

ARTICULOS SOBRE TECNICAS/ PAPERS ON TECHNIQUES

CLONING AND EXPRESSION OF GENES CODING FOR *Haemophilus influenzae* Rf RESTRICTION AND MODIFICATION ENZYMES. PURIFICATION OF THE RECOMBINANT ENDONUCLEASE

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SUMMARY

The genes coding for *Hinf* I restriction and modification enzymes were cloned from the *Haemophilus influenzae* Rf strain using pUC18 plasmid as vector. To select the genes from the library, the classical modification phenotype selection was used. When introduced into *Escherichia coli* HB101 strain, the recombinant plasmid pERHinf4 was able to direct the expression of the restriction phenotype. The crude extracts from the transformed *E. coli* contained roughly twice the amount of restrictase expressed by the natural source. A purification procedure for the restrictase is described which when applied to the recombinant enzyme, renders a preparation free of contaminant exonucleases and phosphatases. The overall recovery of the purification procedure was about 33% of the total activity.

RESUMEN

Utilizando el clásico método de selección para el fenotipo de modificación, se aislaron los genes que codifican para la restrictasa y la metilasa *Hinf* I. Se utilizó el plásmido pUC18 para la construcción de la librería genómica, así como para la expresión de estas proteínas en *Escherichia coli*. El clon obtenido a partir del proceso de selección (pERHinf4) mostró expresión de la restrictasa en la cepa de *E. coli* HB101. Los extractos celulares obtenidos a partir de esta cepa transformada mostraron el doble de la cantidad (U/g de biomasa) de restrictasa que usualmente contienen los de la cepa *Haemophilus influenzae* Rf. En este trabajo describimos un procedimiento de purificación de esta restrictasa recombinante expresada en *E. coli*. Este procedimiento permite obtener una preparación libre de fosfatasa y exonucleasas contaminantes, con un recobrado final de enzima equivalente al 33% de la actividad total presente en el extracto.

INTRODUCTION

Several restriction and modification enzymes have been cloned from *Haemophilus sp.* (Wilson, 1988b; Slatko *et al.*, 1988). The purification of the *Hinf*I endonuclease was undertaken from a recombinant source because of the high cost of purification from natural sources. The enzyme yield of the purification procedure from the natural source is approximately 160 U/g of wet cells and the quality of the enzymatic preparation obtained is very variable because of the presence of exonuclease and phosphatase activities.

The molecular cloning of the *Hinf* I restriction and modification enzymes coding genes has already been reported (Chandrasegaran *et al.*, 1988). These authors increased the expression levels in extracts from 150 U/g of wet cells to 300 000 by using a multicopy vector.

The cloning of the *Hinf* I RM (Restriction and Modification) genes based on selection for the modification phenotype (Lunnen *et al.*, 1988; Wilson, 1988a; Chandrasegaran *et al.*, 1988) is reported here. The restrictase was expressed in *E. coli* HB101 strain at a level of 50 000 U/g of wet cells. A purification procedure is described for the recombinant enzyme which allows an overall recovery of 33%. The enzyme preparations obtained from this protocol were free of spurious phosphatase and exonuclease activities.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The *Haemophilus influenzae* Rf strain was obtained from the C.I.G.B. collection (No.49). It was grown in an orbital shaker, using a growth medium containing, per liter, 10 g of casein hydrolyzate, 5 g of yeast extract, 4.4 g of K_2HPO_4 , 2 g of NaCl, 2 g of dextrose, 2 mg of NAD and 10 mg of haemin.

The *E. coli* strain HB101 [F^- , Δ (*gpt-proA*)62 *leu supE44*, *ara14 gal K2 lacY1* Δ (*mcrC-mrr*) *rpsL20* (*Str*^r) *xyI-5 mtl-1 recA13*] (Maniatis *et al.*, 1982) transformed with the plasmid pERHinf4 (described at Results and Discussion section) was grown in an LBA medium containing, per liter, 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and 50 μ g/mL of ampicillin. Cells were harvested when $OD_{600} = 0.8$ by centrifugation at 4 000 x g for 30 min at 4°C, and stored at -20°C.

Enzymes and reagents

Restriction and modification enzymes were obtained from HeberBiotec SA (Havana, Cuba). Calf intestinal phosphatase (CIP) was obtained from Boehringer Mannheim (Germany). Enzymes were used according to the manufacturer's recommendations.

All reagents used were of analytical grade quality.

