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Radiation-Induced Polymerization for the Immobilization of Penicillin Acylase

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ABSTRACT

The immobilization of *Escherichia coli* penicillin acylase (EC 3.5.1.11) was investigated by radiation-induced polymerization of 2-hydroxyethyl methacrylate at low temperature. A leak-proof composite that does not swell in water was obtained by adding the cross-linking agent trimethylolpropane trimethacrylate to the monomer–aqueous enzyme mixture. Penicillin acylase, which was immobilized with greater than 70% yield, possessed a higher K_m value toward the substrate 6-nitro-3-phenylacetamidobenzoic acid than the free enzyme form ($K_m = 1.7 \times 10^{-5}$ and $1 \times 10^{-5} M$, respectively). The structural stability of immobilized penicillin acylase, as assessed by heat, guanidinium chloride, and pH denaturation profiles, was very similar to that of the free-enzyme form, thus suggesting that penicillin acylase was entrapped in its native state into aqueous free spaces of the polymer matrix.

Index Entries: Penicillin acylase; enzyme immobilization; radiation polymerization; 2-hydroxyethyl methacrylate; polymer matrix.

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INTRODUCTION

Several authors, Kaetsu and collaborators in particular, have reported on the immobilization of enzymes, proteins, cells, and other bioactive substances, such as drugs, by means of the radiation-induced polymerization of suitable monomers (*1–12*). The method is based on the physical entrapment in matrices formed by γ-irradiation of a mixture of monomers such as esters of acrylic and methacrylic acid in the presence of an aqueous solution of the biomaterial after cooling at a very low temperature.

For the immobilization of enzymes, this method has advantages over polymerization induced by chemical initiators because it is highly efficient, working with it is possible at a very low temperature, thereby largely preventing enzyme inactivation, and the porosity of the polymer matrix can be controlled (*4,12*). Furthermore, the use of chemical initiators, potentially harmful to enzyme stability, is avoided. According to the authors, the protein is firmly adsorbed to the polymer at the surface of the pores produced by the ice crystals formed during freezing. For this reason, this immobilization is also described as the adhesion method (*1*).

We here report on the immobilization and properties of penicillin acylase (penicillin aminohydrolase, EC 3.5.1.11) from *Escherichia coli*. Because this enzyme is an important industrial biocatalyst (*13*), the search for simple methods for its immobilization is of continuous interest.

It was discovered that this enzyme could be immobilized into a leakproof polymer using 2-hydroxyethyl methacrylate (HEMA) as monomer, but only in the presence of small amounts of a suitable crosslinking agent. The properties of the immobilized enzyme are also reported and compared with those of the native enzyme.

EXPERIMENTAL

Materials and Methods

The HEMA, obtained from Aldrich-chemie, Germany, was fractionally distilled at reduced pressure under N_2. Trimethylolpropane trimethacrylate (TMPTMA), supplied by Tessil Chimica S.p.A., Italy, was used as received. Penicillin acylase, as a partially purified material [0.5 UI/mg using 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) as substrate], was supplied by DEBI-Sclavo, Milan. It was used either as received or after purification to homogeneity (12 UI/mg), which was achieved using an affinity column of Sepharose-CL-6B with *p*-aminophenylacetic acid as ligand and a linear gradient of phenylacetic acid as eluent. The ligand was bound to Sepharose by the tosyl chloride activation procedure, as described elsewhere (*14,15*).

Enzyme Assay

The activity of soluble penicillin acylase was evaluated on the basis of the hydrolysis of the synthetic substrate NIPAB (*16*), revealed by the increase in absorbance at 405 nm, $\Delta\epsilon_M$ = 8976 L/*M*cm. The activity of the immobilized form was ascertained by suspending the resin in the substrate mixture in a cuvet and evaluating the absorbance after a fixed period of time. We also measured the activity using penicillin-G as the substrate. Here, the amount of 6-aminopenicillanic acid (6-APA) produced in a reaction mixture containing 20 mg/mL of penicillin-G in 0.05*M* phosphate buffer, pH = 7.5 at 37°C, was evaluated following the *p*-dimethylaminobenzaldehyde method (*17*).

