New Methodology for Tosylation of Hydroxylic Supports as Exemplified by the Immobilization of Micrococcal Endonuclease on Agarose

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ABSTRACT

Improvement have been made in a simplified procedure we previously reported (J.Mol.Catal. (1986),38,227 for the activation of tosyl chloride of supports possessing primary hydroxyl groups. The method is simple, can be completed in less than 90 min, yields a broad range of activation degrees, and, since it involves no toxic reagents, may be used for preparing immobilized enzymes to be utilized in food manufacturing and processing. The immobilization of *Staphylococcal Nuclease* has been carried out by this method. The insolubilized derivatives are more active than the native enzyme in the hydrolysis of DNA. The thermal stability of nuclease derivatives is greater than that of the native enzyme. These derivatives remain active at 50°C, and the native enzyme, 39°C. The insolubilized nuclease is more stable against organic solvents such as, dimethylsulfoxide (DMSO) or tetrahydroduran (THF) than the native enzyme.

Index Entries: Tosylation of polysaccharides; agarose; *Staphylococcal endonuclease;* immobilization of enzymes; hydrolysis of DNA.

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INTRODUCTION

Staphylococcal Nuclease (E.C. 3.1.31.1; Mw = 16870) is a well-known enzyme that catalyzes the hydrolysis of DNA and RNA to yield 3′-mono and dinucleotides in the presence of Ca(II) (*1–4*). It has been used in the structural analysis of synthetic DNA (*5*) or polynucleotides (*6*). Recently, we have reported the utilization of immobilized nuclease on corn cob in order to decrease the nucleic acid content in single cell protein concentrates for use in human consumption (*7*).

Toward this end, many enzymatic derivatives were prepared, but low thermal stability was obtained (BrCN-agarose (sepharose) derivatives (*8*)), or potential toxicity, as a result of the presence in the insolubilized derivative of pyridine used in the activation of the support (*9*). These derivatives could not withstand temperatures >40°C for long periods (*8,10*). These facts made us look at a new immobilization method that is more reproducible than the one described before (*8–10*), and without potential toxicity. We modified the activation of agarose and other polysaccharides by tosylation (*11*), using acetone as dehydrating agent. Now we have used a hydrophilic base, such as, ethanolamine. This base is more hydrophilic and stronger than pyridine. It can react with the HCl produced in the activation process more easily than pyridine, thereby avoiding the destruction of agarose by acid hydrolysis. On the other hand, ethanolamine can be efficiently removed by water washings. The hydrophobic base, pyridine, is difficult to eliminate by this method, and so, the insolubilized derivatives produced in its presence are potentially toxic, whereas, the structure of the agarose (polymer of α-D-galactopyranose and 3,6-anhydrous-β-L-galactopyranose) makes it nontoxic and useful in the food industry.

MATERIALS AND METHODS

Agarose (Biogel, A-150m, 100–200 mesh, containing 1% w/v of agarose) was supplied by Bio-Rad laboratories; and Endonuclease from *Staphylococcus aureus* by Boehringer Manheim. Salmon testes DNA Type III (from Sigma) was heat-denatured and used as substrate. Tosyl chloride, >99% purity, and ethanolamine (98%) were from Merck. Acetone was supplied by Sharlau, and dehydrated over molecular sieves 3A from Sigma.

Activation of Agarose

Drying of Agarose

Wet gel, 5 mL, was mixed and mechanically stirred with 10×5 mL bidistilled and deionized water for 5 min in order to remove commercial additives. After filtration, the gel was washed successively with 10×5 mL

of water, twice with 10×5 mL water/pure acetone mixtures: 3/1 (v/v) and 1/3 (v/v). Finally, the filtrate was washed twice with 10×5 mL of pure dry acetone. Then, the gel was dried in vacuo at 120°C overnight. This procedure gave us reproducible dry agarose for the functionalization.

