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Affinity partitioning of restriction endonucleases

Application to the purification of EcoR I and EcoR V

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ABSTRACT

Partitioning of restriction endonucleases between two liquid aqueous phases can be strongly influenced by group-specific ligands included in the two-phase system. Three restriction endonucleases, namely EcoR I, EcoR V and BamH I, were partitioned within an aqueous dextran–polyethylene glycol (PEG) system. The enzymes could be extracted into the upper PEG phase by using either triazine dyes or herring DNA as affinity ligands. The influence of the endogenous bacterial nucleic acids, concentration of polymer-bound dye and concentration of sodium chloride on the system were examined. A partial purification of EcoR I (up to 52-fold) and EcoR V (up to 37-fold) was achieved using a combination of affinity partitioning and ion-exchange chromatography, providing an extremely fast and economical method for the isolation of restriction endonucleases free from contaminating nuclease activities.

INTRODUCTION

Site-specific restriction endonucleases are widely used in the analysis of DNA molecules. The ability to cleave DNA at specific sequences is the fundamental technology responsible for the rapid development of genetic engineering. Today, more than 1000 restriction endonucleases have been identified from different bacterial sources [1].

Although several procedures have been published regarding their purification, most of them involve lengthy protocols, including precipitation with salts [2] followed by column chromatography on different media, such as phosphocellulose [2,3], hydroxylapatite [4], DEAE-cellulose [3,5], DNA–cellulose [2], Cibracron Blue F3G-A–agarose [6,7], heparin–agarose [8,9] and/or gel filtration [10].

However, recently a rapid two-step chromatographic procedure for the isolation of these enzymes [11] and an inexpensive scheme involving triazine dye adsorbents [12] have been reported.

Aqueous two-phase systems have been used for the purification of a vast number of biological macromolecules such as proteins, enzymes and nucleic acids [13]. These

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systems are obtained by dissolving two polymers in water at appropriate concentrations, *e.g.*, dextran and polyethylene glycol (PEG) [13,14]. The partitioning of proteins depends on a number of parameters, including concentration and molecular weight of the polymers, salt and buffer added to the system, pH and temperature [15]. Further, the partitioning of enzymes and other proteins can be selectively influenced by covalently binding of affinity ligands to one of the phase-forming polymers. This method, called affinity partitioning [16–18], has been widely used for the effective extraction of enzymes and for ligand–protein binding studies [19]. The most popular ligands in affinity partitioning are triazine dyes, which have been used for the affinity extraction of dehydrogenases, kinases and a number of other enzymes and proteins [20–23].

As we have demonstrated in previous work [12], various triazine dyes interact with restriction endonucleases and dye-ligand chromatography can be effectively used for the purification of these enzymes using three-step procedures.

This work was carried out to study the behaviour of restriction endonucleases in aqueous two-phase systems in the presence of dye ligands bound to PEG and to develop procedures which are economical and faster than those published previously. The influence of added non-specific DNA in the system was also examined.

EXPERIMENTAL

Materials

Yeast extract, tryptone and brain heart infusion were obtained from Difco (Detroit, MI, U.S.A.) and dextran T-500 (mol. wt. 500 000) and prepacked MonoS HR 5/5 (5 × 50 mm) cation-exchange high-performance liquid chromatographic (HPLC) column from Pharmacia (Uppsala, Sweden). DNA from herring testes, polyethyleneimine (PEI) and Nonidet P-40 (NP-40) were purchased from Sigma (St. Louis, MO, U.S.A.), agarose for gel electrophoresis and λDNA from Bethesda Research Labs. (Bethesda, MD, U.S.A.), polyethylene glycol PEG 6000 (mol. wt. 6000–7500) from Serva (Heidelberg, F.R.G.), DEAE-cellulose (DE52) from Whatman (Maidstone, Kent, U.K.) and the Procion dyes Blue MX-4GD and Navy H-ER (ICI, Manchester, U.K.) were provided by Dr. Y. D. Clonis (University of Patras, Greece). All other chemicals were of analytical-reagent grade.

