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Behavior and Application of Immobilized Micrococcal Nuclease[a]

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INTRODUCTION

It is important in the insolubilization of enzymes on a support that the insolubilization method being used can yield an activated matrix with a broad range of activation degrees; that is, a support with very low activation would yield enzyme insolubilized through a single bond, whereas the protein in a highly activated support would be bound through multiple linkages. This multipoint attachment leads to an enzyme that is more stable against deleterious agents (i.e., heat, organic solvents, etc.).

Micrococcal endonuclease (EC 3.1.31.1) is a well studied[1] extracellular phosphodiesterase from *Staphylococcus aureus* that hydrolyzes either DNA or RNA to produce 3′-mononucleotides and dinucleotides and that requires Ca^{2+} for activity. In our laboratory, we have been interested in the study of the behavior of this enzyme insolubilized on agarose activated by cyanogen bromide.[2–4] At present, we are extending our knowledge to the behavior of nuclease insolubilized both on agarose activated with tosyl chloride and on other supports as well. By using tosyl chloride, we have shown[5] that it is possible to obtain a high range of activation degrees in agarose, namely, from 1 to 40 tosyl arms per 10 nm^2 of support surface area (taking 10 nm^2 as the cross-sectional area of the nuclease molecule).[1] Therefore, this method is very well suited for preparing insoluble derivatives in which the enzyme is bound through multiple linkages to the matrix.

The daily intake of nucleic acids from single-cell protein (SCP) concentrates for human consumption must be limited. This is necessary because of the risk of gout due to either the deposit of uric acid crystals in the joints or the formation of uric acid stones in the urinary tract. Many different methods, some of them using rather drastic treatments, have been proposed for the reduction of the nucleic acid content of SCP

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concentrates, but no agreement has yet been reached about how they affect toxicity or digestibility of the resulting protein.[6-8]

The long-term goal of our studies with staphylococcal nuclease is to apply immobilized nucleases to the hydrolysis of nucleic acids in SCP. It is important to carry out the hydrolysis process at temperatures that are not too high so that the protein digestibility is not impaired; however, the temperatures must still be high enough to prevent contamination by the normal mesophilic microorganisms. In this discussion, we present data of the hydrolysis of DNA, used as a model substrate, at temperatures up to 45 °C.

MATERIALS AND METHODS

The procedures for our experiments were as follows. Agarose gel beads (Bio-Gel A-150m, 100–200 mesh, from Bio-Rad Laboratories) were activated with *p*-toluenesulfonyl chloride following our modifications[5] of the method developed by Mosbach and co-workers.[9] Then, tosylated agarose suspended in 0.1 M $NaHCO_3$/Na_2CO_3 buffer, pH 9.0, was mixed with nuclease solution (mol. wt. = 16,800, from Boehringer Mannheim) and was left at 25 °C to obtain the insoluble enzyme derivatives.

As substrate, we used Sigma salmon testes DNA Type III in its denatured form after 30 min at 100 °C and this was followed by rapid cooling.[10] Initial activities of the soluble and insoluble enzymes were measured by charting in graph form the increase in A_{260} at 30 °C on 0.1 M Tris-HCl buffer, pH 8.8, containing 0.1 M $CaCl_2$.[10] An activity unit is the amount of enzyme causing a change of 1.0 A_{260} min^{-1}. The hydrolysis of DNA was carried out in a 35-mL batch reactor, with aliquots removed periodically to measure the increase in A_{260}. The percentage of hydrolysis attained was calculated on the premise that a 33% increase in the DNA initial absorbance represents 100% hydrolysis. The DNA concentration level in the reactor was 2.5 mg/mL; in addition, in order to start the reaction, 2 mL of agarose-enzyme gel was added.