Construction of a *Haemophilus influenzae* Rf genomic library in pUC18

High molecular weight DNA was isolated from *Haemophilus influenzae* Rf (Caserta *et al.*, 1987) and cleaved completely with *Pst* I endonuclease. The region between 2.3 and 4.3 kb was purified from agarose gels and ligated into a pUC18 vector prepared by digestion with *Pst* I and alkaline phosphatase treatment.

After transformation into *E. coli* HB101, each plate was flooded with an LBA medium and the colonies were scraped together and pooled. Cells were harvested and plasmids were extracted by the alkaline-SDS procedure and further purified by precipitation with polyethylene glycol (Maniatis *et al.*, 1982).

Selection for the modification phenotype

Plasmid DNA from the library (4 μ g) was digested to completion at 37°C for 1 hour with 10 units of *Hinf* I endonuclease and *E. coli* HB101 was transformed with the product of the digestion. A new pool of plates was done and their plasmids were purified and digested again with *Hinf* I endonuclease as explained before. Surviving colonies were individually picked up and cultured overnight in 5 mL of LBA medium. Cells were harvested and plasmids were purified by the miniprep procedure (Maniatis *et al.*, 1982). Plasmids were analyzed for resistance to *Hinf* I cleavage and for the presence of insert.

Hinf I restriction endonuclease assay

Escherichia coli cultures harboring the recombinant plasmids were grown overnight in 300 mL of LBA medium. Cellular extracts were prepared by sonication, and incubated with 1 μ g of λ DNA in the presence of 50 mM NaCl, 10 mM TrisHCl pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol for 1 hour at 37°C in a final volume of 50 μ L. One unit of activity was defined as the amount of enzyme required to cleave the substrate to completion under the conditions described. The products of the digestion with different amounts of extract were analyzed by ethidium bromide staining of agarose gels.

Protein determinations

Protein concentrations were determined as described by Bradford (Bradford, 1976). The method is based on the interaction between the Commassie blue reagent and the peptidic bridge of the proteins.

Purification of recombinant *Hinf* I endonuclease from *E. coli* HB101 transformed cells

A kinetic study was performed to determine the optimal O.D. for harvesting the *E. coli* cultures containing the pERHinf4, concerning the production of the restrictase. After harvesting the cells by cen-

trifugation at 4°C, the following procedure was performed. The cell paste (6 g wet weight) was suspended in 12 mL of the PC buffer (10 mM KPO_4 , 10 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 7) with 0.5 mM PMSF, 1 mM benzamide and disrupted in the French press. The lysate was clarified by centrifugation at 27 000 x g for 2 h at 4°C. The supernatant was precipitated using ammonium sulfate (70%) and centrifuged at 12 000 x g for 10 min at 4°C. The pellet was suspended and dialyzed for 12 h against the PC buffer with 150 mM NaCl. The extract was loaded onto a Phosphocellulose P-11 (Whatman, 2.6 x 40 cm column) with a bed volume of 100 mL, previously equilibrated with the PC buffer containing 150 mM NaCl, at a flow rate of 25 mL/h. The column was washed with 1 L of the PC buffer with 150 mM NaCl and eluted with a linear gradient of NaCl (0.15-0.8 M). The *Hinf* I activity was eluted between 0.3 and 0.49 M NaCl. Pooled fractions were dialyzed against the PC buffer for 4 h and then loaded onto a Heparin-Sepharose column (Pharmacia, 1 x 10 cm) with a bed volume of 10 mL, previously equilibrated with the PC buffer, at a flow rate of 20 mL/h. The column was washed with 100 mL of the PC buffer and eluted with a linear gradient of NaCl (0-0.8 M). The *Hinf* I activity was eluted between 0.25 and 0.35 M NaCl. Fractions were pooled, concentrated with polyethylene glycol (Mw. 20 000) and dialyzed against the concentration buffer (50 mM KCl, 10 mM TrisHCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, pH 7.4). This preparation was stored at -20°C.

Phosphatase and exonuclease assays

The presence of phosphatase and exonuclease contaminating activity in the final preparation of recombinant and natural *Hinf* I endonuclease was performed, as previously described (González, 1990).

RESULTS AND DISCUSSION

Isolation of clones expressing the *Hinf* I R⁺M⁺ phenotype

Approximately 10 000 recombinant colonies from the *Pst* I library were screened for the modification phenotype. When plasmid DNA from the library was overdigested with *Hinf* Y and transformed into *E. coli*, a population of resistant colonies was obtained. After a second round of overdigestion and transformation, a few hundred resistant colonies were obtained. One of the recombinant plasmids, the pERHinf4, contained the 3.9 kb insert (figure 1 and 2) corresponding to the chromosomal fragment which harbors the methylase and restrictase genes (Chandrasegaran *et al.*, 1988).