Two-phase systems

Phase systems were prepared from stock solutions of the polymers in water. The PEG 6000 solution was 40% (w/w) and the dextran T-500 20% (w/w). The polymer solutions were weighed and mixed with salt, buffer, water and sample to the final weight, usually 4 g. All partitioning experiments were carried out at 4°C. Triazine dye derivatives of PEG were prepared according to Johansson [15]. The partition coefficient, K, is defined as the ratio of the concentrations of the component in the upper and lower phases. Enzyme concentrations were determined by measuring the activity expressed in units/μl. The concentration of herring DNA was determined photometrically at 260 nm (an absorbance of 1 corresponds to 50 μg/ml).

Crude extract preparation

Escherichia coli RY 13 (EcoR I) and *Escherichia coli* J62 pLG74 (EcoR V) were

grown at 37°C until the late log phase on a medium containing 5 g/l of yeast extract, 10 g/l of tryptone and 5 g/l of NaCl. *Bacillus amyloliquefaciens* (BamH I) was grown at 37°C until the late log phase on a medium containing 37 g/l brain heart infusion. All cell types after harvesting by centrifugation were dissolved in 20 m*M* Tris–HCl buffer (pH 7.4). The cells were disrupted with a French Press (SLM Instruments) and centrifuged at 4°C for 1 h at 100 000 *g*. With *Escherichia coli* RY 13 (EcoR I), the clarified crude extract was dialysed against buffer A [20 m*M* Tris–HCl (pH 7.4)–40% (v/v) glycerol] and stored at −20°C. With *Escherichia coli* J62 pLG74 (EcoR V), the clarified crude extract was treated with PEI in order to remove nucleic acids by precipitation [24]. Solid ammonium sulphate was added to the PEI supernatant to a final concentration of 75% of saturation. After centrifugation, the precipitate was dissolved in 20 m*M* Tris–HCl buffer (pH 7.4), dialysed against buffer A and stored at −20°C. In the case of *Bacillus amyloliquefaciens* (BamH I), the clarified crude extract was divided into two parts. One part was dialysed against buffer A and stored at −20°C. The other part, as with the EcoR V extract, was treated with PEI, precipitated with ammonium sulphate (75% of saturation), dialysed against buffer A and stored at −20°C.

Enzyme assays

Routine assays for locating EcoR I or EcoR V endonuclease activity during the chromatographic runs were performed as described elsewhere [12]. One unit of enzyme activity is defined as the amount of enzyme required to produce a complete digest of 1 μg of λDNA at 37°C for 1 h in a total volume of 50 μl. The purity of the final enzyme preparations with respect to non-specific nucleases was evaluated by the "overdigestion" and "cut–ligate–recut" tests [12]. Agarose gel electrophoresis of DNA fragments was performed as described earlier [12]. Protein determination was performed by the Bradford method using Coomassie Brilliant Blue G and bovine serum albumin as protein standard [25].

RESULTS AND DISCUSSION

Effect of sodium chloride

It is well known that the partitioning of DNA is strongly dependent on the salt composition of the system [26]. Therefore, the effect of NaCl on the partitioning of the restriction enzymes was studied in relation to the presence or absence of endogenous DNA.

A crude sample containing EcoR V was treated with PEI in order to remove nucleic acids whereas that containing EcoR I was not. Crude samples containing BamH I (treated and untreated with PEI) were also used.

Without the addition of NaCl, more than 90% of the enzyme EcoR V was in the lower phase, whereas 95% of the enzyme EcoR I was found in the upper phase. When the concentration of NaCl in the system is raised from 0 to 150 m*M*, the partitioning of EcoR V was affected only slightly, whereas the partitioning of EcoR I was changed dramatically and 95% of the enzyme was transferred into the lower phase (Fig. 1A).

Similar results were obtained with BamH I (Fig. 1B). Working with extract that had been treated with PEI (as with EcoR V), the partitioning of the enzyme was not affected by NaCl, whereas with the untreated extract the behaviour of the enzyme was similar to that of EcoR I.