RESULTS

As a result of our methodology,[5] the tosylation of Bio-Gel A-150m readily yielded very different degrees of activation (from 1 to 40 tosyl groups per 10 nm^2). In this

TABLE 1. Properties of the Agarose-Nuclease Derivatives

Support Activation (no. of tosyl groups per 10 nm^2)	E_{gel}[a] (μg nuclease/mL gel)	Derivative	Initial Activity[b] (units/mg)
6.5	4.1	N-Ag 1a	540
6.5	8.9	N-Ag 1b	170
10.4	4.7	N-Ag 1c	320
10.4	8.8	N-Ag 1d	240

[a] E_{gel} = concentration of the insolubilized enzyme in the gel particles.
[b] The specific activity of soluble nuclease is 1710 units/mg.

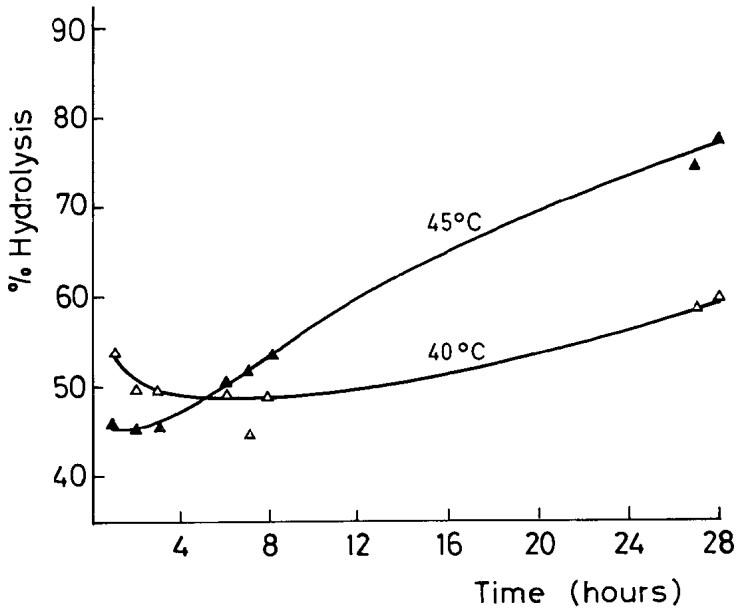

FIGURE 1. Course of DNA hydrolysis by insolubilized nuclease. Reaction medium: Tris buffer. Derivative: N-Ag 1b.

study, we have compared two support activations in which E_{gel} has been varied from 4.1 to 8.9 μg/mL (0.24 to 0.53 μM). The properties of the insoluble derivatives obtained are given in TABLE 1. It can be seen that the initial activities of the insolubilized nuclease derivatives towards a macromolecular substrate (denatured DNA) were substantially lower than those found in the native enzymes. However, these derivatives were much more resistant to moderately high temperatures (see below).

The time course of DNA hydrolysis at two temperatures is shown in FIGURE 1. The extent of hydrolysis after 24 h at 45 °C was much higher than at 40 °C. The decrease at 40 °C in % hydrolysis was due to interference in the assay because of the high Ca^{2+} and DNA concentrations present (this interference was much more apparent at 30 °C). Furthermore, 50 °C appeared to be a reaction temperature too high for the Bio-Gel A-150m support.

The number of possible (covalent) linkages between enzyme and support, on the one hand, and the load of enzyme per unit volume of support, on the other hand, are the most important characteristics of insolubilized enzymes because they enhance the stability and productivity of the biocatalyst. Therefore, we next evaluated the behavior of the four insoluble derivatives in DNA hydrolysis at 45 °C (TABLE 2). Clearly, the derivative N-Ag 1c, which had the larger enzyme load on the most activated support, showed the poorer results (although its initial activity value was better than that of other derivatives; cf. TABLE 1).

In previous research,[11] we investigated the behavior of the initial rates of DNA hydrolysis in both the absence and the presence of several organic solvents. The best results were obtained when the reaction mixture contained 2% of the organic solvent.

TABLE 2. DNA Hydrolysis at 45 °C with Insolubilized Nuclease

Derivative	% Hydrolysis	
	After 1 h	After 24 h
N-Ag 1a	48	76
N-Ag 1b	46	78
N-Ag 1c	42	63
N-Ag 1d	47	75

Hence, we have studied the time course of hydrolysis in the presence (2%) of dimethyl sulfoxide (DMSO) or tetrahydrofuran (THF) (FIGURE 2). It was evident in this enzyme system that reaction mixtures of Tris–dimethyl sulfoxide (98:2) yielded more hydrolysis than plain buffer.

