DEOXYRIBONUCLEASE I COVALENTLY COUPLED TO POROUS GLASS

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1. Introduction

The preparation of several insolubilized enzymes by covalent linking to porous glass was reported recently [1–3]. We describe here the properties of DNAase I coupled to porous glass – an enzyme derivative surpassing the soluble enzyme in its ability to degrade DNA.

2. Materials and methods

Porous 96% silica glass, 790 Å ± 10% pore size, 100 mesh (Corning) was cleaned by heating at 85° for 3 hr in 0.1 N HNO_3, dried in an oven at 90° and heated at 700° for 3 hr under oxygen. The cleaned glass was treated with a 10% (v/v) solution of γ-aminopropyltriethoxysilane in toluene as previously described [1–3]. The alkylaminosilane glass was converted to an arylamine derivative by reaction with *p*-nitrobenzoylchloride, followed by reduction of the nitro groups with $Na_2S_2O_4$. The arylamine derivative was diazotized [4] and allowed to react for 4 hr with a 1% solution of pancreatic DNAase I (Calbiochem, Los Angeles, California) in 0.1 N $NaHCO_3$ adjusted to pH 8.5. The final product, containing 12 mg of protein per g of glass (according to the difference in total nitrogen content before and after coupling the enzyme to glass determined by the Kjehdahl method), was washed with an excess of distilled water and stored dry at 4°.

To determine the properties of the insolubilized enzyme, 0.25 to 3.4 g of the DNAase-glass derivative was packed into disposable chromatographic columns (catalogue No. 96010 or 96020, BioRad Laboratories, Richmond, California) and washed with an appropriate buffer solution. Ten ml of a solution containing 0.51 μCi/ml of ^{3}H-labeled calf thymus DNA (specific radioactivity 76.2 μCi/mg; Worthington Biochemical Corp., Freehold, New Jersey) in the same buffer was placed on top of the columns and was allowed to displace the DNA-free buffer within the column and the adjoining tubing. The outlet tubing from the bottom of the column was connected with the top of the chromatographic column, and the solution of DNA was recirculated through the DNAase-glass derivative at speeds between 0.1 to 1.9 ml/min with a polystaltic pump (Buchler Instruments, Fort Lee, New Jersey). At time intervals, corresponding to a completed single (multiple) passage(s) of the DNA solution through the column, 0.225-ml samples were withdrawn from the outlet tubing and analyzed for total and trichloroacetic acid-(TCA) precipitable radioactivity [5]. Results were expressed in percentages of TCA-precipitable radioactivity, considering the TCA-precipitable radioactivity of the original DNA solution (0.225 ml) as 100%. Measurement of radioactivity was performed as described previously [6]. After the last passage through the column, a 2-ml aliquot of the DNA solution was further analyzed by gel filtration on Sephadex G-100 in K25/45 columns (Pharmacia, Uppsala, Sweden). Tris-buffered saline (0.14 M NaCl, 0.01 M tris-acetate, pH 7.0; TB) was used as eluant and fractions of 5 ml each were collected. Aliquots of 0.2 to 0.5 ml were counted for radioactivity.

3. Results and discussion

In the process of degradation of DNA by DNAase I,

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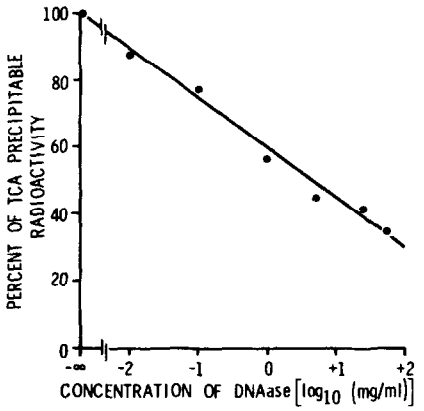

Fig. 1. Conversion of ^{3}H-labeled calf thymus DNA into TCA-soluble degradation products by increasing amounts of soluble DNAase I. The enzyme was added to a solution of DNA (0.17 μCi/ml) in a Britton-Robinson buffer pH 5.5 [8] containing 10^{-3} M $MgCl_2$. The reaction was allowed to proceed for 30 min at 36° and was stopped by adding 0.2 ml of a TCA solution (100 g/100 ml). Under optimal conditions (0.05 M tris, 0.005 M $MgCl_2$, 0.0013 M $CaCl_2$) [11], similar results were obtained, but the amount of TCA-soluble radioactive material released from DNA was about 1.25 times higher than that shown on the figure.