Immobilization

Procedure A

Mixtures of 1 mL HEMA and 1 mL penicillin acylase solution in 0.1*M* phosphate buffer, pH = 7.5, were placed in glass tubes, degassed, frozen at −78°C, and exposed to the γ-rays of a 3000-Ci ^{60}Co source at a dose rate of 0.80 Gy/s for different lengths of time. After irradiation, the vials were opened and the contents either sliced into thin disks or ground into small granules (0.1–0.2 mm diameter), and both were finally washed by stirring in 20 mL of phosphate buffer solution for either 20 min or 5 h.

Procedure B

The enzyme–monomer mixture (1 mL) was injected drop-wise with a syringe into 50 mL of stirred petroleum ether and cooled to −78°C to freeze the mixture into small, bead-like particles (*11*). The suspension was directly irradiated, as reported above.

Microscopic Examination

Portions of the polymer sample were sliced with a microtome at −20°C and examined with a light microscope at different magnifications and photomicrographs were taken.

RESULTS AND DISCUSSION

Influence of Irradiation Time and Crosslinking Agent on Matrix Porosity and Enzyme Leakage

It was possible to investigate penicillin acylase immobilization by radiation-induced polymerization of HEMA because no appreciable enzyme loss occurred when an aqueous buffered solution of enzyme was mixed with an equal volume of HEMA at 0°C and when the mixture was frozen to −70°C and then thawed. Experiments on immobilization were

initially performed following procedure A, using a homogeneous enzyme preparation at a protein concentration of 0.8 mg enzyme/mL. A mixture containing a 1:1 ratio of HEMA and enzyme solution was chosen since it appeared the most suitable for the immobilization of other enzymes (*3,9*).

Irradiations at a dose rate of 0.80 Gy/s were carried out at 1, 3, 4, 6, and 8 h; photomicrographs showing the structure of the polymer matrix at 3 and 4 h of irradiation are reported in Fig. 1. The microscopic structure is very similar to that already reported by Kaetsu (*18*) in radiation-induced polymerization of the HEMA-water system. However, in the case of enzymes, such as α-amylase, glucoamylase, thermolysin, antibodies, and enzyme-labeled antibodies (*3,8,9*), the polymer matrix obtained at a 50% HEMA concentration gave a limited leakage of the biocomponent only in the first few washings. Penicillin acylase, similarly entrapped, is rapidly washed off the matrix. Figure 2(A) reports the enzyme activity released from the matrix and the activity of the matrix for the sample obtained after 4 h of irradiation. Less than 30% of the enzyme is entrapped in the resin after the first 4 washings, but, since there is a continuous leakage, about 10% of the enzyme is left after 12 washings. The enzyme is lost even more rapidly when the irradiation time is reduced; larger pores in the matrix are obtained. The matrices obtained under these conditions are also sticky and swell in water and are therefore unsuitable for filtration and for packing a column to be used as a reactor.

On the other hand, although an increase in irradiation time to 6 or 8 h reduced the resin porosity, it did not reduce significantly the leakage of penicillin acylase; a brown material was produced and the total activity of the resin was reduced, probably because of partial denaturation of the enzyme during irradiation. At present, it is not clear why, in apparently identical conditions, penicillin acylase is washed off, whereas other enzymes are retained in the matrix. One possible explanation may be that efficient retention of the enzyme in the HEMA composite is required in the matrix at the surface of the pores (*1–3,6*) in addition to entrapment in aqueous spaces with pores of a suitable size. It therefore seems reasonable that efficient immobilization is dependent on the properties of the enzyme, such as size, shape charge, and hydrophilicity as well as on those of the polymer.

The addition of small amounts (0.5, 1, 2, and 3%, with respect to the monomer) of TMPTMA, a crosslinking agent for the three double bonds present in the molecule, causes a significant improvement in the properties of the resin. In Fig. 1(c) the optical micrograph of the resin shows a reduced porosity with respect to the sample irradiated for the same time without TMPTMA. The resultant resin now has a more glass-like structure and can therefore be more easily ground into a powder suitable for filtration.