DISCUSSION

Because of the high molecular weight of nucleic acids, we are studying the agarose gel of lowest agarose concentration (i.e., of larger pores) as a matrix for nuclease immobilization. Bio-Gel A-150m, which is commercially available, contains 1% (w/v) agarose and its exclusion limit is 150×10^6 daltons. Previously,[5] we estimated that its

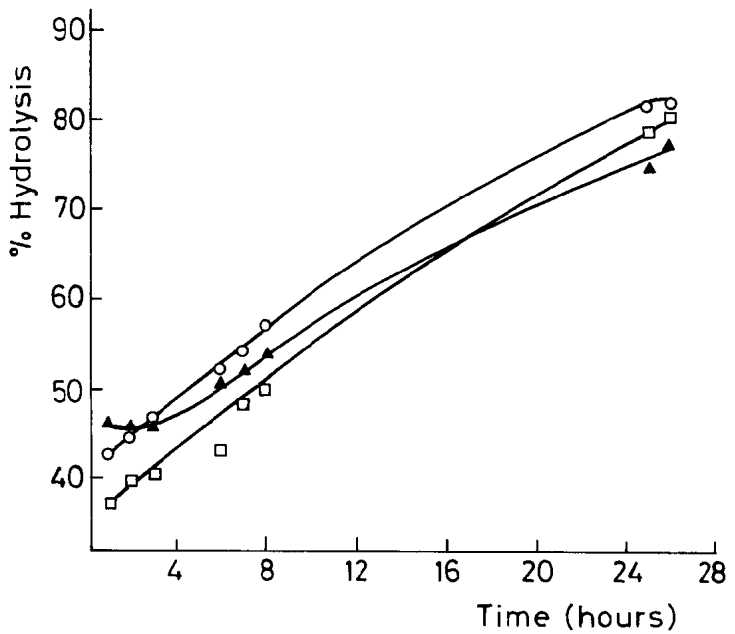

FIGURE 2. Time course of the percentage of hydrolysis in several reaction mixtures at 45 °C. ▲ = Tris buffer; ○ = Tris-DMSO (98:2); □ = Tris-THF (98:2). Insoluble nuclease derivative: N-Ag 1b.

surface area and average pore diameter were 17 m^2 mL^{-1} and 330 nm, respectively. Presently, we are in the course of evaluating other types of supports differing in chemical nature and porosity.[12]

The data reported here show that nuclease insolubilized on tosylated agarose can be used for long periods of time in the hydrolysis of DNA at 45 °C. Derivatives previously obtained using CNBr-activated agarose as support could not be employed at temperatures above 37 °C.[3] On the other hand, native nuclease is sensitive to prolonged exposure to temperatures ≥40 °C.[13] In these conditions, a conformational change that disturbs the active site takes place.[11] Therefore, insolubilization of staphylococcal nuclease on tosylated agarose offers a good resistance to temperature in the conditions of catalysis.

With the development of biotechnology, it has become absolutely necessary in many instances to introduce organic solvents in the reaction mixture. In an earlier work, we studied the effect of several organic solvents (dimethylformamide, dimethyl sulfoxide, tetrahydrofuran, acetonitrile, and methanol) on the initial activity of DNA hydrolysis by soluble and insolubilized nucleases.[14] In this report, the presence (2%) of DMSO or THF in the hydrolysis reaction mixture has been investigated (FIGURE 2). While DMSO is a most promising solvent for the study of nucleic acid hydrolysis, THF [whose dielectric constant ($\epsilon = 7.4$) and partition coefficient[15] (log P = 0.49) are very low] offers no improvement to the catalysis in plain Tris buffer.

