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DNase Activity of Micrococcal Endonuclease Insolubilized on Corn Cob

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ABSTRACT

The endonuclease from *S. aureus* has been immobilized on ground maize cob, previously activated with tosyl chloride. Pretreatment of the support in acid before tosylation yielded the best insoluble enzyme derivatives. The catalytic activity has been evaluated as percent of total hydrolysis attained in a batch reactor using DNA as a model substrate. The derivatives prepared are very resistant to high temperatures under conditions of catalysis (24 h at 45°C). For these long reaction times, the extent of hydrolysis in the presence of small amounts of organic solvents (dimethyl sulfoxide at 2%) is larger than in plain buffer (Tris). This type of derivative could be very useful for the removal of nucleic acids from single-cell protein concentrates.

Index Entries: Enzyme immobilization; maize cob, ground; enzyme supports; support activation by tosylation; organic solvents, influence of. . .in enzyme activity; nucleic acid removal.

INTRODUCTION

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. The

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enzyme requires Ca^{2+} for activity. In our laboratory, we have been interested in the study of this enzyme immobilized on agarose activated by cyanogen bromide (2–4). In our investigation directed toward the search for better methods of support activation, we have shown that, by using *p*-toluenesulfonyl chloride ("tosyl chloride") (5), it is possible to obtain a high degree of activation in agarose. Therefore, this method should be well suited for preparing insoluble derivatives in which the enzyme is bound through multiple linkages to the matrix. This multipoint attachment will lead to an enzyme that is more stable against deleterious agents.

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 owing 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 concentrates (6–8), but no agreement has yet been reached about how they affect toxicity or digestibility of the resulting protein.

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 of 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 paper, we present data of the hydrolysis of DNA, used as a model substrate, at temperatures of 45°C, by micrococcal nuclease immobilized on corn stover activated with tosyl chloride.

EXPERIMENTAL

MATERIALS

Micrococcal endonuclease from *Staphylococcus aureus* was purchased from Bochringer; salmon testis DNA (type III) and bovine serum albumin, from Sigma; Tris, from Ega-Chemie; and citric acid monohydrate, trisodium citrate dihydrate, $CaCl_2 \cdot 2H_2O$, and tosyl chloride (TsCl), from Merck. The organic solvents dimethyl sufoxide (DMSO) and tetrahydrofurane (THF) were furnished by Scharlau.

Corn cob particles (EU-Grits, 0.84–0.59 mm, from Eurama, Manbourget, France) were kindly provided by P. Monsan, (BioEurope, 31400 Toulouse). The specific surface area of the particles was 0.083 m^2/g.

METHODS

Previous Treatment of the Support

Twenty mL of hydrolysis medium (0.1 *N* HCl; 1 *N* NaOH; 0.1 *M* bicarbonate buffer, pH 9.0, 0.5 *M* NaCl; or bidistilled water) were added to

2 g of support and the mixture was stirred at room temperature for 24 h. The solid was filtered and washed with 120 mL of distilled water and then with 120 mL of pure dry acetone.

Preparation of Activated Support

In order to avoid the hydrolysis of the tosyl chloride, 1.5 g of previously treated cob particles were further dehydrated with 30 mL of pure dry acetone. Then, tosylation was effected essentially following our modifications (*5*) of the method developed by Mosbach and coworkers (*9*). More details are given in a Spanish patent (*10*).

For the analysis of tosyl group content, 0.1 g of activated support were hydrolyzed in 2 mL of 0.1 *M* bicarbonate buffer, pH 9.0, for 24 h at room temperature. And then, absorbance of the solution was determined at 256.8 nm in a Shimadzu model 160 spectrophotometer.

Insolubilization of the Enzyme

One g of activated support was suspended in 10 mL of Na_2CO_3/$NaHCO_3$ buffer (pH 9.0, 0.5 *M* NaCl). Then, 200 μL of enzyme solution (containing 22.5 μg of nuclease) in storage buffer (citric/citrate buffer, pH 6.4, 10 m*M* $CaCl_2$, 0.1% albumin) were added, and the mixture was gently stirred at 30°C during 1.5 h. The solid was filtered and washed with 10 mL of water. The amount of enzyme immobilized was obtained from the difference between the activity of the soluble enzyme added to the suspension of activated matrix and the activity recovered in the filtrate and washings. In control experiments with unactivated support, 100% of the activity of the enzyme remained in the filtrate and washings.