Tosylation of Dry Agarose

The tosylation of 1 g of dry agarose was carried out using 10–20 g tosyl chloride (TsCl) (*see* Table 1), and an amount of ethanolamine between 0.01 and 2 mols (1–3 mL) per gram of agarose. The mixture was stirred magnetically for 20 min at 25°C. The solid was then filtered and washed twice with dried acetone (10×10 mL) to remove the unreacted TsCl and the ethanolamine, washed twice with 1/3 and 3/1 (v/v) water/acetone mixtures, and twice more with bidistilled and deionized water (pH 7.0). In these washings, the ethanolammonium chloride produced in the activation of the agarose, and the remaining ethanolamine, were completely removed from the activated gel. The activated gel was stored at 4°C. The deactivation of thie gel at 4 mo was 10%, lower than that reported by Mosbach et al. (*12*) for the gel activated by similar organic reaction in the presence of pyridine, and dried with 1,4-dioxane (25% at the third month).

Determination of the Activation Degree of the Gel

The amount of tosyl groups in the activated agarose were determined by hydrolysis with 0.01*M* NaOH during 12 h period at room temperature. Then, the amount of free tosylate anions (TsO^-) were determined by UV spectroscopy in a UV-visible spectrophotometer Varian model Cary 219. $\lambda = 260$ nm. The molar absorptivity obtained was $\epsilon = 900\ (M \times cm)^{-1}$. The calibration equation was (1):

$$A_{260} = (-0.006 \pm 0.002) + (900 \pm 31)\,[TsO^-] \qquad (1)$$
$$N = 10 \quad r = 0.996$$

where: A_{260} is the absorbance (D.O.) at 260 nm of the solution, $[TsO^-]$ is the concentration (mol/L) of tosylate anion in the solution.

Hydrolysis of Activated Glucose at Different pH Values

The percentage of the tosylated groups of agarose (CH_2-OTs) that hydrolyzed at different pH values were determined by UV spectroscopy, $\lambda = 260$ nm. Two different calibration equations were used. At pH ≥ 7.0, equation (1), and at pH < 7.0, equation (2)

$$A_{260} = (-0.0003 \pm 0.002) + (491 \pm 7)\,[TsOH] \qquad (2)$$
$$N = 11 \quad r = 0.999$$

[TsOH] is the concentration (mol/L) of tosylic acid in the solution from the hydrolysis of tosylate groups in acid media.

Insolubilization of *Endonuclease S. aureus*

Tosylated agarose in 0.1*M* $NaHCO_3/Na_2CO_3$ buffer, pH 9.0, 0.5*M* NaCl was mixed with nuclease solution. The ratio μg Enzyme/mL gel was 8.0 in all cases, and the total volume of reaction was 10 mL. After stirring gently at 30°C for 20 min, the solid was recovered by filtration and washed with 20 mL of water. Then 20 mL 0.1*M* Tris-HCl buffer, pH 8.8, were added to destroy the remaining tosyl groups of the agarose, that may cause multiattachment of the enzyme on the support, giving enzymatic derivatives with low enzymatic activity. Afterward, the following cycle of washing was repeated:

1. 10 mL of 0.1*M* NaOAc/HOAc, pH 4.0 (0.5*M* in NaCl);
2. 10 mL bidistilled water, and
3. 10 mL of Tris-HCl buffer, pH 8.8 (0.5*M* in NaCl).

The insolubilized enzymatic derivatives were stored until use at 4°C, in 20 m*M* citrate buffer, pH 6.4, 10 m*M* $CaCl_2$ with 0.1% bovine serum albumin (*10*).

The amount of bound enzyme was determined by the difference between the activity added and the activity measured, in the washings. In control experiments with unactivated agarose, 100% activity was recovered in the washings. The results obtained by determination of protein in the washings by means of the Lowry method (*13*) agreed with the results obtained from the enzymatic activity assays.

Enzymatic Activity

Native Enzyme

The DNase activity of native nuclease was determined according to the method described by Anfinsen et al. (*4*), that, based on the increase in the absorbance at 260 nm (A_{260}), the hydrolysis of DNA observed was caused by the hyperchromic effect of the resulting nucleotides vs DNA. Heat denatured DNA (30 min at 100°C) was used as substrate, because higher DNase activity was observed vs heat denatured DNA than vs native DNA (*4,14,15*). The standard assay was carried out in a UV cuvette with 1 cm of ptical pathlength. DNA concentration was 16–79 μg/mL. T = 30°C, [Ca(II)] = 0.01*M*. Nuclease concentration 0.224 μg/mL. The reaction media were:

3 mL 0.1*M* Tris-HCl buffer pH 8.8
2.64 mL Tris-HCl buffer + 0.06 mL DMSO
2.64 mL Tris-HCl buffer + 0.06 mL THF

The units of enzyme activity were determined according to (3)

$$\text{Units} = (\Delta A_{260}/\text{min}) \times V_\gamma \quad (3)$$

The enzyme specific activity was determined by (4)

$$\text{Specific activity} = (\Delta A_{260}/\text{min}) \times V_\gamma / V_{enz.} \times [E] \quad (4)$$

where: ΔA_{260}/min is the slope of the curve ΔA_{260} vs time at t=0; V_γ is the total volume (3 mL) of the reaction in cuvette; V_{enz} is the volume (mL) of the enzyme solution added to the cuvette (mL); [E] is the enzyme concentration (0.224 μg/mL) in cuvette.

Insolubilized Enzyme

The activity of the insolubilized enzyme was measured in a similar way than described in the native one. The units were determined by the equation (3). The specific activity was determined by (5)

$$\text{Specific activity} = (\Delta A_{260}/\text{min}) \times V_\gamma / \delta \times V_{enz.} \times [E] \quad (5)$$

where: ΔA_{260}/min, V cuvette, V_{enz} and [E] have the same significance than in equations (3) and (4).

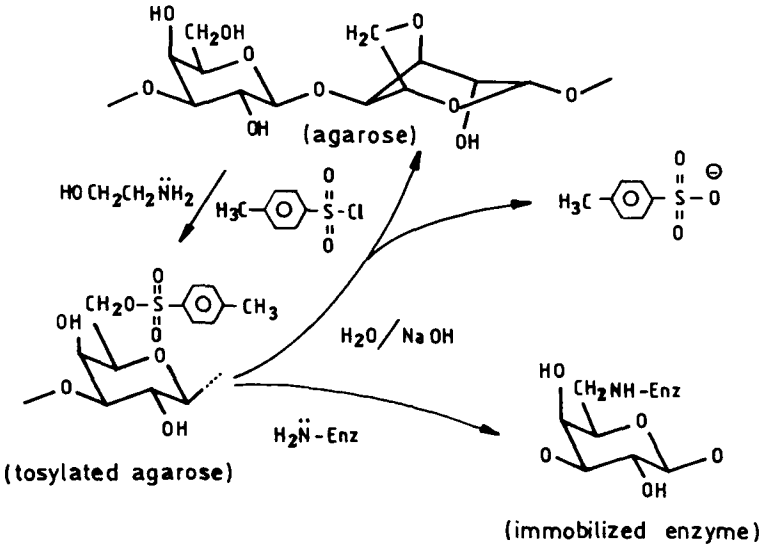
δ represents the effective volume of the insolubilized derivative solution, and is obtained by leaving to sediment during 24 h the storage mixture in which the solution where the insolubilized derivative is kept. $\delta = (V_f/V_i)$, where V_i is the volume (mL) of the agarose-enzyme mixture at zero time, and V_f is the volume (mL) of the agarose-enzyme derivative after sedimentation. In our case, $\delta = 0.4$.

RESULTS AND DISCUSSION

Activation of Agarose

The activation of agarose by reaction with TsCL takes place in the primary alcohol groups of galactose that are less hindered and more reactive than the other hydroxylic groups to react with the TsCl by an esterification process (*9*) in these mild conditions.

In Table 1, we show the results obtained in several experimental conditions. The activation degree of the biogel has been expressed also as number of tosyl groups per 1000A^2 (the cross sectional area of a molecule of nuclease). This value can easily be obtained from the number of tosyl



Scheme 1.

Table 1
Activation of Agarose by Tosylation Method Assay

	I	II	III*	IV	V*
Dried agarose (g)	1	1	1	1	1
Reaction volume (mL)	50	50	50	50	50
Volume of ethanolamine (mL)	3.15	1	1	—	1
Tosyl chloride (g)	10	10	10	10	10
Moles of TsO-H_2C/1000A^2	0.3	3.7	11	2.4	11

groups per gram of wet gel, using the density of agarose (p = 1.021 g/mL gel), its pore fraction (85%), and its surface area (Sarea = 17 m^2/g·gel) (*9*).

