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Dye-Ligand Chromatography for the Resolution and Purification of Restriction Endonucleases

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ABSTRACT

The resolution of restriction endonucleases from the same microorganism is conventionally achieved by lengthy fractionation protocols. We now report effective single-step procedures that exploit dye-ligand chromatography for the resolution and purification of restriction enzymes. After suitable initial screening, we demonstrated that resolution of two restriction activites can be achieved in one chromatographic step, and further purification can subsequently be effected using selected dye-adsorbents. Accordingly, we resolved in one step, Hpa I from Hpa II, Hind II from Hind III, and Sac I from Sac II. Furthermore, a three-step chromatographic procedure has been developed to purify EcoRV suitable for commercial exploitation, as judged by the "overdigestion" and "cut-ligate-recut" quality control tests.

Index Entries: Dye chromatography; affinity chromatography; enzyme purification; restriction endonucleases; Sac I; Sac II; Hpa I; Hpa II; Hind II; Hind III; Sph I; EcoRI; EcoRV.

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INTRODUCTION

The commercial realization of recent advances in recombinant DNA technology ultimately depends to a large extent on the availability and, thus, the purification technology of enzymes and proteins. In this context, restriction endonucleases (*1,2*) play a major role in genetic engineering and, consequently, a great deal of attention has been paid toward their purification technology. Fractionation protocols incorporate, usually, precipitation with salts (*3*), followed by a combination of column chromatographic steps involving media such as phosphocellulose (*3–6*), hydroxylapatite (*3,5*), DEAE-cellulose (*4,7*), DNA-cellulose (*3*), Cibacron Blue F3G-A-agarose (*8–10*), heparine-agarose (*11,12*), and/or gel filtration (*10*). More recently, an expensive but highly effective approach has been adopted (*6*) whereby a conventional adsorbent and an HPLC-ion exchanger are combined to allow rapid purification of restriction endonucleases in two steps. Since immobilized dye-ligands are inexpensive chromatographic media and have been shown to specifically interact with a wide range of nucleotide-binding enzymes (*13–19*), we have investigated the applicability of dye-ligand chromatography as a means of fractionating restriction endonucleases both economically and effectively. Accordingly sixteen reactive dyes were immobilized onto agarose and the resultant adsorbents were screened for their ability to both purify and resolve restriction enzymes from bacterial extracts. The most promising dyed adsorbents can, subsequently, be combined in order to construct fractionation protocols that allow effective purification of restriction endonucleases, for example, as we have demonstrated for EcoRV. Such procedures, which employ inexpensive and facile-to-prepare adsorbents, are particularly useful when enzymes are required in some quantity. The endonucleases involved in this study were, for the resolution experiments, Sac I and II, Hpa I and II, and Hind II and III, whereas those for the purification procedures were EcoRV, Sph I, and EcoRI.

MATERIALS AND METHODS

Materials

Yeast extract and tryptone were obtained from Difco (Detroit, MI), bovine serum albunim from Miles (USA), and glycerol from Merck (Darmstadt, FRG). Agarose for gel electrophoresis, λ DNA, and λ Hind III were from Bethesda Research Laboratories (Bethesda, MD), Sepharose-4B was from Pharmacia (Uppsala, Sweden), whereas the triazine dyes (Procion range, ICI, Manchester, UK) were provided by Y. D. Clonis (University of Cambridge, UK).

Growth of Cells

Escherichia coli RY 13 (EcoRI) and *Echerichia coli* J62 pL G74 (EcoRV) were grown at 37°C, *Streptomyces phaeochromogenes* (Sph I) and *Streptomyces achromogenes* (Sac I, II) were grown at 30°C; these cell types were grown until late log phase on a medium containing (%, w/v): yeast extract, 0.5; tryptone, 1.0; and NaCl, .5. *Haemophilus influenzae* Rd exo- (Hind II, III) and *Haemophilus parainfluenzae* (Hpa I, II) were grown at 37°C until late log phase on a medium containing: brain heart infusion, 37 g/L; NAD, 2 mg/L; and hemin, 10 mg/L. All cell types after harvesting by centrifugation were stored −70°C.