The immobilized nuclease derivative thus obtained was left in 10 mL of Tris buffer (in this paper, Tris buffer stands for 0.1 *M* Tris, pH 8.8, 10 m*M* Ca^{2+}) at room temperature during 1.5 h in order to destroy the tosyl groups that did not react with the nuclease. Then, the immobilized derivative was washed, consecutively, with 10 mL acetic/acetate buffer (pH 4.0, 0.5 *M* NaCl), 10 mL of water, 10 mL of Tris buffer, and 2×10 mL of water. The insolubilized nuclease derivatives were stored at 4°C in 10 mL of storage buffer per g of solid.

Enzymatic Hydrolysis

Soluble nuclease activity toward denatured DNA was measured in Tris buffer by graphically following the increase in A_{260} at 30°C (*1*) in a Varian Cary 219 spectrophotometer.

The hydrolysis of DNA (at a concentration of 2.5 mg/mL) was carried out in a thermostatized 35-mL batch reactor. Before starting the reaction with the addition of 1 g of corncob-enzyme, the absorbance of the starting DNA solution was measured using appropriate dilutions. Periodically, samples were taken from the reactor in order to monitor the progression of the enzymatic hydrolysis. The percentage of hydrolysis attained was

Table 1
Influence of Pretreatment of Corn Cob in the Tosylation

g of Support	Pretreatment	TsCl added, g	Reaction time, h	μmol Tosyl per g of support
1.0	—	1.0	20	64
1.5	0.1 N HCl	2.0	24	26
1.5	Bicarbonate	2.0	24	26
1.5[a]	1 N NaOH	2.0	24	41
1.5	H_2O	2.0	24	34

[a] After the pretreatment, the weight decreased to 1.0 g.

calculated on the premise that a 33% increase in the initial absorbance of this denatured DNA represents 100% hydrolysis ((*1*) and our unpublished observations).

RESULTS

Activation of the Matrix

The tosylation of untreated corn cob was carried out following a methodology similar to that previously described for agarose (*5*). In a series of experiments using 1.0 g of corn cob particles, the reaction time was varied from 1 to 20 h, and the amount of TsCl from 0.1 to 2.5 g. In all cases, the pyridine (in mL) was equal to the TsCl added (in g). An increase of the activation time and the amount of the added TsCl increases the activation of the solid. The extreme values of tosylation obtained, per g of support, were: 10 μmol tosyl for 0.5 g of TsCl and 1 h; and 107 μmol tosyl for 2.5 g of TsCl and 20 h of reaction. Corn cob particles contain 25–35% cellulose and 20–30% lignin (*11*). The tosylation of the reactive groups in cellulose and lignin is rapid during the first hour, slowing down afterward. In no case was destruction of the particles by high concentrations of TsCl observed, opposed to that found with agarose (up to 70% weight loss (*5*)). The values of tosylation obtained with high amounts of TsCl and pyridine may be overestimated because of the fact that pyridine, which has an appreciable absorbance at 257 nm, is very difficult to remove completely from the solid by washing with solvents that do not hydrolyze the tosyl arms formed.

On the other hand, pretreatment of the particles lowers the amount of tosyl groups bound, even when using more TsCl and longer reaction times (*see* Table 1). This is probably owing to the removal of small molecules (alcohols, phenols, and so on) that are susceptible to be tosylated; indeed, the filtrate after the pretreatment is slighly yellow (with HCl or water), yellow (with bicarbonate), and red (with NaOH). Only the pre-

Table 2
Hydrolytic Activity of Nuclease Immobilized on Corn Cob

Derivative	Organic solvent in the buffer, %	Percent DNA hydrolyzed at 1h	8h	24h
M—HCl—N	0[a]	74	76	81
	0	78	90	93
	2(DMSO)	75	95	100
	2(THF)	47	86	91
M—H_2O—N	0	77	91	92
	2(DMSO)	73	93	97
	2(THF)	47	89	95
M—NaOH—N	0	57	82	88
	2(DMSO)	54	86	91
	2(THF)	48	79	94
M—CO_3—N	0	64	85	86
	2(DMSO)	60	91	96
	2(DMSO)[b]	52	90	92
	2(THF)	44	88	90
M—N	2(DMSO)	46	86	91

[a,b]The experiment was carried out at 30°C (*a*) or 50°C (*b*); all the others at 45°C.

treatment with NaOH led to destruction of the structure of the solid (from a weight of 1.5 g down to 1.0 g). Apparently, the phenolic compounds in lignin are easily solubilized, and then are oxidized to colored products.

