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LIGATION OF RESTRICTION ENDONUCLEASE-GENERATED DNA FRAGMENTS USING IMMOBILIZED T4 DNA LIGASE

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T4 DNA ligase was covalently coupled to Sepharose 4B using 2,2,2-trifluoroethanesulfonyl chloride activation. The immobilized DNA ligase could catalyze the joining of restriction endonuclease-generated DNA fragments with sticky ends as well as blunted ended DNAs. Immobilization provided an increased stability. At 4° C, the immobilized ligase remained active for at least three months. Nucleic acid synthesis and *in vitro* DNA recombination should be the main fields of application for such immobilized DNA ligase.

INTRODUCTION

DNA ligases have become indispensable reagents for *in vitro* DNA recombination (1). The unique ability of T4 DNA ligase to catalyze the joining of DNA fragments with "sticky ends" as well as blunted ended DNAs has made this enzyme the most extensively used ligase in nucleic acid research (2,3). The enzyme acts by transmuting the high-energy pyrophosphate linkage of ATP into a phosphodiester bond between the 5'-phosphoryl and 3'-hydroxyl termini of DNA.

Immobilization of enzymes and other biomolecules is a widespread and well-known technique in biochemistry (for review see (4)). Also in the "cloning technology", the use of immobilized enzymes should be advantageous, as it allows easy removal of the enzymes from the reaction mixtures as well as their subsequent reuse. In this report we wish to describe the immobilization of such an enzyme, T4 DNA ligase, to Sepharose using the CNBr method as well as the recently described procedure involving 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) (5). The latter method was chosen since stable bonds are formed between the matrix and the protein and also because coupling can proceed under mild conditions. Apart from the practical advantages offered by immobilization, such preparations should also provide an insight into questions relating to enzyme mechanism involved in DNA ligation, as discussed later.

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MATERIAL AND METHODS

Enzymes: T4 DNA ligase and restriction endonucleases *Eco*RI and *Sma*I were all obtained from Boehringer Mannheim. *Taq*I was purchased from Bethesda Research Laboratories Inc.

DNAs: The plasmid pBR 322 was purified from cleared lysates by centrifugation to equilibrium in CsCl-ethidium bromide gradients (6). Lambda DNA was purchased from P-L Biochemicals Inc.

Tresyl chloride activation of Sepharose: Activation of Sepharose 4B (Pharmacia) was done according to Nilsson and Mosbach (5). The wet Sepharose was transferred to dry acetone by washing with 10 gel volumes of distilled water, 30:70, 60:40 and 80:20 of acetone:water, 2 x 10 volumes of acetone and finally with 3 x 5 volumes of dry acetone (dried over molecular sieve, 100 g/3 l acetone). In a typical experiment 10 g moist Sepharose was then transferred to a beaker containing 1 ml dried acetone and 0.4 ml pyridine. During magnetic stirring 0.2 ml tresyl chloride (Fluka AG, Buchs, Switzerland) was added dropwise for 1 min. After reaction for 10 min. at room temperature the gel was washed with 2 x 100 ml acetone and then gradually transferred back to 1 mM HCl by reversing the washing scheme described above using 1 mM HCl instead of water. The activated gel was stored at 4° C in 1 mM HCl and samples were taken when required during a period of one month.

The degree of activation was determined by elemental analysis of sulphur.

CnBr activation: CnBr activation of Sepharose 4B was done according to March et al. (7) using dimethylformamide instead of acetonitrile. The reactive cyanate groups were determined by the method described by Kohn and Wilchek (8). The activated gel was used immediately.

Enzyme immobilization: 40 U of T4 DNA ligase were dissolved in 0.1 M NaH_2PO_4, pH 7.5; containing 1 mM EDTA; 50 µM ATP; 10 % glycerol to a final volume of 200 µl and kept at 4° C. A wet tresylated or CnBr activated Sepharose preparation was washed with the above cold phosphate buffer, gently sucked dry, and 100 mg moist gel was thereafter transferred to the enzyme solution. The resulting gel slurry was mixed "end over end" at 4° C for 16 hrs.