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Effect of Salts on Enzyme Stability

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We propose the modification of the biocatalyst environment with additives as a viable approach to the stabilization of enzymes. The effect of polyols (ethylene glycol, glycerol, erythritol, xylitol, and sorbitol), polymers (PEG, dextrans), and carbohydrates (sucrose, lactose) has already been investigated.[1-3] In addition, a mechanism of enzyme stabilization has been proposed.[4] The influence of different salts (KCl, NaCl, LiCl) on invertase, glucose oxidase, and lysozyme stability is investigated in this study. Enzyme half-life is taken as a criterion of enzyme stability, and we define the protective effect as the ratio of the enzyme half-life in the presence of additive to the enzyme half-life without any additive. Moreover, with the help of Raman spectroscopy, the effect of these salts on the modification of the water molecule organization is observed.

In the presence of LiCl, NaCl, and KCl, a definite maximum effect on the thermal stability of invertase is observed (TABLE 1). This stability maximum varies with the salt concentration (i.e., situated at 1.0 M for LiCl, 2.0 M for NaCl, and 3.0 M for KCl) and with the nature of the cation (K^+, Na^+, Li^+). In the case of KCl (3.0 M), the half-life of invertase is increased by a factor of 1300. TABLE 2 shows the evolution of the stability of the glucose oxidase with an increase in the salt concentration. The maximum protective effect is observed in the presence of KCl (2.0 M).

In the case of lysozyme, the nature of the cation has no effect on the enzyme stability. The half-life of lysozyme is multiplied by a factor of five in the presence of LiCl, NaCl, and KCl (1.0 M). The difference in the protective effect observed between these three enzymes can be related to their hydrophilic/hydrophobic balance. In the most hydrophilic enzyme studied, namely, invertase, we observed that the effect of the cations was most evident. Both effects diminish when the enzyme becomes increasingly hydrophobic in glucose oxidase and lysozyme phases.

We used Raman spectroscopy in order to characterize the effect of solvent (water) induced by the salts on enzyme stability. Raman spectra are obtained with a T800 Coderg triple monochromator and using the 488.0-nm exiting line (400 mW, 19 °C) of an argon ion laser. Raman intensities $I_{\parallel}$ and $I_{\perp}$ are obtained for incident and scattered polarization either parallel or perpendicular. We present in FIGURE 1 the isotropic (below) and anisotropic (above) parts of the Raman spectra of water and a KCl 2.0 M aqueous solution.

TABLE 1. Effect of Salt Concentration on the Stability of Invertase[a]

	Salt (M)	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Protective Effect	LiCl	35	48	43	8.5	—	—	—
	NaCl	81	254	—	388	240	60	2.1
	KCl	36	138	240	600	1300	—	—

[a]Denaturation temperature: 70 °C.

The isotropic and anisotropic parts, I_I and I_A, are related to $I_\parallel$ and $I_\perp$ as

$$I_I = I_\parallel - (4/3)I_\perp \qquad \text{and} \qquad I_A = I_\perp.$$

All these spectra are corrected for the Bose-Einstein population factor. The Raman spectrum of water has been studied extensively;[5-8] the main conclusions can be summarized as follows:

(I) The broad band between 3000 and 3700 cm^{-1} is attributed to intramolecular stretching of the OH covalent bond (ν_1). Its shape is due to (a) the perturbation of this covalent bond by the presence of OH . . . H intermolecular bonds and (b) the Fermi resonance between ν_1 and $2\nu_2$ (ν_2 is the bending frequency of H_2O).

(II) The low intensity peak at about 1640 cm^{-1} is attributed to the bending of the water molecule (ν_2).

(III) The low frequency part is correlated to intermolecular librational or vibrational motions.

The water organization is due to hydrogen bonds and, consequently, this is related directly to low frequencies (III) and indirectly to high frequencies (I and II).

The spectra of the KCl (2.0 M) aqueous solution exhibit important modifications compared to those of pure water:[9]

(1) The shape of the high frequency band (3000–3700 cm^{-1}) strongly depends on the concentration. The variations of different parameters (frequencies, heights, widths) versus concentration are linear within experimental errors. This evolution shows a decrease of the Fermi resonance and a decrease of the number of hydrogen bonds between water molecules.

