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PREPARATION OF IMMOBILIZED SEPHAROSE-MICROCOCCAL NUCLEASE DERIVATIVES: ACTIVITY AND STABILITY

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Micrococcal nuclease has been covalently attached to CNBr-activated Sepharose 4B by coupling through three different enzyme functions: (*a*) amino groups; (*b*) carboxyl groups; and (*c*) tyrosyl or histidyl residues. On the basis of coupling yield and catalytic efficiency, Sepharose-(NH_2) nuclease derivatives were chosen for further activity and stability studies. The activity of the insoluble enzyme has been evaluated with macromolecular (DNA) and small (synthetic nucleotide) substrates; with the latter the enzyme retains 70% of native enzyme activity. Good enhancement of enzyme stability in the 4–40°C range has been observed.

INTRODUCTION

One of the greatest potential applications of immobilized enzymes is the degradation of macromolecular substances (proteins, nucleic acids, polysaccharides) in food and related industries (1). The immobilization of hydrolytic enzymes is therefore of great interest. In our laboratory we have been working on the system formed by the support agarose and the enzyme micrococcal nuclease, both of which have been extensively studied. Agarose has been used in gel filtration (2), protein insolubilization (3), and biospecific affinity chromatography (4). Micrococcal nuclease (EC 3.1.4.7), an extracellular phosphodiesterase from *Staphylococcus aureus* that can hydrolyze either DNA or RNA to produce 3'-mononucleotides and dinucleotides is a very well-known enzyme (5–9). Anfinsen and coworkers have elucidated the primary structure, the catalytic mechanism, and details of the active site (9).

In this paper we describe the preparation and properties of the enzyme covalently attached to CNBr-activated Sepharose 4B. In order to select the most suitable derivative, we have tested three coupling methods which bind the enzyme by three different types of functions: (*a*) amino groups; (*b*) carboxyl groups; and (*c*) tyrosyl or histidyl residues. The Sepharose-(NH_2)

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nuclease derivatives were chosen for subsequent studies of activity (with natural and synthetic substrates) and of storage stability at several temperatures.

MATERIALS AND METHODS

Materials

Materials included CNBr-activated Sepharose 4B (bead size, 40–200 μm) from Pharmacia; nuclease (mol. wt. 16,800) from Worthington, 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide, and calf thymus DNA (mol. wt. $1–1.3\times10^6$) from Sigma, and thymidine 3′-phosphate-5′-(p-nitrophenyl phosphate) ("nitrophenyl-pdTp," mol. wt. 523) from Ash Stevens, Inc., Detroit, Mich.; p-nitrobenzoyl azide was synthesized following the procedure described by Naegeli et al. (10). All other reagents were from commercial sources. Deionized water was used throughout, and glassware used for the enzyme solutions was siliconized.

Preparation of Insolubilized Derivatives

The amount of enzyme insolubilized was obtained by difference from the activity of the soluble enzyme added to the suspension of Sepharose beads and the activity recovered in the filtrate and washings. In control experiments with inactivated Sepharose, 100% of the activity of the enzyme remained in the filtrate and washings. After coupling, the Sepharose-nuclease was filtered and washed with the buffer used and with 1 M NaCl.

Nuclease Insolubilized Directly on the CNBr-Activated Sepharose 4B (Sepharose-(NH_2) Nuclease). The coupling procedure was that recommended by Pharmacia Fine Chemicals. CNBr-activated Sepharose 4B and nuclease, in 0.1 M $NaHCO_3$ buffer, pH 9.0, were mixed and left at 25°C with gentle stirring. The Sepharose-nuclease was filtered and then washed several times with buffer and with 1 M NaCl. The remaining activated groups in the support that may not have reacted with the enzyme were blocked by immersion of the derivative in 1 M ethanolamine, pH 9.0, for 1 h. The Sepharose-nuclease was then washed several times alternately with 0.1 M acetate buffer, pH 4.0, containing 1 M NaCl, and with 0.1 M borate buffer, pH 8.8, containing 1 M NaCl, and finally with deionized water.

Carbodiimide-Activated Nuclease Bound Through an Amino Spacer to Sepharose (Sepharose-(COOH)nuclease). The preparation of aminoethyl-Sepharose and coupling of carbodiimide-activated nuclease were carried out according to Cuatrecasas (11).

Nuclease Bound Through a Diazo Spacer (Sepharose-(tyr, his) Nuclease). The preparation of p-aminobenzamidoethyl-Sepharose, its diazotation and subsequent coupling of nuclease were made following the procedures of Cuatrecasas (11). The remaining activated groups in the matrix were blocked by immersion of the derivative in 0.05 M histidine pH 8.0 for 1 h.

Enzyme Assays

Initial activity of the soluble and insolube enzymes were measured by following graphically the increase in absorbance at 260 nm and 30°C of heat-denatured DNA (7) or at 330 nm and 25°C of nitrophenyl-pdTp (8), using a Zeiss PMQII spectrophotometer equipped with a 2-cm pathlength cuvette with magnetic stirrer. Heat denaturation of DNA was accomplished as reported by Cuatrecasas et al. (7). When using DNA as substrate, a unit of activity is that amount of enzyme causing a change of 1.0 A_{260} per min; the specific activity of the soluble enzyme is 40,000 U/mg.