Residual reactive tresyl groups on the Sepharose were removed by adding 0.1 M dithiothreitol or in the case of CnBr, by adding 0.1 M 2-mercaptoethylamine (2 hrs, 4° C). After coupling, the gel was washed free of uncoupled enzyme by washing four times in 0.1 M NaH_2PO_4, pH 7.5; 1 mM EDTA; 10 mM dithiothreitol (DTT); 0.5 M NaCl followed by two washes in 0.1 M NaAc, pH 5.0. Finally the gel was equlibrated in 66 mM Tris-HCl, pH 7.6; 6.6 mM $MgCl_2$; 10 mM DTT; 10 % glycerol and stored at 4° C.

Enzyme activity determinations: The DNA ligase activity was determined by the $^{32}PP_i$ exchange assay devised by Weiss et al. (9). One unit is the enzyme activity which exchanges 1 nmol $^{32}PP_i$ into Norit adsorbable material within 20 min. at 37° C.

DNA joining: The reactions were carried out in volumes ranging from 30 to 300 µl containing 20 µg/ml DNA; 66 mM Tris-HCl, pH 7.6; 6.6 mM $MgCl_2$; 10 mM DTT; 500 µM ATP; 30-50 mg moist DNA ligase gel. The gel slurry was incubated by gentle agitation at 20° C in 1.5 ml plastic tubes. After 16-18 hrs the immobilized ligase was removed by centrifugation.

Agarose gel electrophoresis: Performed on a horizontal slab gel of 1 % agarose in 0.05 M Tris-acetate-EDTA buffer (pH 7.8).

RESULTS

Catalytic properties: As substrates for our study two standard DNAs were used; plasmid pBR 322 and lambda DNA which had been cleaved with the re-

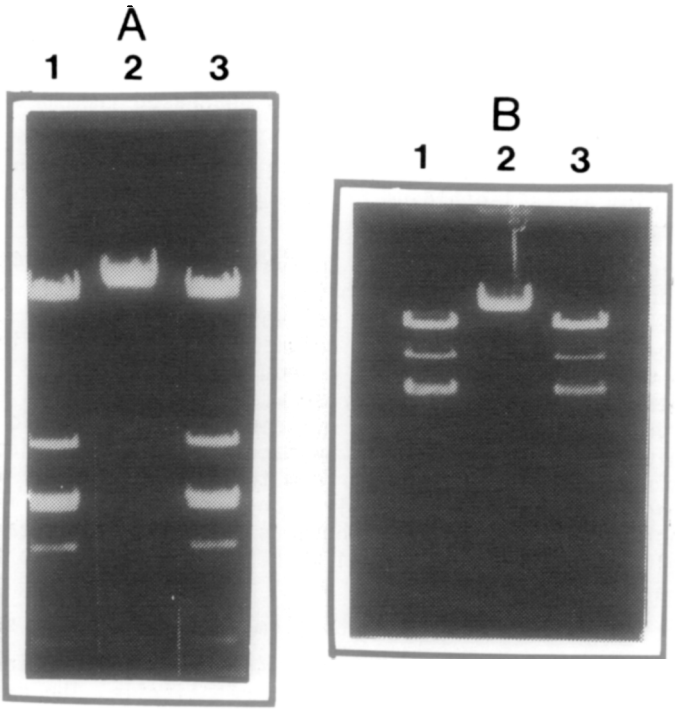

Fig. 1. Ligation/recut assay of *Eco*RI (panel A) and *Sma*I (panel B). Lambda DNA was used as substrate. Lane 1, 1.0 µg lambda DNA cleaved with respective restriction enzyme; lane 2, ligated products (1.0 µg) of immobilized ligase; lane 3, 1.0 µg ligated lambda DNA recut with the same restriction enzyme.

striction enzymes *Eco*RI and *Taq*I to generate fragments with "sticky ends". Blunted ends, i.e. termini without any single stranded overlaps, were obtained by cleaving lambda DNA with *Sma*I. The ligated products obtained with the immobilized ligase after incubation over night at 20^{o} C were subjected to electrophoretic separation on agarose gels. As shown in figure 1, the immobilized ligase could effectively ligate both blunted ends as well as DNAs with sticky ends. In order to check the nucleotide integrity after ligation an aliquot was recut with the same nuclease as previously used (figure 1). No change of the typical cleavage patterns were obtained, indicating that immobilization of T4 DNA ligase does not interfere with the normal catalytic properties of the soluble enzyme.