Insolubilized Nuclease Derivatives

The five activated supports described in Table 1 were contacted with 22.5 μg of nuclease to obtain the corresponding immobilized enzyme derivatives (*see* Methods). They are designated: M-N; M-HCl-N; M-CO_3-N; M-NaOH-N; and M-H_2O-N, respectively.

Enzymatic Hydrolysis

In previous research (*12*), we measured the initial rates of DNA hydrolysis in Tris buffer in both the absence and the presence of organic solvents; the best results were obtained when the reaction mixture contained 2% of solvent. The deviation from 260 nm of the λ_{max} of DNA absorbance was also measured in the aqueous-organic media (*13*).

In the present paper, the hydrolytic capacity of immobilized nuclease has been analyzed in a batch reactor using as a model substrate DNA in large saturating excess. The reaction conversion (% of hydrolysis) in Tris buffer containing 2% THF; 2% DMSO; and 0% organic solvent, was mea-

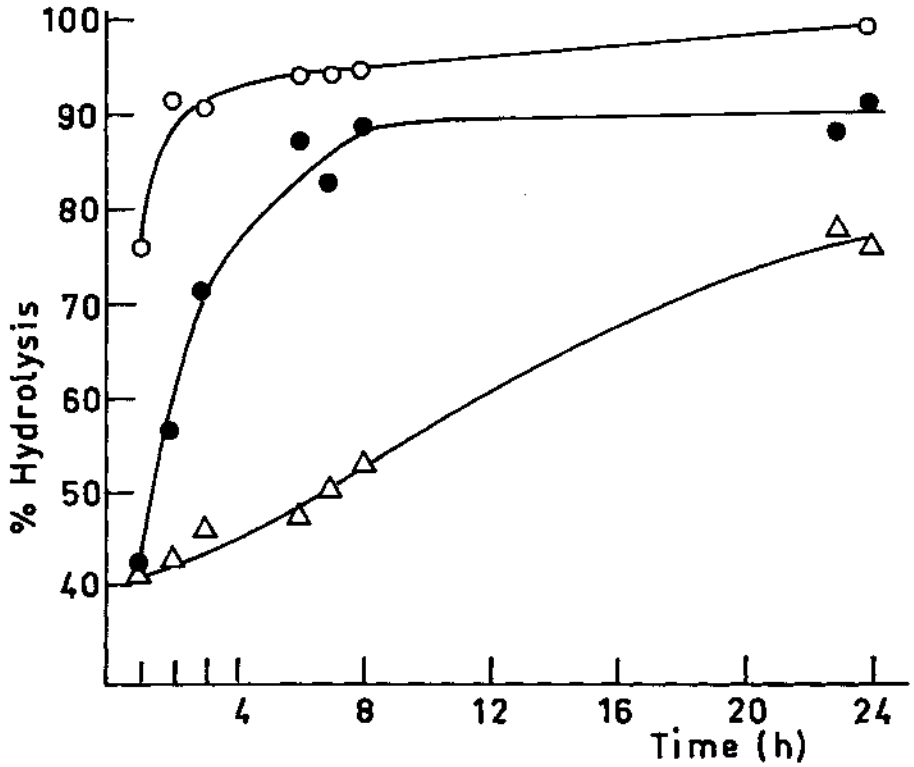


Fig. 1. Comparison of the performance of nuclease immobilized on three different supports in the hydrolysis of DNA vs time. Reaction medium: Tris buffer containing DMSO (2%); temperature: 45°C. Derivatives: ○———○, M—HCl—N; ●———●, resin-nuclease, △———△, agarose-nuclease. For details, *see text*.

sured as a function of time. The absorbance was read at 259, 260, and 259.5 nm, respectively. The results obtained appear in Table 2. In all cases, the best reaction medium for 1 h hydrolysis was Tris buffer containing no solvents. However, after 8 or 24 h the presence of DMSO yields larger degrees of hydrolysis. Lower values were obtained at 50 vs 45°C (cf., lines 12 and 13). The presence of THF yields smaller conversion figures than just plain buffer. These results are similar to those found for nuclease immobilized on agarose (*14*) and on Merrifield resin (*15*).