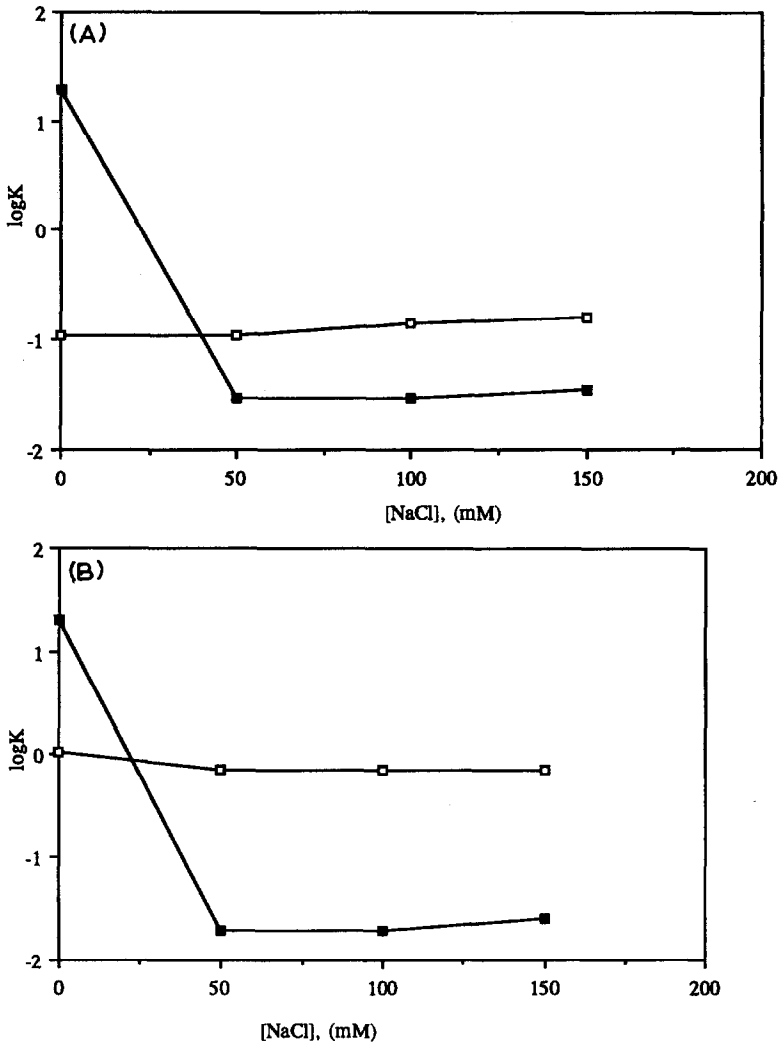

Fig. 1. Effect of NaCl on the partitioning of restriction endonucleases. System: 7.5% (w/w) dextran, 5.3% (w/w) PEG, 25 m*M* lithium phosphate buffer (pH 6.8) and increasing concentrations of NaCl. Temperature, 4°C. (A) Partitioning of the enzymes (□) EcoR V and (■) EcoR I. The crude sample containing EcoR V was treated with PEI in order to remove nucleic acids by precipitation. (B) Partitioning of the enzyme BamH I. Two crude samples were used, (□) after PEI treatment and (■) without PEI treatment.

From these results, we suggest that in the absence of endogenous DNA, NaCl has no effect on the partitioning of the restriction enzymes examined. However, in the presence of endogenous DNA, NaCl has an indirect effect on the partitioning, probably owing to the interaction of restriction enzymes with DNA.

In order to follow the partitioning of bacterial DNA, the phases of the systems were analysed by agarose gel electrophoresis. As expected, in systems without NaCl the DNA was detected in the upper phase, whereas in systems with NaCl it was detected in the lower phase (data not shown).

Effect of dye–PEG derivatives

A triazine dye, Procion Blue MX-4GD, which is known to interact with the restriction enzymes EcoR V and EcoR I [12], was coupled to PEG and further studied in affinity partitioning experiments. The dye–PEG was found to be effective in extracting the enzyme EcoR V into the PEG-rich phase with a maximum increase of 2 units in $\Delta \log K$, *i.e.*, the difference in the logarithm of the K values with and without dye-liganded polymer (Fig. 2). However, with EcoR I, the dye–PEG was not able to extract the enzyme into the upper phase. Even when 7% dye–PEG (of total PEG) was included in the system, 70% of the enzyme was still in the lower phase, whereas with 3% dye–PEG (of total PEG) less than 5% of EcoR V was found in the lower phase (Fig. 3A).