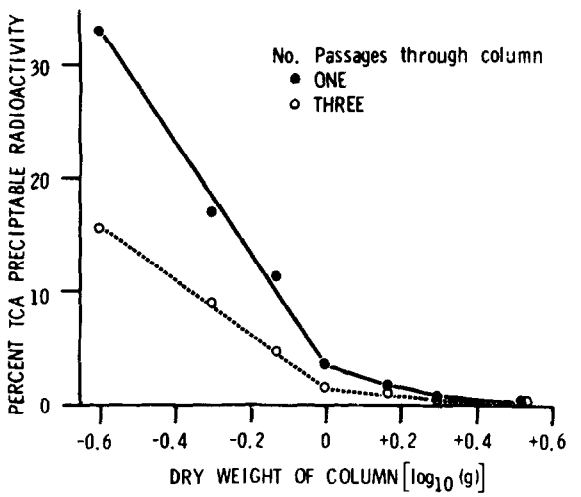

Fig. 2. Conversion of ^{3}H-labeled calf thymus DNA into TCA-soluble degradation products by increasing amounts of DNAase I coupled to porous glass. The DNA solution (7 ml) in Britton-Robinson buffer pH 5.5 containing 10^{-3} M $MgCl_2$ was passed through columns of the insolubilized enzyme (0.25 to 3.4 g, corresponding to 3 to 41 mg of DNAase I, i.e., to 0.43 to 5.9 mg of DNAase I per ml of the substrate solution). The temperature was 36° and the flow rate, 0.33 ml/min.

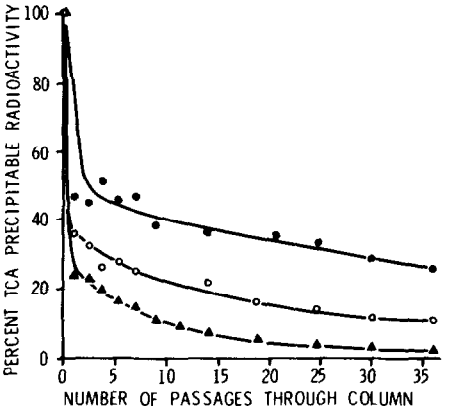

Fig. 3. Effect of the number of passages of DNA solutions in different buffers through a DNAase I glass column (2.4 g) on the extent of degradation of DNA. The flow rate was 0.35 ml/min.

●—● (a) TB, 22°, no $MgCl_2$ added (pH 7.0).
○—○ (b) TB, 10^{-3} M disodiumethylenediaminetetraacetate, 10^{-3} M sodium arsenite, 36° (pH 7.0).
▲—▲ (c) TB, 10^{-3} M $MgCl_2$, 36° (pH 7.0).

DNA fragments are produced which represent more resistant substrates than the original DNA. Therefore the rate of the enzymatic reaction slows down considerably and can be restored only by the repeated addition of a very large amount of DNAase I. As a result of this phenomenon, called autoretardation [7], the proportion of TCA-precipitable radioactive material decreased only linearily when ^{3}H-labeled DNA was treated with logarithmically increasing amounts of DNAase I (fig. 1). If it were possible to design a system in which partly degraded DNA would be continuously in contact with the fresh enzyme, the autoretardation could be obviated, at least partly. This might be accomplished by passing a substrate solution through a column of insolubilized DNAase I. Moreover, the occurrence of suitably sized pores in the insolubilized enzyme may segregate DNA molecules of different sizes. This ensures that the smaller molecules, but not the larger ones, will be in contact with the fresh enzyme (inside the pores) and, consequently, will be further degraded with greater efficiency.