This plasmid was selected to assay the expression of *Hinf* I endonuclease activity. The extract prepared from 300 mL overnight cultures was found to contain detectable *Hinf* I endonuclease activity (figure 3).

Expression levels of the *Hinf* I recombinant endonuclease in *E. coli*

The extract prepared from 300 mL of transformed *E. coli* cultures expressed at least 10 000 U/g of wet cells (figure 3). When the culture was stopped at the optimal O.D. calculated from the kinetic study, a yield of about 50 000 U/g of wet cells was obtained (figure 4).

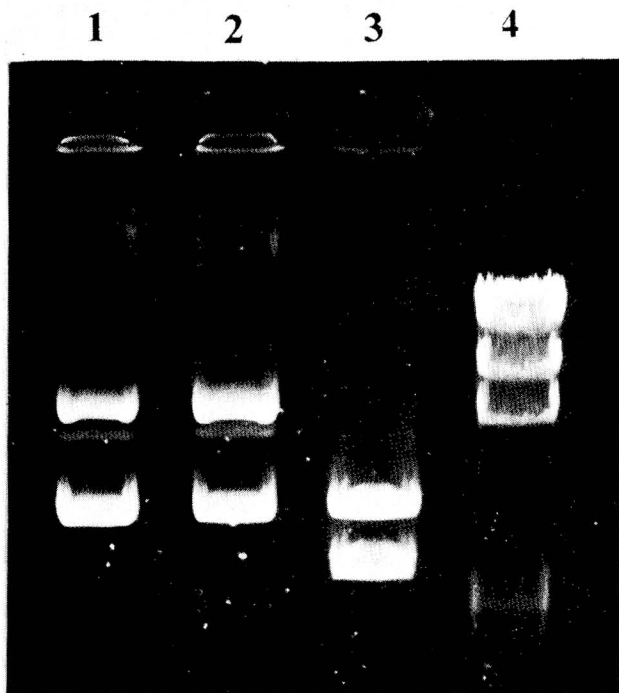


Fig. 1. *Hinf I* and *Pst I* digestion of the plasmid pERHinf4. Lane 1: undigested plasmid; lane 2: plasmid digested with 10 U of *Hinf I*; lane 3: plasmid digested with 5 U of *Pst I*; lane 4: lambda DNA digested with *Hind III* as molecular weight markers.

This time point corresponded to 6.5 h. At longer time points, the enzymatic activity was masked by the presence of non-specific nuclease activities.

This level of expression was much higher than the one obtained when crude extracts from the natural source were tested. In our hands, the crude extracts from the natural source never showed levels of activity above 27 000 U/g of wet cells. However, this recombinant strain

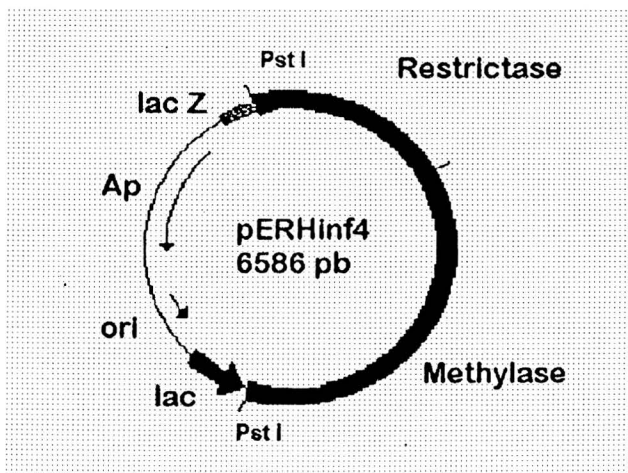


Fig. 2. Map of plasmid pERHinf4. Ap:β-lactamase gene; lac: lac promoter; ori: ColE1 origin of replication.

showed levels of expression about six times lower than the one described by other authors (Chandrasegaran *et al.*, 1988) using the same vector. This difference might be attributed to the fact that they employed a different strain to measure the expression (*E. coli* MM294).

Purification of recombinant *Hinf I* restrictase from *E. coli*

To the author's knowledge, there is no reported procedure for the purification of the *Hinf I* restrictase. Here is shown a purification procedure which allows the obtainment of an enzymatic preparation free of exonuclease and phosphatases activities. Table 1 shows the results of the different purification steps when the recombinant restrictase was purified from *E. coli* extracts. The final specific activity achieved was always around 49 000 U/mg. The overall yield was about 19 000 U/g of wet cells, as opposed to the very low yield (160 U/g) obtained when the restrictase was purified from *H. influenzae* extracts using the procedure described in Materials and Methods for the recombinant enzyme.