In general, each of the treatments assayed were found to be better than no treatment at all. The best results were obtained with HCl and the less favorable ones with NaOH; this is in agreement with the greater capacity of bases, as opposed to acids, to decompose the lignin component in corncob. The decreasing order of suitability of the pretreatments resultd to be HCl > H_2O > bicarbonate > NaOH.

In Fig. 1 the performance, at 45°C, of the best immobilized nuclease derivative, M-HCl-N, is compared with two others, N-Ag 1b and S_{12}max, studied in our laboratory. N-Ag 1b is a derivative (*14*) containing 8.9 μg

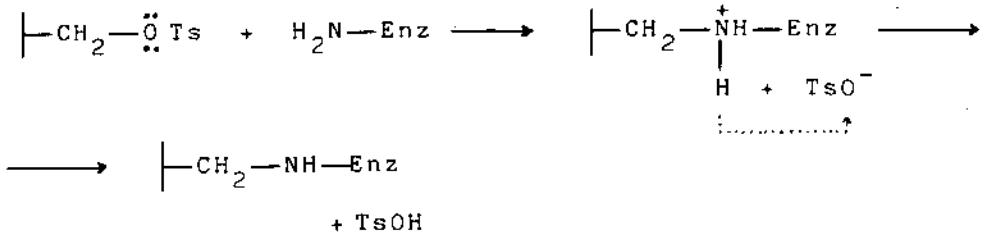
nuclease/mL of 1% agarose gel previously activated with TsCl. S_{12}max contains (*15*) 45.5 μg nuclease/g Merrifield's peptide resin (1% cross-linked). We can observe that R-HCl-N is by far the best immobilized derivative: after 1 h the hydrolysis is 77% (vs 42% for the others); 90% conversion is attained in 2 h, whereas S_{12}max (with double loading of enzyme) needs approx 15 h; in the case of N-Ag 1b (although with a low load of protein) the reaction proceeds very slowly and even after 24 h is still at a level below 80%.

DISCUSSION

After the removal of the cereal grains in a maize ear, the remaining core is a cheap and hard residue, which is very resistant to heating and to microbial contamination. Therefore, once ground into particles of convenient size, it can become a good candidate for supporting enzymes and other ligands.

There are two major classes of groups susceptible of tosylation in corn cob:

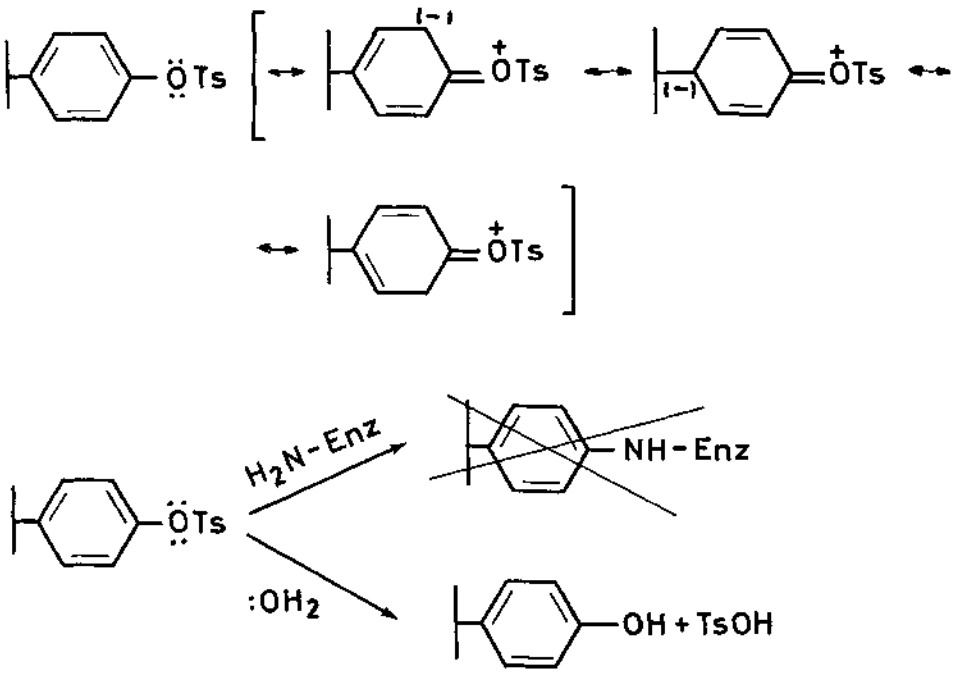
Primary alcohols in cellulose. These functions are readily reactive, and the tosyl arms so built in the matrix are good leaving groups toward amino groups in a protein