Figure 2(B) shows the penicillin acylase activity of both washings and of the matrix after repeated extensive incubation with buffer. Un-

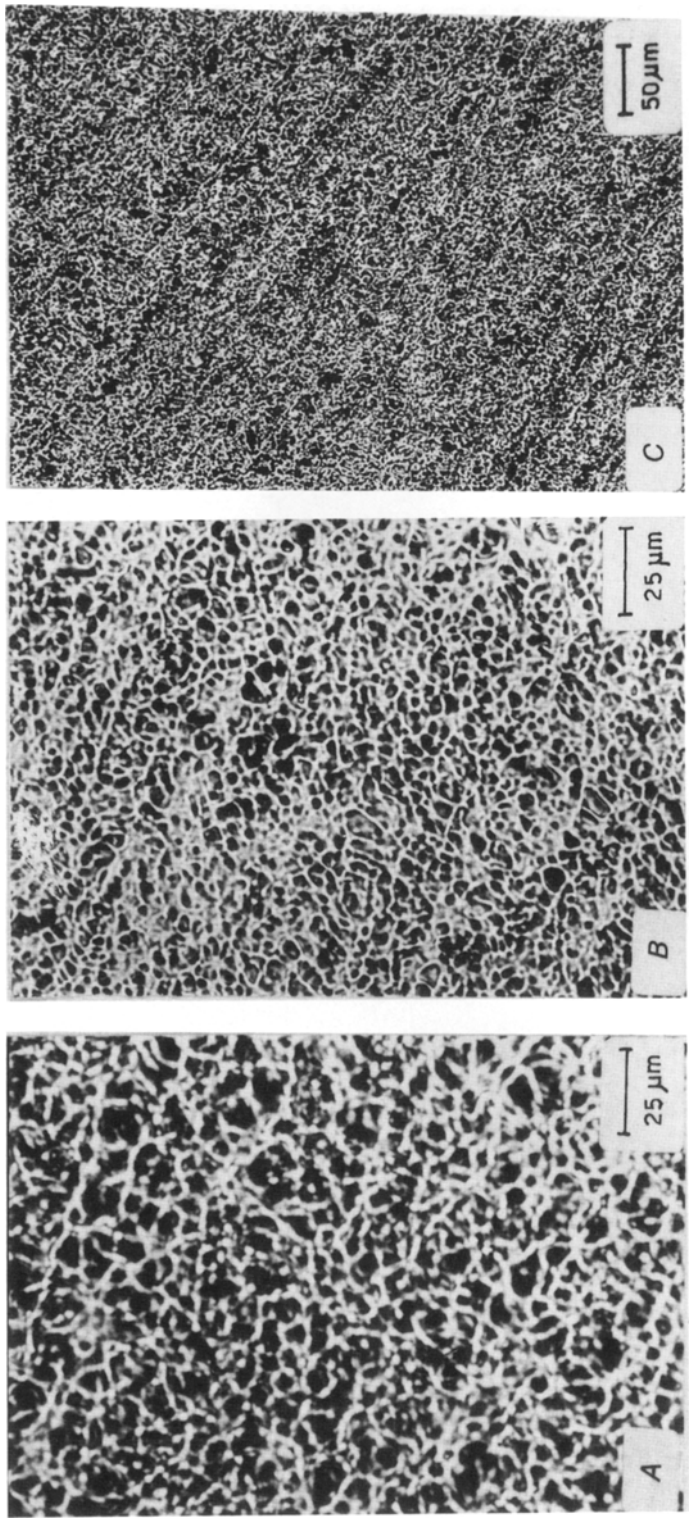

Fig. 1. Micrographs of pore structure of the polymer matrix obtained by radiation-induced polymerization in the 1:1 v/v ratio of HEMA and penicillin acylase solution in 0.1*M* phosphate buffer, pH = 7.5, at −78°C and at the dose rate of 0.80 Gy/s for 3 h (a) and 4 (b) h of irradiation. Micrographs for the resin obtained after 4 h of irradiation in the presence of 1% TMPTMA (c).