The influence of another PEG-bound dye, Procion Navy H-ER, on the partitioning of the enzyme BamH I is shown in Fig. 3B. Similarly the dye–PEG was able to extract BamH I into the upper phase only if the nucleic acids had been removed from the crude extract. However, with EcoR I, at higher NaCl concentrations the dye–PEG can more effectively extract the enzyme into the upper phase (Fig. 4), suggesting that there is a salt-dependent competition between the dye and bacterial DNA for binding to restriction endonucleases.

Effect of non-specific DNA

All the sequence-specific DNA-binding proteins exhibit a non-specific binding to any DNA. Further, immobilized non-specific DNA (calf thymus DNA, salmon sperm DNA, etc.) has been used for the isolation of several restriction endonucleases [2]. In an effort to study the affinity partitioning of restriction endonucleases in aqueous two-phase systems, we used as a ligand non-specific DNA (herring testes DNA).

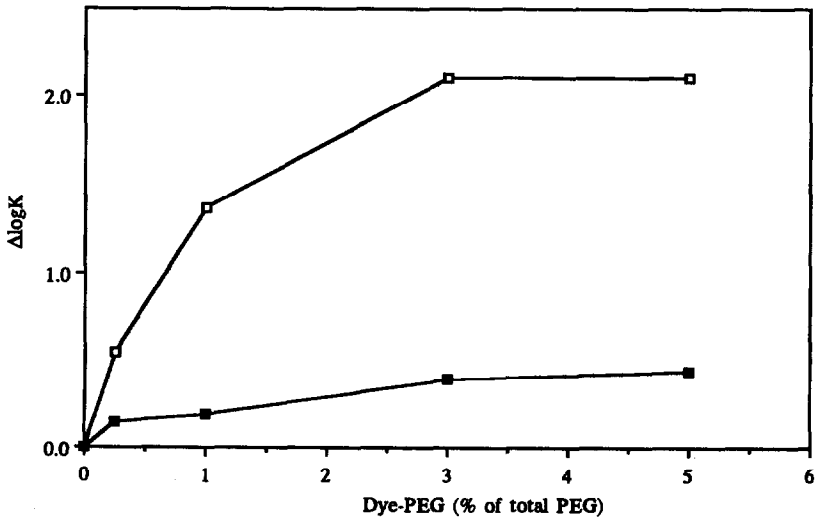

Fig. 2. Change in the partition coefficient of the enzyme EcoR V as a function of PEG-bound Procion Blue MX-4GD concentration. (■) Protein; (□) EcoR V. System: 7.5% (w/w) dextran, 5.3% (w/w) PEG, 25 mM lithium phosphate buffer (pH 6.8). Temperature, 4°C.