Phenolic groups in lignin. These groups can be tosylated but they are not reactaive vs NH_2 functions because of the conjugation of electrons of oxygen to the aromatic ring, which makes quasidouble the oxygen-ring bond. This character renders this bond very stable and no linkage of the enzyme to the solid is possible (*see* Scheme 1).

Hence, only the primary alcohols can be used for the immobilization of the enzyme. The pretreatment of the corncob shall cause destruction of phenolic groups—generally as gallic esters—therefore, the extent of activation will be lower than in the untreated solid (cf., Table 1). Monsan and coworkers have used corn cob activated by other procedures as a support for enzymes (*11,16*). These workers also found that pretreatment is water yielded insoluble derivatives that are more active than those prepared with the untreated matrix.

In the last decades, a growing interest in the development of cheap sources of protein to feed a rising world population has developed. In



Scheme 1. Inability of tosylated phenols to react with amino groups in proteins and other ligands.

this respect, the use of SCP concentrates offers promise, provided that the content of nucleic acids is reduced drastically. In the 1970s, the Protein Advisory Group of the United Nations considered 2 grams of nucleic acid the maximum safe intake per person per day (*17*). Since SCP concentrates from yeasts and bacteria contain from 5 to 15% nucleic acid (*18,19*), if this concentration could be reduced one order of magnitude, an adult could eat up to 150 g a day of microbial proteins.

Our long-term goal of reducing the nucleic acid content in SCP with immobilized nucleases carries implicit the separation, after the hydrolysis, of the nucleotides from the proteins. This can be effected by precipitation of the protein at acid pH (from 4 to 5); the polynucleotides—if they are small enough—remain in solution. Preliminary studies carried out in our laboratory with micrococcal nuclease insolubilized on agarose (activated by CNBr), at 25°C, using the supernatant obtained from a sample of brewer's yeast cells (J. M. Guisán and A. Ballesteros, unpublished), appear to indicate that protein and polynucleotide separation can be achieved by acid precipitation when the percent hydrolysis attained is over 90%. Micrococal nuclease is a suitable candidate for attaining high extents of hydrolysis and at the same time for producing polynucleotides of small size, because of its endonucleolytic (also exonucleolytic) action. Other (co)immobilized nucleases (such as, spleen phosphodiesterase) could cooperate in the process of nucleic acids removal from SCP. The

productivity of reactors different from the batch reactor studied in the present paper would also offer an interesting field of research.

The immobilized nuclease derivatives prepared in the present study have the ability to catalyze during long times at 45°C, a temperature at which there is no contamination by mesophilic microorganisms. These derivatives have better qualities than those prepared earlier in our laboratory (*3,5*); they represent a very good stabilization with respect to native enzyme, unable to catalyze at ≥ 40°C for long periods of time (*20*).

It should be noted that the immobilized derivates work better in plain Tris buffer for short reaction times. However, the presence of a small proportion of an organic solvent (e.g., DMSO or other to be tested) can positively influence the hydrolysis reaction when long contact times are needed. When selecting a solvent, one must cast always an eye on its properties. In our case, the presence of DMSO apparently stabilizes the active site of the enzyme since the extent of hydrolysis was found to be higher after 24 h. This might be owing to its high polarizability and hydrogen-bond basicity values (1.00 and 0.76, respectively, as opposed to 0.58 and 0.55 for THF (*21*)).

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