Coupling efficiency: Immobilization of DNA ligase using tresyl chloride was studied under various conditions. As seen from Table 1, the coupling yield was dependent upon the degree of activation. Coupling efficiency for the alternative CNBr gels with the same degree of activation (i.e. tresyl and cyanate groups, respectively) appeared to be almost identical, i.e. 14-16 %. Linkage between the protein and the matrix is believed to be through amino

Table 1. Coupling efficiency for T4 DNA ligase at different degrees of tresyl chloride activation

Amount of tresyl chloride added in the activation step (μmol/g moist gel)	Amount of tresyl groups formed on the gel after activation (μmol/g moist gel)*	Amount of T4 DNA ligase added (U/g moist gel)	Enzyme activity yield (%)
23	3.3	100	6.3
90	15	100	14.7
180	25	100	9.4
23	3.3	200	7.7
90	15	200	8.7
180	25	200	17.1
90	15	400	13.1
180	25	400	16.5**
230	35	400	11.0
180	25	400 (no ATP added)	5.5

* determined by elemental analysis of sulphur
** as described in Material and Methods

or mercapto groups on the protein. The reactive lysine residue responsible for the ATP binding of the ligase was found to be critical in the immobilization step. If this amino acid residue was not protected through adenylation, the coupling yield was decreased substantially (Table 1).

Stability: T4 DNA ligase in its soluble form is a labile enzyme. At 20° C, i.e. a normal temperature used for DNA ligations, the enzyme loses most of its activity within 24 hrs. As shown in figure 2, stability was greatly improved upon immobilization (tested by the pyrophosphate exchange assay). When stored at 4° C, the immobilized enzyme remained active for at least three months, during which time it was assayed repeatedly for DNA ligation. Immobilization by CNBr was found to give a less stable product having a long-term stability of about one month when stored at 4° C.

Diffusional limitations: Compared with the soluble DNA ligase longer incubation times were necessary for the immobilized enzyme. Dealing with macromolecular nucleic acids, a complete ligation required incubation for 16-18 hrs for the immobilized system while the soluble only needed 1-2 hrs (figure 3). Most likely diffusional hindrance is responsible for the observed differences. However, the reaction rate should not be a critical step in recombinant DNA work as long as the ligation time does not exceed the normally used overnight period.

DISCUSSION

The most commonly used procedure for coupling enzymes to agarose gels involves activation by CNBr. However, the linkages formed are known to be labile and

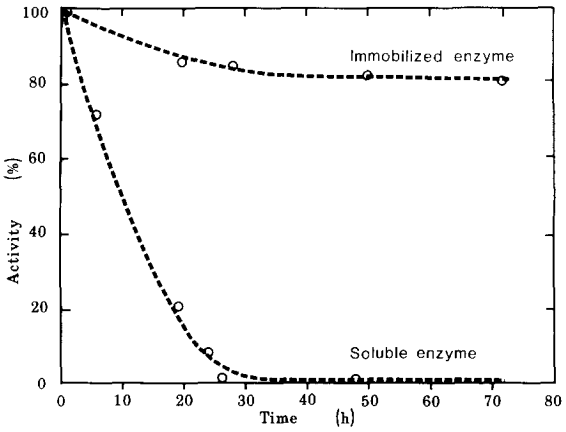

Fig. 2. Stability of soluble and immobilized T4 DNA ligase. 0.5 U of the enzyme was incubated at 20° C in 66 mM Tris-HCl, pH 7.6; 6.6 mM $MgCl_2$; 10 mM DTT. Activities were determined by the pyrophosphate exchange assay.

additional charged groups might be introduced on blocking the activated matrix causing non-specific adsorption (10). As shown for the immobilized T4 DNA ligase, the tresyl chloride activated gels give rise to preparations of increased stability over those of CNBr treated gels.