From the data in Table 1, one can see that a large range of activation values can be obtained by varying the experimental conditions. An excess of TsCl vs ethanolamine is necessary to get good activation values (entries I and II). Nevertheless, the presence of the base is necessary to avoid the destruction of the agarose structure as a result of the hydrolysis of the glycoside bonds by the HCl produced in the activation process. (Recovered agarose 0.9 g, assay II vs 0.6 g assay IV.)

The highest activation degrees were obtained in experiments III* and V*. In these cases, Whatman type 4 filter paper was used instead of the regular filter paper. The Whatmann paper led to a quicker filtration diminishing the contact time between the activated gel and the aqueous solutions used to turn the gel to aqueous conditions. This fact slows

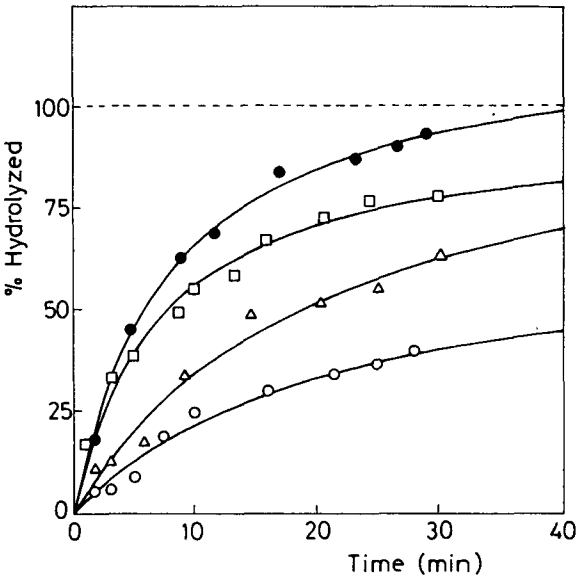


Fig. 1. Percentage of activated groups of the tosylated agarose (CH_2-OTs) hydrolyzed at different pH values. ● pH 9.72; □ pH 8.02; △ pH 7.01; ○ pH 5.98.

down the hydrolysis of tosyl groups ($-CH_2OTs$) in activated agarose and therefore, the activation degree is greater in III* than in II. This very simple observation has not been reported before (*8,12*). We think that the low reproducibility, and low or medium activation values obtained by these workers could be caused by this simple fact.

No greater activation degree was obtained using double amount of TsCl (experiments III* and V*). This fact could be related to the destruction of the biopolymer structure by the excess of activating reagent. On the other hand, similar results were obtained with agarose 100–200 mesh and 50–100 mesh, therefore, the particle size did not affect the activation degree obtained in this support.

Hydrolysis of Tosylated Agarose

As we previously said, the activated groups of the tosylated agarose can be hydrolyzed by the water of the reaction mixture medium where the insolubilization of the enzyme is carried out, giving $-CH_2-OH$ groups that cannot react with the enzyme, to give the insolubilized derivatives. It is interesting, therefore, to study the stability of the activated matrix vs pH in order to know the optimal pH for immobilization regarding the support. Evidently, this pH may, or may not coincide with the optimal pH regarding the enzyme. In the latter case, a compromise must be found.

The hydrolysis of activated agarose containing 2.3 tosyl arms/1000A^2 was carried out at different pH values. The data in Fig. 1 show that, at the

Table 2
Percent of Enzyme Bound to the Agarose
in the Insolubilized Derivatives

Amount TsO/1000A^2	Percent bonded enzyme
0.3	69
1.5	69
2.4	69
3.7	65
11.0	67

same reaction time, the hydrolysis increases with the pH. Thus, this activation method is better for immobilizing enzymes for long periods at pH <7.0, because the hydrolysis of tosylated agarose takes place slower at this pH than at pH >7.0. This fact agrees with the nucleophilic character of the nucleotides:

H_2O in acidic medium = weak nucleophil = slow hydrolysis
HO^- in basic mediu = strong nucleophil = quick hydrolysis.