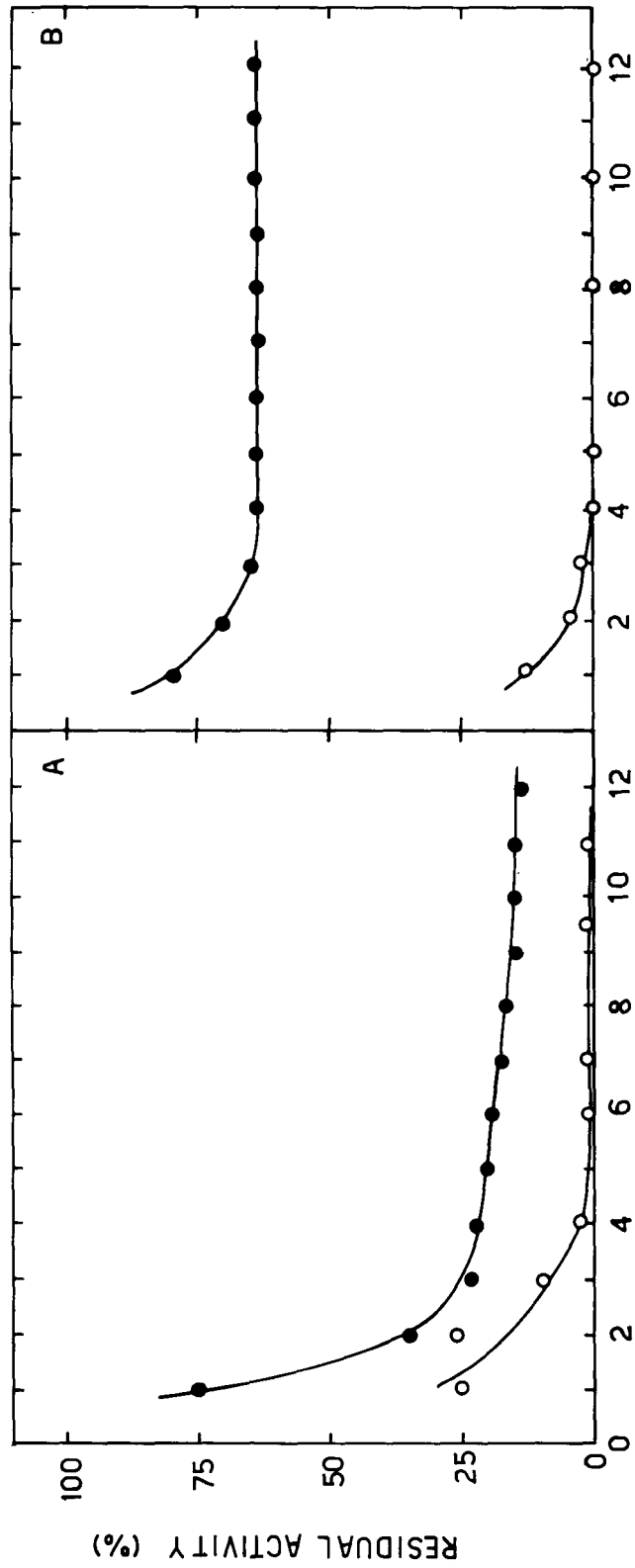

Fig. 2. Penicillin acylase activity of a resin obtained by a 4-h period γ-irradiation of a mixture of an aqueous solution of penicillin acylase (0.8 mg/mL) and HEMA in the absence (A) and presence (B) of TMPTMA. In (A), the resin was finely sliced and washed several times with buffer (20 mL) for 20 min. In (B), the resin was finely ground and repeatedly washed with 20 mL buffer for 5 h. After each washing, the activity was assayed in the supernatant (○) and resin (●). The 100% corresponds to the activity of the enzyme mixture before irradiation.

bound penicillin acylase activity, representing a total of about 25% of the starting material, was found only in the first three washings. This means that about 75% was permanently entrapped in the polymer matrix. On the other hand, the activity of the polymer decreased during the first three washings and remained constant at about 62% for the following cycles, with an apparent overall enzyme activity loss of 13%. No appreciable difference in enzyme release was observed in samples obtained at 0.5, 1, 2, and 3% TMPTMA.

The reason for the increased retention of penicillin acylase in the crosslinked matrix does not appear to be related to the reduced size of the pores, since matrices with pores of the same size were also obtained when HEMA was irradiated without TMPTMA for longer periods of time. In this case, no improvement in retention was observed. The increased stability of the polymer matrix may thus be related to the tridimensional arrangement of the matrix network. The HEMA–TMPTMA resin did not in fact show the tendency to swell in water, a phenomenon observed with the HEMA resins; with other enzymes, the swelling appeared to be involved in the release from the matrix (*6*).