Soluble and insoluble enzymes were stored in 20 mM citrate buffer, pH 6.4, 10 mM $CaCl_2$, and 0.1% bovine serum albumin. Amounts of Sepharose-nuclease were measured and transferred using push-button micropipettes. All the measurements of activity were made at the maximal stirring speed of the Zeiss device in order to minimize the external diffusional limitations. The amount of insolubilized derivative in the assays was always maintained in the region of linearity between activity and amount of derivative.

RESULTS AND DISCUSSION

We have prepared three different types of derivatives, following the procedures outlined above. The preparation conditions were: gel volume, 1 ml; total volume of the reaction mixture: 25 ml; amount of enzyme to be insolubilized: 15–20 μg. The results are presented in Table 1. In the case of Sepharose-(COOH) nuclease, in control experiments with carbodiimide, the enzyme lost 50% of its activity in 24 h; hence the corresponding values in Table 1 are only approximate.

The attainment in a short time (1 h) of 100% nuclease binding agrees with the *a priori* expectation because of the high reactivity of the cyanate groups in the CNBr-activated Sepharose (12) and the number of amino groups present in the nuclease [23 lys plus the amino-terminal ala (9)]. In the case of Sepharose-(tyr, his) nuclease, 8 h is required for total binding of the protein. According to Anfinsen et al. (9), the enzyme has three histidine

TABLE 1. **Preparation of Insolubilized Nuclease Derivatives**

Derivative	Reaction conditions	Enzyme in the reaction mixture (μg)	Enzyme[a] bound (%)	Spec. activity[a] (U/mg)	Catalytic[a] efficiency (%)
Sepharose-(NH_2) nuclease	25°C 1 h	16.2	100	514	1.28
Sepharose-(tyr, his) nuclease	4°C 8 h	20.0	100	6.0	0.015
Sepharose-(COOH) nuclease	25°C 24 h	19.0	~30	~85	~0.2

[a]Determined using DNA as substrate.

residues and seven tyrosines [three of them inaccesible to nitration with tetranitromethane (13)].

The catalytic efficiency is defined as the ratio of the specific activity of the insolubilized enzyme to that of the soluble one. The different efficiencies obtained (0.015 to 1.28%) may be explained by the involvement of several amino acids in the active center (9). Of all the amino residues, only lys 84 and lys 116 participate in the catalysis. Concerning the activity of Sepharose-(tyr, his) nuclease, only two tyrosines (85 and 116) participate in the active site. Sepharose-(COOH) nuclease species are less interesting because of the deleterious effect of the coupling procedure on the enzyme; however, it was, *a priori*, a method worth trying because the enzyme has 18 acid residues, with only 3 responsible for the catalytic activity (9).

All the properties listed in Table 1 (reaction time, coupling yield, and catalytic efficiency) favor the Sepharose-(NH_2) nuclease derivative as the best for subsequent studies with the nuclease insolubilized on Sepharose. Since it is not as yet possible to predict the influence of each type of binding on the three-dimensional structure of the enzyme or on the accessibility of the active site, even in the case of a well-characterized enzyme like micrococcal nuclease, studies such as those reported above are necessary. Sometimes it will also be advisable to test more than one support.

Activity of Sepharose-(NH_2) Nuclease with Different Concentrations of Enzyme on the Support

The insolubilization reaction was carried out at 25°C. In all cases the reaction time (time required for insolubilization of at least 95% of the soluble enzyme) was under 2 h. Figure 1 depicts the efficiency values

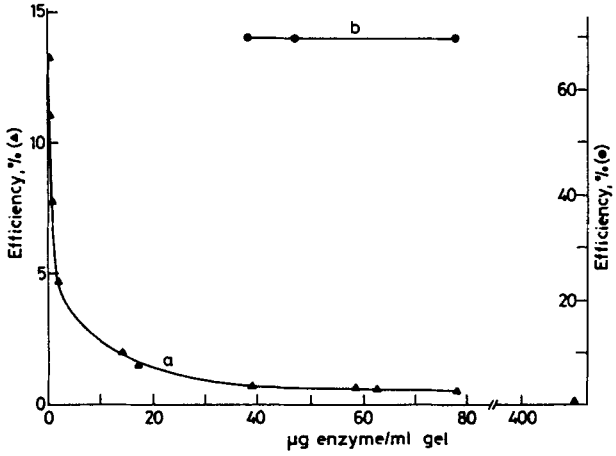

FIG. 1. Catalytic efficiency of Sepharose-(NH_2) nuclease toward two different substrates. The assay mixture consisted of 25 mM tris-HCl buffer, pH 8.8, 10 mM $CaCl_2$, substrate, and insolubilized enzyme. (a) 50 μg/ml of heat-denatured DNA. (b) 0.1 mM nitrophenyl-pdTp. These substrate concentrations were the most suitable for routine assays, giving 80% of maximal velocity.