Nevertheless, the insolubilization of enzymes such as *Micrococcal Endonuclease* whose insolubilization pH is greater than 7.0, can be carried out at short contact times. Owing to the short reaction time and the large molecular structure of the diffusing enzyme, one can anticipate that the protein may be bound through a single linkage.

Immobilization of Nuclease

The insolubilization of nuclease was carried out at room temperature, and pH 9.0, by a method described previously by Ballesteros et al. (*8,16*), but reducing dramatically the immobilization time to 20 min. It has been reported (*10,17*) that, the immobilization on BrCN-activated agarose through acid residues, pH of the immobilization mixture 4.7, gave an insolubilized derivative that lost 20% activity in 3 h. However, when the enzyme was immobilized at pH 9.0, 100% of enzymatic activity remained after 24 h. Therefore, we have carried out the immobilization at this pH, although, this pH produced greater hydrolysis of tosyl groups of the agarose than acidic pH.

From the data in Table 2, we can deduce that the percentage of fixed enzyme is identical in all cases in spite of the different degrees of activation in the agarose. This fact can only be explained assuming that, at this small concentration of nuclease, the diffusion of the enzyme molecules toward the active points in the support is the limiting factor in the process (*18*). In these conditions, the enzyme would be attached to the agarose by one, or even, two points at the same time that the small OH^- and/or H_2O

Table 3
Enzyme Engineering of Native Nuclease vs Heat Denaturized DNA
[Ca(II)] = 0.01*M*; [E] = 0.445 μg/mL

T(°C)	Medium Tris buffer[a] organic solvent	Km·10^{-2} (mg/mL)	k_{cat}·10^{-6} (units × mL × (min × mg^{-1}))
30	100	1.5 ±0.2	5.9±0.2
30	98/2 DMSO	1.4 ±0.2	5.1±0.5
30	98/2 THF	1.0 ±0.1	4.5±0.4
35	100	1.2 ±0.2	5.4±0.6
35	98/2 DMSO	0.9 ±0.3	4.3±0.5
35	98/2 THF	0.85±0.09	4.1±0.3
40	200	2.5 ±0.3	6.8±0.5
40	98/2 DMSO	0.71±0.09	3.8±0.4
40	98/2 THF	2.6 ±0.3	3.3±0.4
45	100	1.1 ±0.5	4.7±0.7
45	98/2 DMSO	1.1 ±0.5	4.6±0.4
45	98/2 THF	1.1 ±0.5	2.5±0.3

[a]Tris-HCl buffer 0.1*M* pH = 8.8.

species hydrolyze the rest of the activated groups in the gel. These bond points are lower than 5/1000A[2] that make inactive the enzyme according to the literature (*8,16*). Owing to the fact that the more activated gels carry to similar yields in bonded enzyme than the medium activated agaroses, we do not recommend high activation treatments that produce partial destruction of the biogel.

DNase Activity of Native Enzyme

The enzymatic activity of the native enzyme was determined in pure Tris-HCl buffer, and in the presence and absence of DMSO or THF at 2%. The DNase activity of native nuclease was determined as increasing in Absorbance at 260 nm, using a solution of heat denatured [DNA] = 0.8 μg/mL and [E] = 0.554 μg/mL in cuvette. The results obtained, using Lineaweaver-Burk representation, are shown in Table 3.

We can observe that k_{cat} values slightly increase in pure Tris-HCl buffer, in the absence of organic solvents between 30 and 40°C. Nevertheless, at T > 45°C, the k_{cat} value diminishes as was reported by Chaiken (*19*). This fact has been related to a conformational change in the enzyme at T ≥ 40°C, observed by UV spectroscopy (*20*). This effect is not observed in the presence of 2% DMSO that stabilizes the enzyme at T ≥ 40°C as has been shown in a previous paper (*20*). The k_{cat} values remains constant within the experimental error with this solvent. Tris-HCl 98%–THF 2% diminishes the enzymatic activity with respect to pure Tris-HCl buffer.

This fact is related to a conformational change of the protein that closes the pocket of the enzyme as we have detected by UV spectroscopy (*20*). On the other hand, the apparent Km values remain constant within the experimental error. Nevertheless, the very complex process studied by us makes this value ambiguous in order to obtain mechanistic conclusions.