Preparation of Gel for Agarose Electrophoresis of DNA Fragments

Agarose (2.5 g) was suspended in a 250 mL final volume of a solution containing 225 mL H_2O and 25 mL of the following buffer: .5*M* Tris-HCl, pH 8.0, .02*M* EDTA, and .2*M* sodium acetate. The suspension was heated until boiling under gentle shaking and was then cooled to approximately 60°C before adding 30 μL ethidium bromide (5 mg/mL H_2O). The warm agarose solution was poured onto an appropriate glass plate, left for 1 h at room temperature to set, and stored in the above buffer diluted 10 times.

Enzyme Assays

Routine assays for locating endonuclease activity during the chromatographic runs were performed as follows: into an Eppendorf plastic tube were pipetted 2 μL of appropriate enzyme buffer (10 times concentrated) as recommended by New England Biolabs (*20*), 2 μL λDNA (.5 mg/mL) or Hind III digest of λDNA (for Sac I and II only), 3 μL enzyme sample and 13 μL water. The mixture was incubated at 37°C for 1 h or 3 h (for Hpa I and II, Hind II and III, and Sac I and II only) before adding 2 μL of a mixture containing 50% (v/v) glycerol, .25*M* EDTA, and .45% (w/v) bromophenol blue. The mixture was finally incubated for a further 10 min at 70°C prior to application to the electrophoresis agarose gel (*6*). The latter was usually run at 80–100 V for approx 1 h and the bands were visualized under ultraviolet light.

One unit of enzyme activity (1 U) was defined as the amount of enzyme required to produce a complete digest of 1.0 μg DNA at 37°C for 1 h in a total reaction volume of 50 μL. Protein determination was performed by the Bradford method using Coomassie Brilliant Blue G and bovine serum albumin as protein standard (*21*).

Quality Control Tests

The purity of the final EcoRV preparation with respect to nonspecific nucleases was evaluated by the "overdigestion" and "cut-ligate-recut"

tests. The stock solutions employed were the same as those described under Enzyme Assays.

Overdigestion Test

Into an Eppendorf tube were pippeted 5 μL appropriate enzyme buffer (*20*), 5 μL BSA solution (1 mg/mL), 4 μL λ DNA (.25 mg/mL), and varying amounts of purified enzyme sample (1–5 μL). The assay mixture was brought to 50 μL final volume by adding H_2O and was then incubated at 37°C for 16 h followed by 10 min at 65°C. The overdigestion fold was calculated by multiplying the incubation time (16 h) by the maximum number of units that give a clear DNA cleavage pattern specific for the enzyme.

Cut-Ligate-Recut Test

Cut: Into an Eppendorf tube were added 30 μL buffer, 45 μL λ DNA (15 μg), enzyme sample (100 U), and 220 μL H_2O. The mixture was incubated for 16 h at 37°C followed by 10 min at 65°C before adding 12 μL NaCl (5*M*) and 750 μL ethanol. The mixture was then immersed for 10 min in liquid nitrogen, centrifuged (10 min) in a bench microcentrifuge, and dried in a desiccator (10 min).

Ligation: The dried precipitate was taken up in a mixture containing 15 μL 50 m*M* Tris-HCl buffer, pH 7.4, 4 μL ATP (7 m*M*), 3 μL of a 10-fold concentrated T4 DNA ligase buffer (*20*), 3 μL T4 DNA ligase (.6 Weiss unit), and 5 μL H_2O, followed by incubation for 1 h at 16°C and 10 min at 65°C.

Recut: A sample (4 μL) from the ligation step was mixed with 2 μL of 10-fold concentrated EcoRV buffer (*20*), 2 μL purified enzyme (40 U), and 12 μL H_2O before incubating at 37°C for 1 h followed by 10 min at 65°C.

Enzyme Chromatographic Procedures

All dyed gels were prepared as described earlier (*15,22*). Chromatographic runs were performed in a cold room (4°C) with columns of 3 mL (1.3×2.3 cm) for all analytical (ie screening) runs. For preparative runs (ie preparation of EcoRV) the following column volumes were used: Blue HE-RD, 28 mL; Blue MX-4GD, 12 mL; and Navy H-ER, 5 mL.