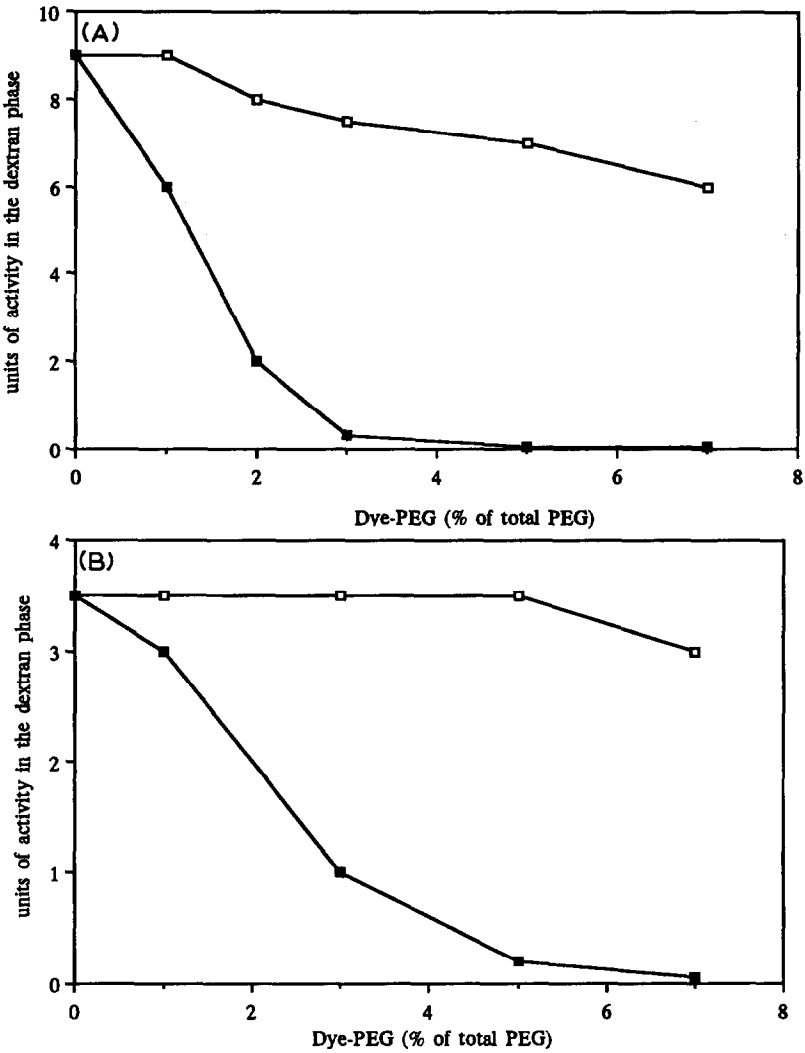

Fig. 3. Effect of dye–PEG derivatives on the partitioning of restriction endonucleases. The same system was incorporated as in Fig. 2, containing 50 m*M* NaCl. Temperature, 4°C. (A) Activity of the enzymes (■) EcoR V and (□) EcoR I remaining in the dextran phase after the addition of increasing amounts of PEG-bound Procion Blue MX-4GD. The crude sample containing EcoR V was treated with PEI. (B) Activity of the enzyme BamH I remaining in the dextran phase after the addition of increasing amounts of PEG–bound Procion Navy H-ER. Crude sample (■) after PEI treatment and (□) without PEI treatment.

As expected, the partitioning of herring DNA is influenced by the salt composition of the system. The DNA is totally transferred into the lower phase when 100 m*M* NaCl is included in the system, as shown in Fig. 5.

In order to examine the influence of non-specific DNA on the partitioning of restriction endonucleases, increasing amounts of herring DNA were added to a two-phase system containing a crude sample of EcoR V (treated with PEI). In Fig.

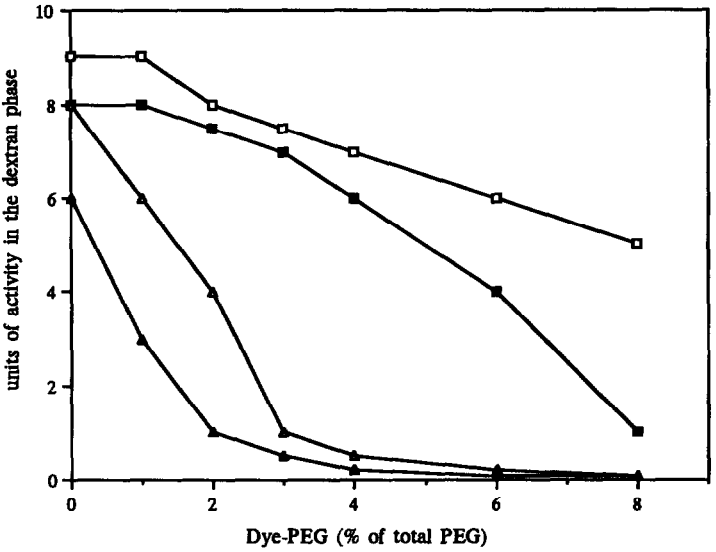

Fig. 4. Activity of the enzyme EcoR I remaining in the dextran phase after the addition of increasing amounts of PEG-bound Procion Blue MX-4GD. The experiment was performed at four different NaCl concentrations: (□) 50; (■) 100; (△) 200; (▲) 300 m*M*. The same system was incorporated as in Fig. 2. Temperature, 4°C.