(2) The lowest frequency part of the spectra (<100 cm^{-1}) remains practically antisymmetric with increasing concentration of KCl, whereas the 200-cm^{-1} feature, which is totally antisymmetric in pure water, becomes more and more depolarized. The behavior of this band, which is attributed to intermolecular

TABLE 2. Effect of Salt Concentration on the Stability of Glucose Oxidase[a]

	Salt (M)	0.5	1.0	1.5	2.0	2.5
Protective Effect	LiCl	1.0	1.9	3.3	1.2	1.0
	NaCl	1.4	3.4	4.0	15.0	9.1
	KCl	1.4	3.4	3.8	16.0	15.5

[a]Denaturation temperature: 60 °C.

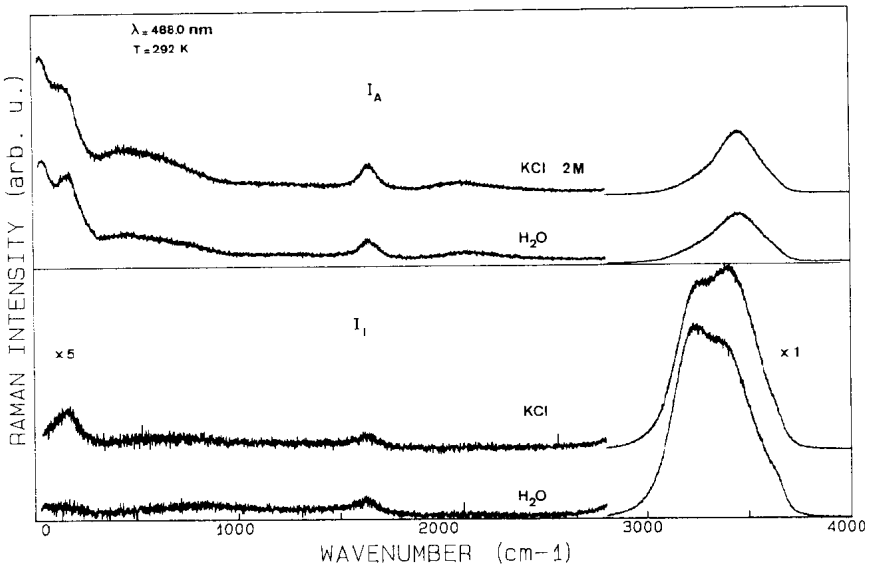

FIGURE 1. Isotropic and anisotropic components of Raman spectra of H_2O and KCl (2.0 M).

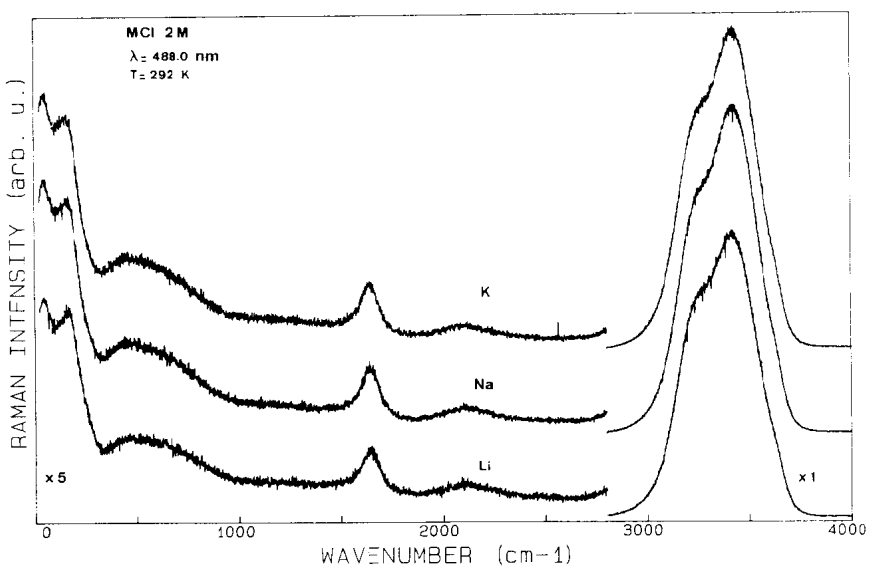

FIGURE 2. Raman spectra (I_I) of LiCl, NaCl, and KCl (2.0 M) aqueous solutions.

hydrogen bond stretching, explains the change in symmetry of the microenvironment of the water molecule. The development of the new band in the isotropic part at almost constant frequencies may lead to the conclusion that a new vibration is involved. This new vibration can be related to water-ion interactions.