Analytical Procedures

Frozen cell paste (Table 1) was thawed with Tris-HCl buffer, 20 m*M*, pH 7.4, containing 10 m*M* β-mercaptoethanol (buffer A) and 25 μg PMSF/mL, sonicated in an ice bath for a total of 15 min (30s×30) and then centrifuged at 4°C for 1 h at 35×10^3 rpm. The supernatant was diluted with buffer A to the final volume indicated in Table 1 and the crude extract at this stage contained the amount of protein shown in Table 1. A sample of the crude extract (Tables 1 and 2) was loaded on each column previously equilibrated in buffer A, and the column was washed with buffer until no absorption at 280 nm was evident in the effluents and then developed

Table 1
Resolution of Restriction Endonucleases by Dye-Ligand Chromatography[a]

Enzyme	Sac I and II				Hpa I and II				Hind II and III			
Cell paste	5 g, 20 mL				16 g, 50 mL				10.4 g, 30 mL			
Crude extract	140 mg protein, 32 mL				52.4 mg protein, 60 mL				611 mg protein, 64 mL			
	Unbound	Bound and eluted			Unbound	Bound and eluted			Unbound			Bound and eluted
Dye-agarose adsorbents	Enzyme present	Enzyme present	Activity, units	Protein, mg	Enzyme[b] present	Enzyme present	Activity, units	Protein, mg	Enzyme present	Activity, units	Protein, mg	Enzyme present
Yellow H-5G	I + II	I	1600	.56	—	I	2050	2.96	II + III	—	—	II + III
Brown H-2G	I + II	—	—	—	—	—	—	—	II + III	—	—	—
Orange H-GR	II	I	15600	2.29	—	—	—	—	II + III	—	—	II + III
Red HE-3B	I + II	—	—	—	—	I	1950	2.40	II + III	—	—	II + III
Red HE-7B	II	I	1200	2.41	—	—	—	—	III	26800	29.75	II + III[c]
Red H-8BN	II	I	23400	2.31	—	—	—	—	III	21600	30.96	II + III[c]
Red MX-5B	I + II	I	13600	.86	—	I	4050	1.82	II + III	—	—	II + III
Blue HE-RD	II	I	11600	1.66	—	I	3900	2.38	III	26000	29.25	II + III[c]
Blue MX-3G	I + II	I	8200	2.05	—	—	—	—	II + III	—	—	II + III
Blue MX-4GD	II	I	22200	2.11	—	—	—	—	II + III	—	—	II + III
Blue MX-7RX	I + II	—	—	—	—	—	—	—	II + III	—	—	—
Navy H-ER	I + II	I	9700	2.50	—	—	—	—	II + III	—	—	II + III
Green HE-4BD	I + II	I + II	—	—	—	—	—	—	III	29400	36.75	II + III[c]
Olive H-7G	II	I	19200	2.20	—	—	—	—	III	49200	30.67	II + III[c]
Turquoise H-A	I + II	I + II	—	—	—	I	780	.98	II + III	—	—	—
Violet H-3R	I + II	I	4000	.85	—	I	2050	2.70	II + III	—	—	II + III

[a] The following bacterial extracts were used: *Streptomyces achromogenes* (Sac I and II), *Haemophilus parainfluenzae* (Hpa I and II) and *Haemophilus influenzae* (Hind II and III). Column bed 1.3×2.3 cm. Total protein loaded on each column was as follows: Sac, 8.75 mg; Hpa, 3.3 mg; Hind, 38.2 mg.

[b] Enzyme activity is not detectable because of the presence of contaminating nonspecific nucleases.

[c] Hind III is present in trace amounts only.

with 1M KCl in buffer A at a flow rate of 20 mL/h. Fractions (2 mL) with enzyme activity were pooled and assayed for enzyme activity (U) and protein (mg) (*see* Table 1).