The purification of the recombinant enzyme allowed a much cleaner final preparation, without the large amounts of phosphatases and exonucleases usually present at the end of the procedure when natural extracts where employed. The use of this modified *E. coli* as a *Hinf I* source is very convenient taking into consideration, 1) the very tricky culture conditions required by the *H. influenzae*, 2) the higher levels of expression, and 3) the quality of the final preparation, which was devoid of undesirable ac-

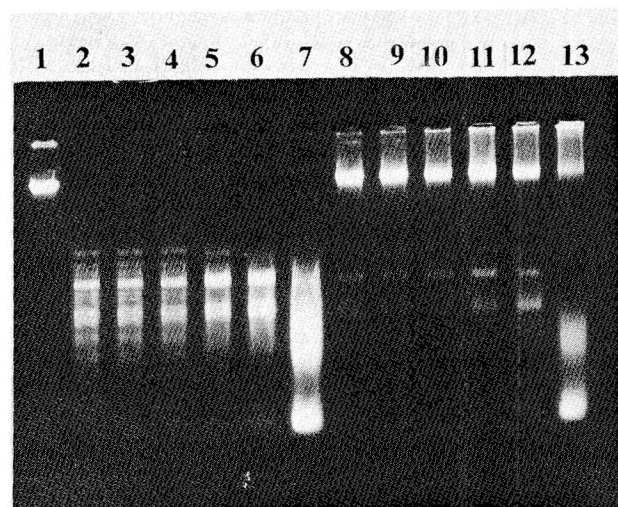


Fig. 3. Recombinant *Hinf I* restrictase activity assay in crude extracts of *E. coli* transformed either with pERHinf4 (lanes 2 to 7) or with pUC18 (lanes 8 to 13). Lane 1: undigested λDNA. Amount of extract added: lanes 2 and 8: 0.2 μL; lanes 3 and 9: 0.25 μL; lanes 4 and 10: 0.33 μL; lanes 5 and 11: 0.5 μL; lanes 6 and 12: 1 μL; and lanes 7 and 13: 2 μL.

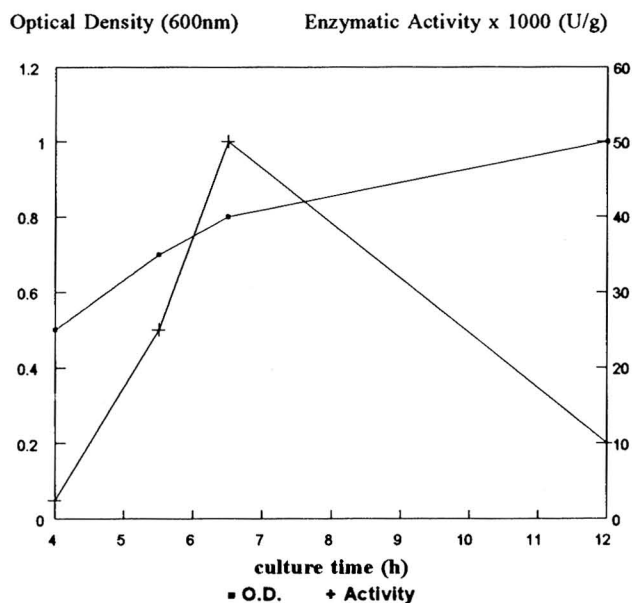


Fig. 4. Kinetics of Recombinant *Hinf* I restrictase production as a function of the optical density of the culture.

Table 1
Summary of the recombinant *Hinf* I restrictase purification procedure.

Fraction	Total activity [U]	Total protein [mg]	Specific activity [U/mg]*	Yield activity [%]**	Purif. (fold) ***
Crude extract	300 000	424	706±177	100	1
P-11	112 000	3.3	33 939±2 785	37±6	48±15
Heparin Sepharose	110 000	2.2	4 9107±1 5783	36±12	69±9

Values shown are the average of three different purification processes ± S.D.

(*) Referred as the ratio total enzymatic activity/total protein.

(**) Referred as percentage of remaining total enzymatic activity compared with the initial amount in the crude extract.

(***) Purification fold calculated as the increase in specific activity compared with the crude extract.

activities. This preparation is suitable for molecular biology applications, as demonstrated by its continuous use in our laboratory.

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