DNase Activity of the Insolubilized Enzyme Derivatives

The results of Table 3 agree with the ones of a previous paper (*20*). We have reported that, the native nuclease is more active in the presence of DMSO/Tris-HCl buffer pH 8.8 (2/98 v/v), and less active in THF/Tris-HCl buffer pH 8.8 (2/98 v/v) than in pure Tris-HCl buffer pH 8.8. These results were explained by the stabilization or alteration, respectively, of the active site by these solvents.

In order to compare the activity and stability of the insolubilized derivatives obtained by the method described above, and the native enzyme, two derivatives obtained from agaroses containing 0.3 and 1.5 tosyl groups per 1000A^2 were used. The DNase activity of the immobilized enzyme was done using the same experimental conditions as in the case of native enzyme. Since the immobilized derivatives contain 8 μg of nuclease per gram of gel, 55 μg of agarose-nuclease derivative were added to 3 mL of the reaction mixture to obtain 0.445 μg Enz/mL. This concentration is equivalent to the one used in the assay with native enzyme. The results are shown in Figure 2.

Both insolubilized derivatives give similar results, as could be expected, since they have the same amount of native enzyme.

On the other hand, the insolubilized derivatives are more active than the native enzyme, this relative stabilization being greater at higher temperatures. Therefore, our immobilization methodology is very adequate for the immobilization of *Micrococcal Nuclease*.

When the hydrolysis of DNA is carried out in the presence of DMSO and THF at 2%, the enzymatic derivative obtained with the more activated agarose (with an average of 1.5 tosyl arms/1000A^2) is more stable and active than the one obtained with agarose with 0.3 tosyl groups per 1000A^2. This finding could be explained assuming that, in this case, the enzyme is attached to the support through a single bond, whereas in the former, the enzyme molecules can be linked through more points. It is well known that, the multipoint attachment yields enzymatic derivatives that are more stable (*21–23*). Both organic solvents improve the DNase activity of the insolubilized derivative as compared to the soluble enzyme. Since we have reported that the nuclease is deactivated by the THF (*20*), the increase in the ratio insolubilized/soluble enzyme activity (Figure 3b) can only be explained by a greater deactivation by THF of the native enzyme

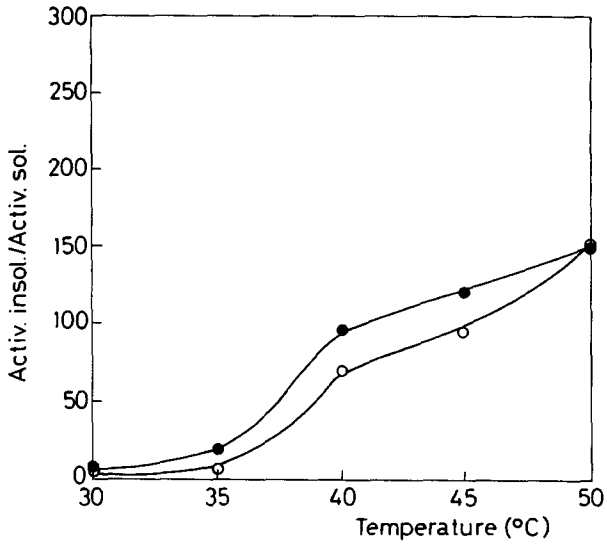


Fig. 2. Relative DNase activity (%) of insolubilized derivatives vs the native nuclease. The insolubilized derivatives were obtained from gels with 0.3 ○ and 1.5 ● tosyl arms per 1000A^2.

than of the insolubilized derivative. Thus, our insolubilization method increases the stability of the nuclease vs THF. Contrariwise, the native nuclease is activated and stabilized by DMSO at 2% (*20*). Hence, the high values obtained for the ratio insolubilized/native enzyme (Figure 3a), may be related to an additional stabilization vs the organic solvent of the insolubilized derivative as compared to the native enzyme. The nature of this activation-stabilization process is presently unknown. The system is being studied by flow microcalorimetry.