Purification of EcoRV

Frozen cell paste (5 g) was thawed with buffer A (40 mL) containing 25 μg PMSF/mL, sonicated in an ice bath for a total of 15 min (30s×30) and then centrifuged at 4°C for 1 h at 35×10^3 rpm. The supernatant (42 mL, 496 mg protein) was loaded onto a Blue HE-RD Sepharose column (2.1×8.0 cm; 28 mL) previously equilibrated in buffer A. The column was washed with buffer until no absorption at 280 nm was evident in the effluents and then developed with a linear gradient of KCl (250 mL total volume, 20–1*M*) in buffer A at a flow rate of 25 mL/h. Fractions (3.5 mL) with EcoRV activity corresponded to ca. .55 *M* KCl in the gradient (No. 60-69) were pooled (35 mL), concentrated in an Amicon concentrator (PM 10 Diaflo membrane; 10×10^3 cutoff) and dialyzed against buffer A (10 mL, 100 mg protein, 500,000 U). This dialyzed sample was subsequently loaded onto a Blue MX-4GD Sepharose column (1.6×6.0 cm; 12 mL) previously equilibrated in buffer A. The column was washed with buffer until no absorption at 280 nm was evident in the washings and then developed with a linear gradient KCl (100 mL total volume, 0–1*M*) in buffer A at a flow rate of 11 mL/h. Fractions (2.3 mL) with EcoRV activity corresponded to ca .75*M* KCl in the gradient (No. 39-47) were pooled (21 mL), concentrated in an Amicon concentrator as above and dialyzed against buffer A containing 5% (v/v) glycerol (4.5 mL, 2.8 mg protein, 360,000 U). This dialyzed sample was then loaded onto a Navy H-ER Sepharose column (1.3×3.8 cm; 5 mL) previously equilibrated in buffer A containing 5% (v/v) glycerol. The column was washed with irrigating buffer until no absorption at 280 nm was evident in the washings and then developed with a linear gradient of KCl (50 mL total volume, 0–1*M*) in irrigating buffer at a flow rate of 7 mL/h (Fig. 1). Fractions (1.5 mL) with EcoRV activity (frac. No 33-39) corresponding to ca .6*M* KCl in the gradient were pooled (10.5 mL, .6 mg protein, 157,500 U), dialyzed against storage buffer, which consisted of Tris-HCl, 10 m*M*, pH 7.4, 50 m*M* KCl, .1 m*M* EDTA, 1 m*M* β-mercaptoethanol, and 50% (v/v) glycerol, and stored at −20°C in the same storage buffer.

RESULTS AND DISCUSSION

Dye-ligand chromatography is becoming an increasingly popular technique for the purification of enzymes and proteins (*23,24,26*), especially those which bind (poly)nucleotides or nucleotide analogs (*15–19*). It is now well documented that triazine dyes at least partly mimic naturally occurring biological heterocycles in binding complementary macromolecules (*13–15,17,18,23,25*). Furthermore, certain significant advantages of

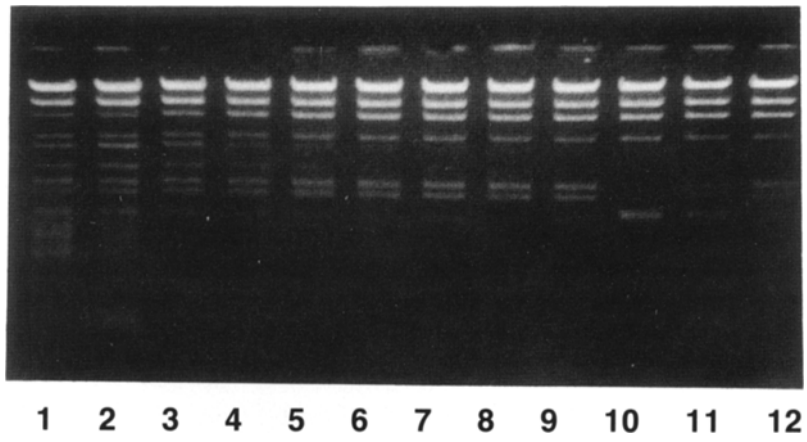

Fig. 1. Resolution of Sac I and II restriction endonuclease on Red H-8BN-agarose column. A dialyzed sample of crude extract of *Streptomyces achromogenes* (8.57 mg protein) was loaded on a 3 mL column of Red H-8BN-agarose. The column was washed with irrigating buffer (fractions 1–8) to remove unbound material and then developed with 1*M* KCl (fractions 9–12) at a flow rate of 20 mL/h. All fractions collected (2 mL each) were assayed for Sac I and II activity. Fraction Nos. 1 and 2 contained only Sac II activity, whereas fractions 10 and 11 only Sac I activity. The characteristic patterns for these two enzymes show five bands (Sac I) and ten bands (Sac II), whereas the six-band pattern corresponds to the substrate Hind III-digested λDNA.

dye-ligands such as their ready availability, low cost, easy immobilization to various matrices, chemical and biological stability, and high capacity of the dyed adsorbents, have contributed in the dramatic expansion of these affinity chromatography media as purification tool for many enzymes both in analytical and the larger scales (*24,26*).