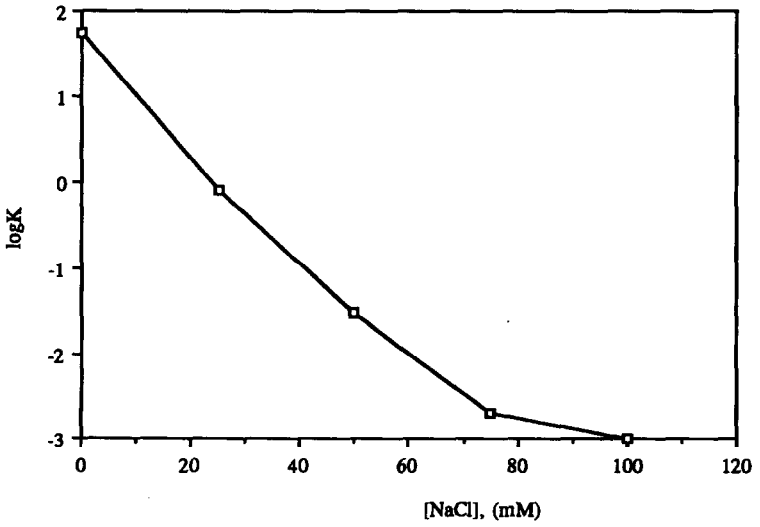

Fig. 5. Effect of NaCl on the partitioning of herring testes DNA. The same system was incorporated as in Fig. 2. Temperature, 4°C.

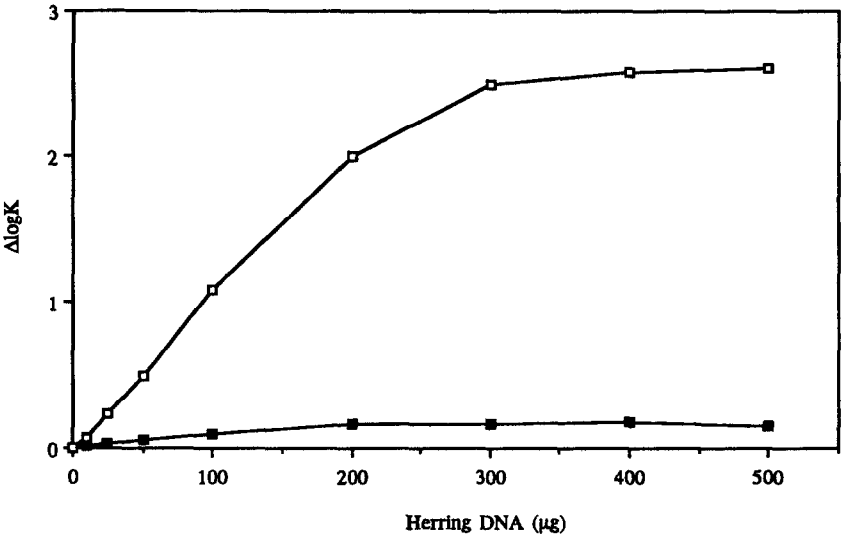

Fig. 6. Change in the partition coefficient of the enzyme EcoR V as a function of increasing concentration of herring testes DNA. (■) Protein; (□) EcoR V. The same system was incorporated as in Fig. 2. Temperature, 4°C.

6 it is shown that herring DNA not bound to PEG is able to extract the enzyme EcoR V into the PEG-rich phase with a maximum increase in $\Delta \log K$ of 2.5 units.

The affinity extraction of the enzyme EcoR V using non-specific DNA as an affinity ligand is illustrated in Fig. 7. The enzyme binds to herring DNA and follows its partitioning within the extraction steps. Using this simple scheme the enzyme can be extracted into the lower phase, in less than 1 h, with a 92% recovery and 4.7-fold purification.

