured DNA. The purified plant endonuclease for apurinic sites is devoid of exonuclease activity; it is slightly inhibited by 10 mM EDTA, and its activity is stimulated by Mg^{2+} or Mn^{2+} ions.

After sonication, the crude nuclear fraction from 250 g of embryos ground in Honda's buffer revealed 10 000 units of endonuclease for apurinic sites, whereas the 100 000 X *g* supernatant contained 31 000 units. The chromatin isolated from these nuclei had about 25% of the activity found in the sonicated nuclei; but, because the yield of chromatin isolation is unknown, this result does not enable to calculate the amount of enzyme in the nucleoplasm. When the chromatin was dissociated into its components, the activity of the proteins was 8 times greater than that of the native chromatin from which they originated; on the other hand, 90% of this activity was associated with the non-histone proteins and it is likely that the low activity of the histone fraction was only a contamination. It can be concluded that the determination of the endonuclease for apurinic sites on sonicated nuclei does not give a true estimation of the nucleus content of the enzyme; the figure obtained is much too low. It finally appears that the endonuclease for apurinic sites is mostly localized in the nucleus and that it is one of the non-histone proteins of the chromatin. The experimental data suggest that the enzyme molecule, when integrated in chromatin, is oriented to work on chromatin DNA rather than on an exogenous substrate.

Chloroplasts were purified from *Phasaeolus multiflorus* leaves and lysed in distilled water. Figure 1b shows the action of this crude extract on normal, alkylated and alkylated-depurinated DNA; here again, the DNA becomes a good substrate only when alkylated sites are replaced by apurinic sites. To know whether the chloroplasts had a special endonuclease for apurinic sites, the half-lives at 40°C of the chloroplastic and nucleo-cytoplasmic enzymes were measured; they were found to be 90 and 240 s respectively, which seems to indicate that the two enzymes are different.

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