The polymerization, according to procedure B, which was devised to obtain in one step the matrix-entrapped enzyme in small particles (*see* Fig. 3), gave enzyme immobilization and leakage similar to those ob-

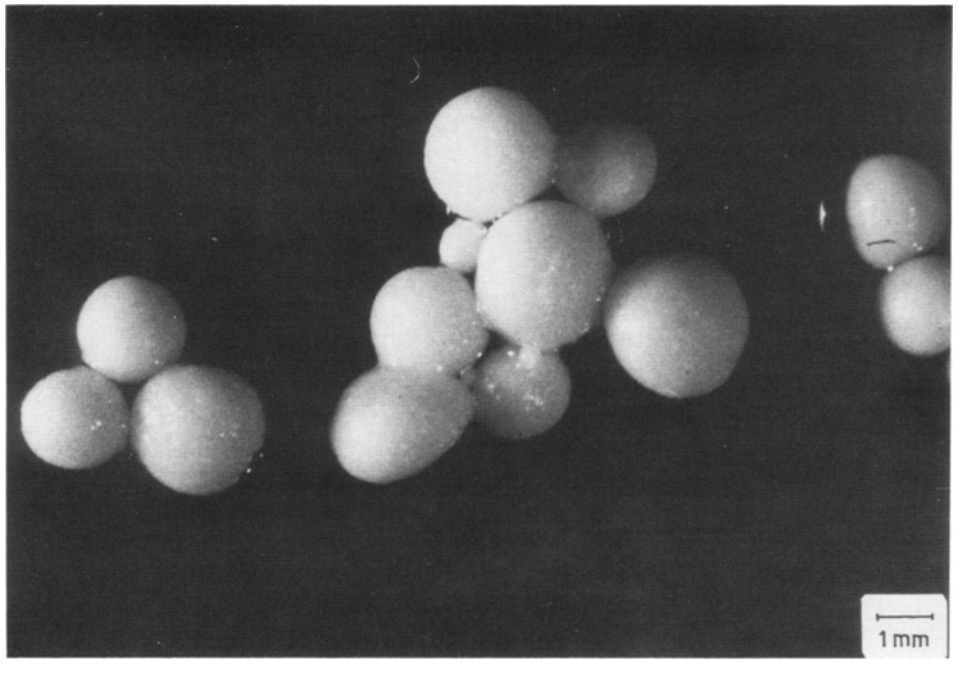

Fig. 3. Optical micrograph of the composite obtained by radiation-induced polymerization of the enzyme–monomer mixture in the presence of TMPTMA injected into precooled petroleum ether.

tained with the method reported for bulk immobilization (procedure A). Penicillin acylase was rapidly released from a matrix prepared from HEMA only, but was retained as the crosslinking agent TMPTMA was added. However, this method appeared to be convenient only for small scale preparations, since a large volume of ether was required to obtain proper dispersion of the enzyme–monomer mixture.

Because penicillin acylase is used industrially as a biocatalyst in the hydrolysis of penicillin-G, immobilization was also performed directly on a crude sample supplied from the market producer. In this case, higher amounts of proteins, up to 10 mg/g of polymer, which approximately corresponded to the activity of 0.4 mg of homogeneous enzyme, were immobilized. Using 1% TMPTMA, over 65% of penicillin acylase activity was permanently retained in the matrix.

Enzymatic and Structural Properties of Immobilized Penicillin Acylase

The K_m values of the native and immobilized enzyme (using NIPAB as substrate) were found to be 1×10^{-5} and $1.7 \times 10^{-5} M$ for the two forms, respectively. The increased value for the immobilized penicillin acylase, already observed with other entrapped enzymes, may be related to a diffusion limitation of the substrate. The specific activity of the immobilized form, 13% lower than the native enzyme, may be related to enzyme inactivation during irradiation or to reduced accessibility of enzyme molecules deeply hidden in the polymer.

The structural stability of immobilized penicillin acylase was studied using pH, temperature, and guanidinium chloride as structure perturbing agents. Figure 4 reports the results obtained with native and immobilized penicillin acylase exposed for a fixed period of time to the denaturant. A very limited increase in thermostability is observed for the entrapped enzyme, whereas the two forms have identical patterns of inactivation toward pH and guanidinium chloride.