The following results indicate that this actually happens when DNAase I covalently linked to porous glass is used. A comparison of results presented in

figs. 1 and 2 shows that the insolubilized enzyme derivative converted DNA into TCA-soluble fragments to a greater extent than did the soluble enzyme. However, autoretardation was not completely eliminated. Passing the substrate solution *n*-times through a column of insolubilized DNAase resulted in a less complete conversion of the substrate into TCA-soluble fragments than did passing the substrate solution once through a column increased *n*-fold in size. Under non-optimal conditions for the enzymatic reaction, repeated passages of the DNA solution through the column failed to convert the entire DNA into TCA-soluble products, the quantity of which seemed to increase asymptotically with the number of passages through the glass-enzyme derivative (fig. 3). However, even under such conditions the products obtained after digestion of DNA consisted of only high molecular weight polynucleotides excluded from Sephadex G-100 and low molecular weight material eluted from the gel in the same fractions as the NaCl marker (fig. 4). By contrast, DNA partially digested by soluble DNAase I consisted of a heterogeneous population of fragments, differing in length, which eluted from Sephadex G-100 in a single broad peak, corresponding to the entire fractionation range of the gel [9].

With the glass-DNAase I derivative, the optimal pH for the enzymatic reaction was between 4.0 and 4.5 (fig. 5), considerably lower than for the soluble enzyme (pH about 7) [10]. A downward shift of the pH optimum, resulting from covalent linking of enzymes to porous glass, was described also for urease [3]. No absolute requirement for the presence of divalent ions was observed with the insolublized enzyme (figs. 3 and 4) in contrast with the soluble enzyme [11]. An increase in temperature of 22° to 65° enhanced the release of TCA-soluble material from the substrate only about 1.5 fold, the enhancement being continuous with increasing temperature.

Histones inhibit the degradation of DNA by DNAase I more efficiently when the insolubilized enzyme is used, probably because the latter cannot compete with histones for sites on the substrate molecule.

DNAase I covalently linked to porous glass may become a useful tool in the preparation of template-free DNA polymerase, DNA-dependent RNA polymerase and viral antigens devoid of any residual genes [12].

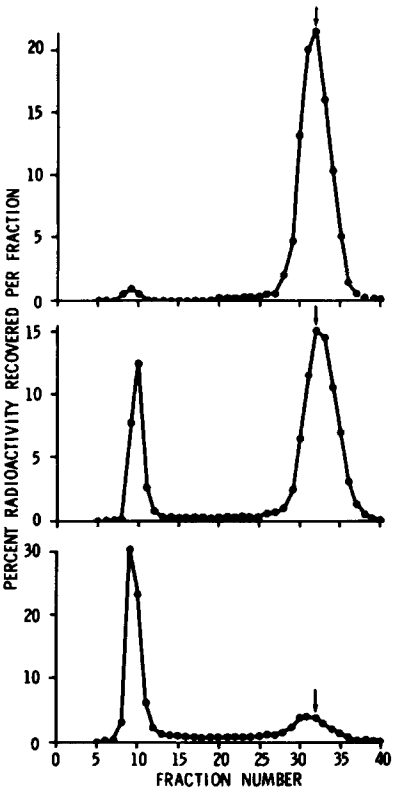

Fig. 4. Gell filtration on Sephadex G-100 of untreated ^{3}H-labeled DNA (bottom), and of the same DNA passed 36 times through columns of insolubilized DNAase I in buffers (b) (middle) or (c) (top) shown in fig. 3. Reaction conditions are also shown in fig. 3. The arrows indicate the position of the peaks of NaCl chromatographed on the same column. The position of the left peaks corresponds to the void volume of the column.

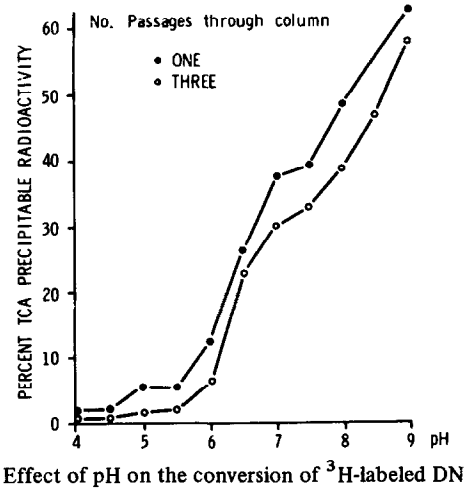

Fig. 5. Effect of pH on the conversion of ^{3}H-labeled DNA into TCA-soluble products by insolubilized DNAase I (1 g). DNA was dissolved in Britton-Robinson buffers [8] containing 10^{-3} M $MgCl_2$. The temperature was 36° and the flow rate, 0.33 ml/min.

Acknowledgments

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