FIGURE 2 presents $I_{\parallel}$ for LiCl, NaCl, and KCl (2.0 M) aqueous solutions. The differences between the spectra of these salts exhibit only small changes. It seems to be very difficult to observe the effect of the cation on water organization. Nevertheless, some parameters can be extracted by means of peak analysis using computer calculations in order to compare the evolution of these spectra. For instance, the frequency of the feature, which is at 189 cm^{-1} in pure water, decreases when adding LiCl, NaCl, and KCl (2.0 M) to reach 182, 177, and 174 cm^{-1}, respectively. This evolution explains the change in water intermolecular interactions and is due to the ionic and size effects of the salts.

An important protective effect is observed for the enzymes, invertase, glucose oxidase, and lysozyme, in the presence of KCl, NaCl, and LiCl. This effect depends on the nature of the enzyme and the concentration of the salt. When the concentration of the salt increases, the protective effect increases, reaches a maximum value, and then decreases (i.e., in the case of NaCl and LiCl). The evolution of the Raman spectrum of salt solutions allows a measurement of water organization. This water structuration is not sufficient to describe the protective effect of the salts on enzymes; it is necessary to take into account the direct interactions between the ions and the enzymes.

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Characteristics of the β-Lactam Synthesizing Enzymes of *Streptomyces clavuligerus*, *Cephalosporium acremonium*, and *Penicillium chrysogenum*

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The biosynthesis of cephalosporins, which involves the formation of penicillins as transitory intermediates, is restricted to a number of eukaryotic and prokaryotic species.[1] The pathway used in the synthesis of desacetylcephalosporin C (DAC) by *S. clavuligerus*, *C. acremonium*, and *P. chrysogenum* in the biosynthesis of isopenicillin N (IPN) is shown in FIGURE 1. The conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N (IPN) has been demonstrated in cell-free extracts (CFX) of all three organisms, and the enzymes that produce DAC from IPN are present in CFX of *S. clavuligerus* and *C. acremonium*. The CFX from these latter two organisms also convert analogues of the tripeptide ACV into antibiotics. Several independent research units are involved in studies on the possible use of these enzymes in the synthesis of novel and natural cephalosporins.

The patterns of formation of these enzymes vary considerably over the course of fermentation. Cells of *S. clavuligerus* produced in 10-L batch fermentations yield CFX that typically contain 1.6 units of IPNS/mL,[2] 0.6 units of epimerase/mL,[3] and 4.5 units of DAOCS/mL (unpublished results). It has been observed (unpublished results) that maintaining the dissolved oxygen content of the culture at saturation throughout the fermentation results in a 3-fold increase both in the level of antibiotics produced and in the specific activity of DAOCS, whereas that of IPNS is only increased 1.5-fold. This procedure does not have any effect on the specific activity of ACVS, which is the initial enzyme in the pathway. However, the cellular content of these enzymes is too low for their exploitation in the *in vitro* synthesis of novel and natural cephalosporin antibiotics; therefore, research is being conducted in several laboratories on the cloning and expression of these enzymes.

Some of the results of studies on these enzymes relative to enzyme engineering of the enzymes in this pathway are summarized in TABLE 1. Recent studies with *C. acremonium* extracts have shown that the initial enzyme in the pathway, ACVS, is a single multifunctional enzyme that acts in a manner similar to those enzymes involved in the synthesis of peptide antibiotics.[4,5] ACVS initially converts L-α-aminoadipic acid and L-cysteine into δ-(L-α-aminoadipyl)-L-cysteine and then adds L-valine to produce