The fact that the immobilization of acylase is not accompanied by an increased enzyme stabilization, as is generally observed in enzyme immobilization, seems to suggest strongly that by polymerization of HEMA in the presence of TMPTMA, penicillin acylase is not bound to the matrix, but entrapped in aqueous spaces of the polymer, where it behaves as the free native enzyme. Other interpretations for this lack of stabilization are also possible; in fact, for other enzymes, stabilization was sometimes related to unpredictable factors, such as interaction between enzyme and polymer. In this context, it is of interest to bear in mind the differences in thermostability of thermolysin entrapped in a polymer obtained from a hydrophobic monomer and that obtained with hydrophilic 2-hydroxyethyl methacrylate; only with the second polymer was the observed thermostability higher than that of the free enzyme (*9*).

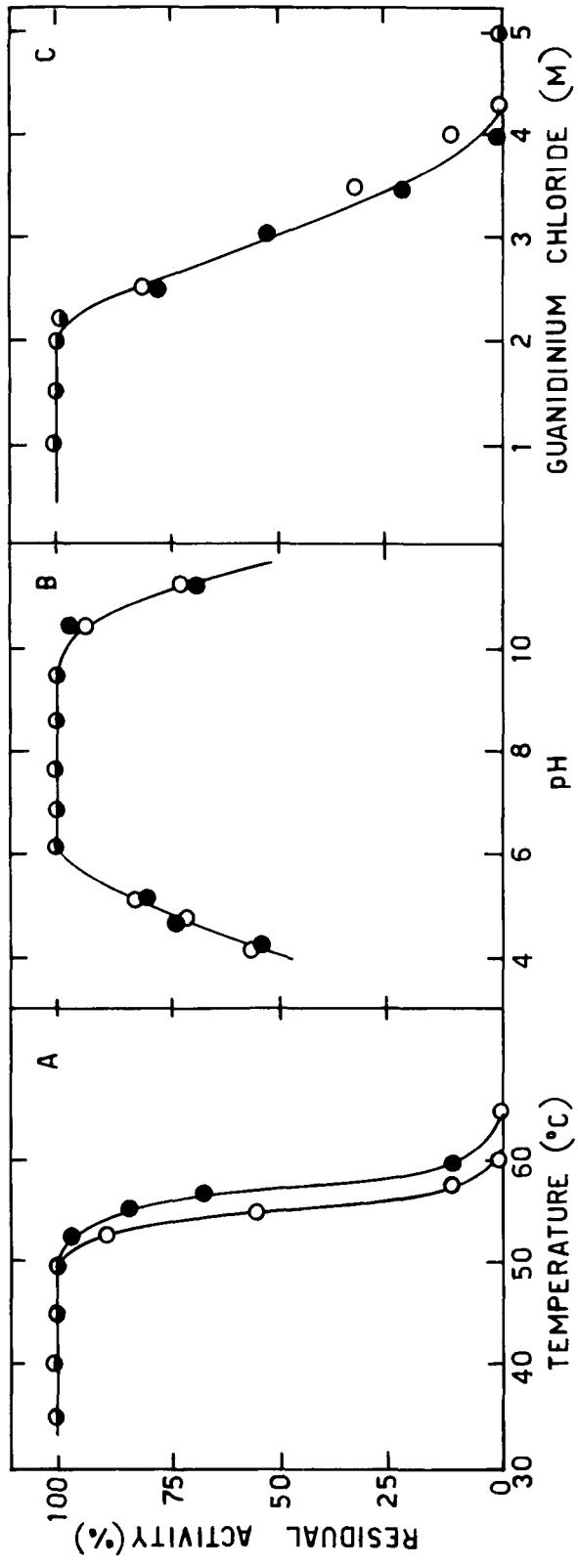

Fig. 4. Stability of native (○) and matrix entrapped (●) penicillin acylase to denaturation. The enzyme was either dissolved or suspended in 0.05*M* phosphate buffer, pH = 7.8, heated for 20 min at the indicated temperature, chilled, and assayed (A). The enzyme was incubated at room temperature for 5 h at various pH values before activity measurements. The following buffers were used: 0.1*M* acetate between pH 3 and 5; phosphate between pH 5 and 8.5; and borate between pH 8 and 11 (B). The enzyme was incubated for 5 min at room temperature in guanidinium chloride solution at the indicated concentration before activity measurements (C).

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