We have also tested other enzymes that are used in "cloning research", such as alkaline phosphatase for dephosphorylation of DNA and the restriction endo-

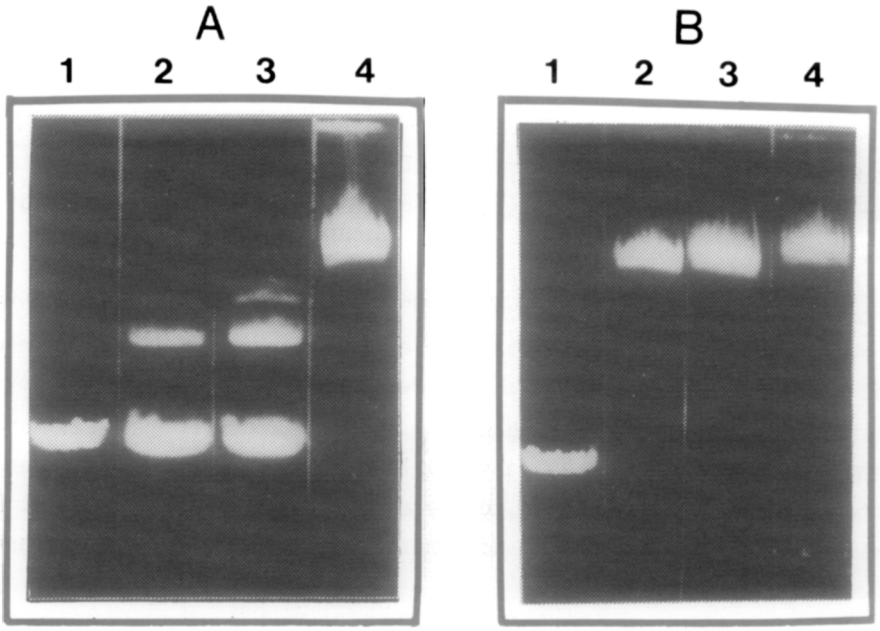

Fig. 3. Comparison of the kinetic properties of immobilized (panel A) and soluble (panel B) DNA ligase. Each reaction mixture contained 5 µg pBR 322 cleaved with *Eco*RI. Aliquots were removed at the following times: lane 1, 0h; lane 2, 1h; lane 3, 2h; lane 4, 16h.

nucleases *Eco*RI and *Taq*I. They all yielded active preparations, however the recovered activity of the restriction enzymes was comparatively low. This was the case both for tresyl chloride as well as for CNBr activated Sepharose (the latter procedure had previously also been applied to the restriction enzymes (11)). This could be explained by the fact that essential lysines are involved in general electrostatic interactions with phosphates in the DNA backbone (12, 13). Apparently, coupling with CNBr or tresyl chloride interferes with critical elements of the active sites of these enzymes. This problem might, however, partially be circumvented by co-immobilizing the enzyme in the presence of a suitable DNA molecule or by immobilizing the enzyme on a matrix with a very low degree of activation.

Immobilized enzymes should greatly simplify work with nucleic acids as the coupled enzymes can easily be removed from the reaction mixtures (no phenol extraction is needed) and thus be reused. Furthermore, when used in enzyme columns in large scale DNA work these reagents have great potential. For instance, oligonucleotides prepared by chemical means could subsequently be ligated on preparative scale using an immobilized DNA ligase column.

Besides the practical advantages offered by immobilizing T4 DNA ligase, we anticipate that this technique will also find application in mechanistic studies of this enzyme. For instance, the actual mechanism of blunt end ligation is still unknown. What we do know is that higher enzyme loading is required to carry out the reaction to completion. It is likely that oligomeric forms of the ligase are involved in the joining of blunted ends as opposed to the involvement of the monomeric form of the enzyme in joining cohesive ends or sealing a nick. Recently, it was found that adenylation of the DNA ligase could affect its oligomerization (14). By immobilizing the ligase at different ATP concentrations affecting the monomer-oligomer ratio, valuable conclusions from the kind of observed activity may be drawn.

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