Purification of EcoR I

Restriction endonuclease EcoR I was purified from an extract of *Escherichia coli* RY 13 cells prepared as described under Experimental. Crude extract (0.5 ml; 5.2 mg of total protein; 40 000 units) was included in the initial two-phase system (No. 0). The composition of the system (4 gram) was 7.5% (w/w) dextran, 5.3% (w/w) PEG, 25 m*M*

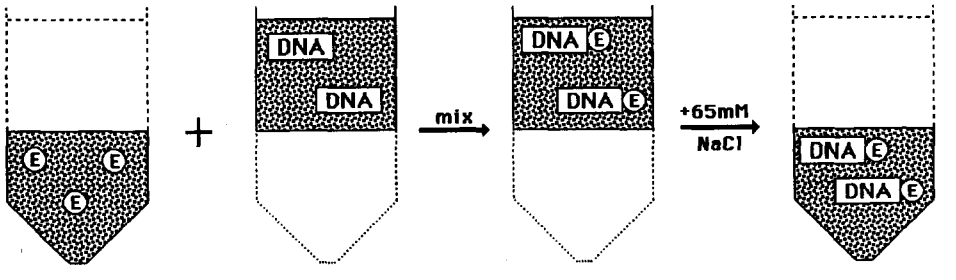

Fig. 7. Scheme for the affinity extraction of the enzyme EcoR V using non-specific DNA as an affinity ligand. The same system was incorporated as in Fig. 2. Temperature, 4°C.

lithium phosphate buffer (pH 6.8) and 50 m*M* sodium chloride. The partitioning was carried out at 4°C using gentle mixing for 30 s and centrifugation for 3–5 min at low speed (1000 *g*). Results from this experiment and from the following partitioning steps are given in Table I. The lower phase of systen 0, containing 90% of the enzyme, was washed by equilibration with pure upper phase of the same composition as above but containing 2% Procion Blue MX-4GD-PEG (of the total PEG amount), giving system No. 1. During the washing step, some dye-binding proteins were extracted into the upper phase, leaving 81% of the target enzyme in the lower phase. As shown in Fig. 4, the enzyme at low concentration of NaCl is not extracted by the dye into the upper phase. Subsequently, the upper phase of system 1 was replaced with a pure upper phase containing Procion Blue MX-4GD-PEG (6% of the total amount of PEG) and 490 m*M* NaCl, yielding system No. 2 (with a final concentration of 300 m*M* NaCl). In system No. 2 the PEG-bound triazine dye was now able to extract the enzyme in the upper phase with an 83% recovery and 4.3-fold purification.

The upper phase of system No. 2 (2.2 ml) was diluted with 10 ml of 20 m*M* Tris–HCl buffer (pH 7.4) containing 10% (v/v) glycerol. The solution was loaded onto a DEAE-cellulose column (8 ml) previously equilibrated in dilution buffer. The column was washed with buffer (40 ml) followed by a linear gradient of 0–0.8 *M* NaCl (80 ml) in dilution buffer. Fractions with EcoR I activity corresponding to *ca* 0.2 *M* NaCl in the gradient were pooled and stored at 4°C.

The final enzyme preparation was subjected to the "overdigestion" test and up to 100 U EcoR I did not produce any non-specific cleavage products after 6 h incubation with 1 μg of λDNA at 37°C, *i.e.*, after 600-fold overdigestion. In the "cut–ligate–recut" test, after more than 10-fold overdigestion of λDNA with EcoR I more than 90% of the DNA fragments could be ligated and recut with this enzyme.

Several purification methods related to the isolation of restriction endonuclease EcoR I have been reported previously [2,4,27]. However, all the procedures involve two or three chromatographic steps and 3–4 days are required for the completion of the preparation.

The purification method presented here is a rapid two-step procedure involving affinity partitioning in aqueous two-phase systems and ion-exchange chromatography with DEAE-cellulose. The whole preparation was completed within 1 day and with 54% recovery. The resulting enzyme preparation was purified 52-fold free from contaminating nuclease activities and is therefore suitable for commercial exploitation.