CONCLUSION

We recently simplified (*9*) the procedure of Mosbach et al. (*12*) for activation of hydroxylic supports TsCl. In the present paper, further improvements are proposed: The substitution of pyridine by ethanolamine, the use of a wide-porosity filter paper, and the elimination of acetone distillation (a drying with molecular sieves is sufficient). Our new methodology presents many advantages:

1. It is simple;
2. Nontoxic solvents are handled, therefore allowing the use of the insoluble derivatives obtained in food technology;

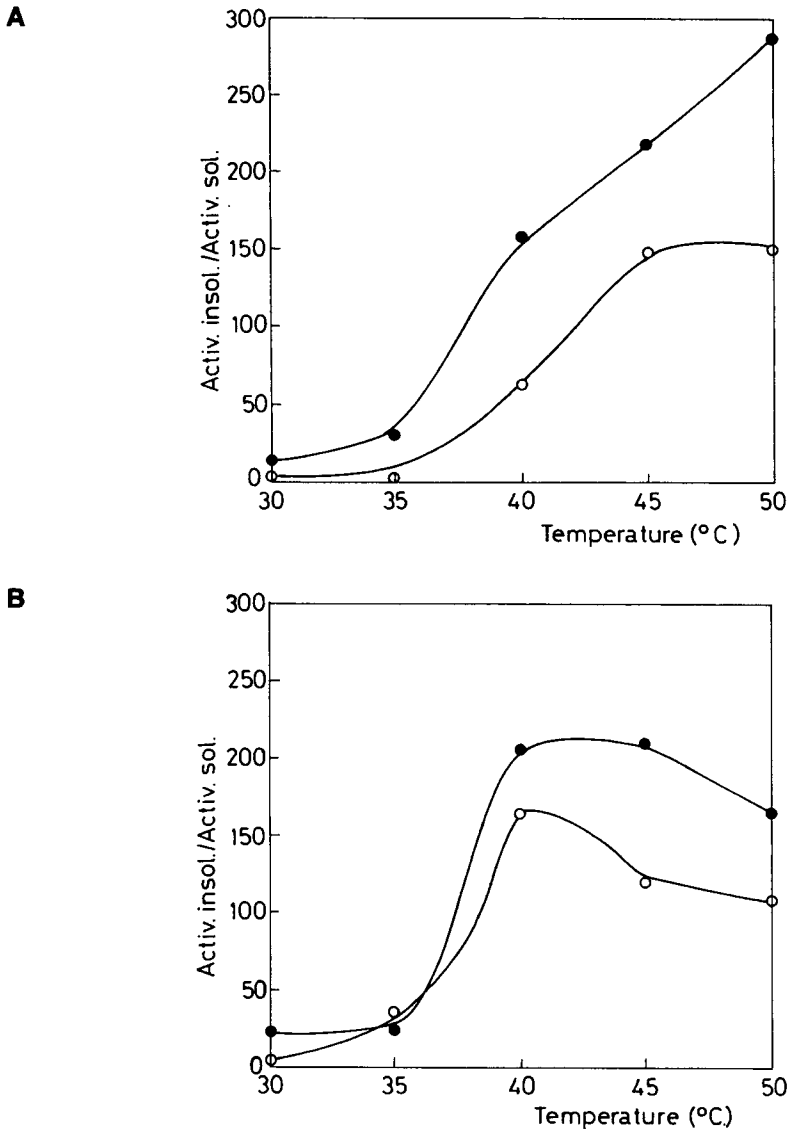


Fig. 3. Enzymatic activity of insolubilized derivatives vs the native nuclease. 3a, 98% Tris-HCl buffer pH 8.8, 2% DMSO; 3b, 98% Tris-HCl buffer pH 8.8, 2% THF. The enzymatic derivative was obtained from a gel with 1.5 ○ and 0.3 ○ tosyl arms per 1000A^2.

3. TsCl is cheap, and does not react with secondary hydroxyl groups of polysaccharide so vigorously as other sulfonic compounds;
4. It can be used on any support having primary hydroxyl group, giving a broad range of degrees of activation; and
5. The whole process of support activation can be completed in less than 90 min.

This methodology gives us a new repetitive activation-stabilization process for *Endonuclease S. aureus.* The insolubilized derivatives of endonuclease are more active and stable than the native enzyme vs the temperature and organic solvents, such as THF and DMSO.

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