obtained as a function of μg of enzyme bound per ml of gel. Using DNA as substrate, efficiency increased from 0.15 to 13% as the amount of enzyme bound onto the support decreased from 410 to 0.1 μg per ml of gel. It was not possible to measure the activity of insolubilized derivatives with lower nuclease content. Efficiency is always higher when the enzyme activity is measured at 6°C, and at this temperature the variation of the efficiency with enzyme concentration is much smaller (14) than at 30°C. All of these facts indicate that diffusional limitations could be the controlling factor in the overall rate of DNA hydrolysis by insolubilized nuclease.

However, if we measure the activity of the derivatives with nitrophenyl-pdTp, we would expect ready access of the small substrate to the immobilized enzyme molecules. In fact, a high and constant value (70%, curve b, Fig. 1) is obtained for the efficiency of three derivatives with enzyme content ranging from 78 to 38 μg/ml gel. Derivatives with lower enzyme content showed no detectable activity toward this substrate. Since from kinetic studies with these derivatives (manuscript in preparation), we have found that there is no diffusional limitation on the hydrolysis of nitrophenyl-pdTp, it is reasonable to extrapolate the 70% efficiency value to the ordinate axis. Chemical modification of the enzyme plus possible physical interactions between enzyme and matrix could account for the reduction in activity from 100 to 70%.

Stability

Melrose in 1971 (15) reviewed the instances in which direct comparisons of the stabilities of soluble and insoluble enzyme pairs were reported. In 30 cases the insoluble enzyme was more stable, in 8 the soluble, and there was little difference in the remaining 12 cases. Several hypotheses have been advanced concerning stability of immobilized enzymes. Mosbach (16) explains the increased stability of some enzymes by multiple-point attachment to the matrix or by the stabilizing effect of the new microenvironment. On the other hand, Goldman et al. (17) interpret the decreased thermal stability of other enzymes as a decrease in the probability of recovering native conformation after thermal perturbation. If we accept all of these possible effects, their relative contribution in each particular system (support, activation method, enzyme), would account for the overall stability observed.

The storage stabilities of soluble nuclease and Sepharose-(NH_2) nuclease at three different temperatures were compared. In every case the enzyme was stored at the indicated temperature as described above, and periodically an aliquot was taken to measure (with nitrophenyl-pdTp, at 25°C) the remaining activity. The results are presented in Fig. 2. A semilogarithmic plot (not shown) of these values follows first-order kinetics. The

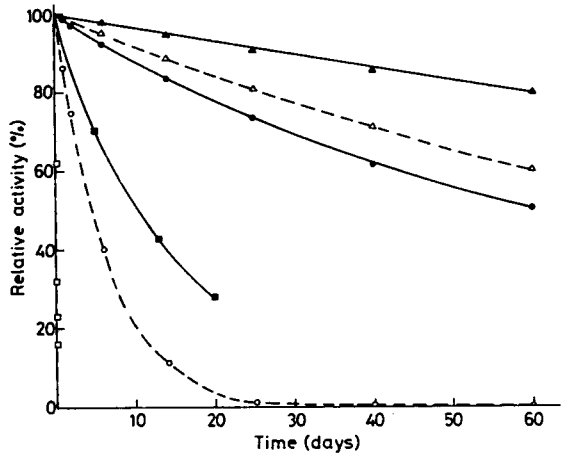

FIG. 2. Storage stability of nuclease (open symbols) and Sepharose-(NH_2) nuclease (closed symbols) at 4 (△ and ▲), 25 (○ and ●) and 40°C (□ and ■). The activity of an insoluble derivative containing 78 μg enzyme/ml gel was measured with nitrophenyl-pdTp. Assay conditions were as in the legend to Fig. 1.

TABLE 2. **Half-life (Days) of Native and Insoluble Nuclease**

	Temperature (°C)			
	−15°	4°	25°	40°
Native	160	80	4.3	0.03
Insoluble	—	160	60	10.7

half-lives of the soluble and insolubilized enzyme from this plot are shown in Table 2, along with the value for the native nuclease at −15°C. The stabilization factor (ratio of the half-life of insoluble to soluble enzyme) increases from 2 to 357 when the storage temperature is raised from 4 to 40°C. Since we have now measured the activity, not with DNA (substrate affected by diffusional limitations) but with nitrophenyl-pdTp (substrate unaffected by diffusional limitations), we are confident that the increased stability observed is real and not the consequence of overloading the matrix with enzyme (16).

The enhancement of storage and thermal stability of this enzyme, together with good retention (70% with the synthetic substrate) of enzymic activity upon insolubilization, could make these Sepharose-(NH_2) nuclease derivatives very useful in continuous operation. Studies on the hydrolysis of high molecular weight natural substrates by the porous Sepharose-(NH_2) nuclease derivatives will be the subject of a coming report.

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