Purification of EcoR V

Restriction endonuclease EcoR V was purified from an extract of *Escherichia coli* J62 pLG74 cells prepared as described under Experimental. Crude extract (1 ml; 15 mg of total protein; 70 000 units) was included in the initial two-phase system (No. 0). The composition of the system (8 gram) was 7.5% (w/w) dextran, 5.3% (w/w) PEG and 25 m*M* lithium phosphate buffer (pH 6.8). The partitioning was carried out at 4°C using gentle mixing for 30 s and centrifugation for 3–5 min at low speed (1000 *g*). Results from this experiment and from the following partitioning steps are given in Table II. The lower phase of system 0, containing 86% of the enzyme, was equilibrated with pure upper phase [of the same composition as above but containing 2.5% Procion Blue MX-4GD-PEG (of the total PEG amount)], giving system No. 1. To the upper

TABLE I

PURIFICATION SCHEME FOR THE ISOLATION OF EcoR I USING THE DYE PROCION BLUE MX-4GD AS PEG-BOUND AFFINITY LIGAND

Purification step	Total protein (mg)	Total activity (units)	Purification factor	Yield (%)
Crude extract	5.2	40 000	1	100
Affinity partitioning				
System 0, lower "phase"	3.02	36 000	1.55	90
System 1, lower "phase"	1.8	32 400	2.34	81
System 2, upper "phase"	0.35	27 000	10	67.5
DE-52 column	0.054	21 600	52	54

phase of system 1 (in which the dye–PEG extracts the target enzyme), 0.5 g of a solid mixture of K_2HPO_4 and $NaH_2PO_4 \cdot 2H_2O$ (1:1.3, w/w) was added, giving a PEG–salt two-phase system (No. 2). The enzyme was recovered into the lower (salt-rich) phase and dialysed against 20 m*M* Tris–HCl buffer (pH 7.4) containing 7 m*M* 2-mercapto-ethanol, 50 m*M* NaCl, 10% (v/v) glycerol and 0.1% (v/v) NP-40 (buffer B). The solution (3 ml) from the latter preparation was applied to a MonoS cation-exchange HPLC adsorbent (50 × 5 mm I.D.; 1 ml) which had been previously equilibrated in buffer B. The column was washed with 4 ml of irrigating buffer and then developed with a linear gradient of NaCl (15 ml total volume; 50–400 m*M*) in buffer B at a flow-rate of 1 ml/min. Fractions with EcoR V activity corresponding to *ca.* 0.15 *M* NaCl in the gradient were pooled and stored at 4°C. The final enzyme preparation was subjected to the "overdigestion" test and up to 100 U of EcoR V did not produce any non-specific cleavage products after incubation for 6 h with 1 μg of λDNA at 37°C, *i.e.*, after 600-fold overdigestion. In the "cut–ligate–recut" test, after more than 10-fold overdigestion of λDNA with EcoR V more than 90% of the DNA fragments could be ligated and recut with this enzyme.

A method for the purification of EcoR V has been described previously [12]. It involves three chromatographic steps based on dye-ligand chromatography and 3–4

TABLE II

PURIFICATION SCHEME FOR THE ISOLATION OF EcoR V USING THE DYE PROCION BLUE MX-4GD AS PEG-BOUND AFFINITY LIGAND

Purification step	Total protein (mg)	Total activity (units)	Purification factor	Yield (%)
Crude extract	15	70 000	1	100
Affinity partitioning				
System 0, lower "phase"	7.2	60 000	1.78	86
System 1, upper "phase"	4.3	55 000	2.74	78
System 2, lower "phase"	3.8	51 000	2.88	73
Mono S column	0.23	40 000	37.3	57

days are required for the completion of the preparation. Similarly to EcoR I, the preparation presented above was completed within 1 day and with 57% recovery. The resulting enzyme preparation was purified 37-fold free from contaminating nuclease activities and is therefore suitable for commercial exploitation.

CONCLUSIONS

Restriction endonucleases can be isolated free from non-specific endonucleases using a combination of affinity partitioning and ion-exchange chromatography. Triazine dyes bound to PEG and free endogenous and herring DNA can be effectively employed as affinity ligands in aqueous two-phase systems. Bacterial DNA affects the partitioning of restriction endonucleases and this property can be exploited for their effective isolation. Owing to the extreme partitioning of DNA, several other DNA-binding proteins could potentially also be effectively extracted.

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