Because of their binding specificity, restriction endonucleases have been subjected to purification by dye-ligand chromatography before (*6,8–10*). On two occasions (*8,9*) it is claimed that restriction endonucleases free from contaminating nucleases could be prepared in a single step on Cibacron Blue F3G-A-agarose. In our laboratory, a single-step procedure has not proven sufficient for preparation of pure restriction endonucleases for commercial use. In the best case we prepare nuclease-free restriction enzymes (e.g., Ban II, Sac I, and Sph I) in two steps by combining dye-chromatography and HPLC (*6*). Likewise, a combination of Cibacron Blue F3G-A-agarose, heparin-agarose and gel filtration was necessary to purify Pal I (*10*). Nevertheless, it seems that dye-ligand chromatography may prove to be a useful technique for the purification of restriction enzymes and, consequently, we have investigated this possibility in some detail. Sixteen triazine dyes were immobilized on CL-Sepharose by nucleophilic displacement of their reactive chlorine(s) under alkaline conditions. The resultant dyed adsorbents were then screened with cell-free bacterial extracts that were the source for the various restriction endonucleases used

in this study. Thus, a sample of dialyzed crude bacterial extract was applied on each column, unbound material was washed off with irrigating buffer until no absorption at 280 nm was evident in the washings, and bound enzyme was then eluted with 1*M* KCl. Collected fractions were assayed for enzyme activity and those which displayed a clear patern specific for the enzyme under study were pooled and assayed both for enzyme activity and protein content. Both the restriction enzyme yields and the corresponding specific activities obtained should be taken into account in evaluating these chromatographic media for incorporation into standard fractionation procedures.

Table 1 summarizes the results from experiments that aimed to test the ability of 16 dyed gels to resolve restriction endonucleases. For 13 gels Sac I enzyme activity could be recovered on elution with 1*M* KCl, and in all but two cases that activity was free of Sac II contaminants. Similarly, for 6 gels Hpa I activity could be recovered on elution with 1*M* KCl, and in every case that activity was free of Hpa II contaminants. Thus, for example, when cell-free *Streptomyces achromogenes* extract was directly applied on Red HE-8BN-agarose column (Fig. 1) unbound material containing Sac II passed through in the washings (fractions 1-8), whereas bound Sac I activity was then eluted free of Sac II with 1*M* KCl (fractions 10, 11).

Likewise, five gels separate effectively Hind III from Hind II, allowing Hind III to flow through without Hind II contaminants and binding Hind II with only traces of Hind III. It seems, therefore, that triazine dye chromatography is an attractive method for resolving restriction endonucleases in a single step, although such enzyme-separation procedures yield restriction activities that may need further purification from nonspecific nucleases. Furthermore, this method may shorten substantially the purification protocols required for Hpa I (6-step) and Hpa II (7-step) (*27,28*) as well as Hind II and Hind III (5-step) (*28*).

Table 2 effectively summarizes the purifying ability of dye-agarose gels for three restriction endonucleases Sph I, EcoRI, and EcoRV. The level of contaminating nonspecific nucleases present in the starting material, i.e., cell-free bacterial extract, drastically interferes with the unit determination of restriction endonucleases, thus, we were not able to determine reliable purification-fold figures. Instead, the carefully measured specific activity of each enzyme was used to evaluate the purifying ability of the various dyed gels. It appears that dye-ligands differ in their ability to purify the three endonucleases tested in terms of final specific activity achieved (Table 2). Thus, for Sph I a range of specific activities from 97 to 212 U/mg was obtained, whereas for EcoRI and EcoRV ranges of 644 to 2700 U/mg and 474 to 2684 U/mg were achieved, respectively. These figures represent an increase over the minimum specific activity obtained for each enzyme by factors of 2.2, 4.2, and 5.7 for Sph I, EcoRI, and EcoRV, respectively. An attempt to arrange the dyed gels in order of increasing purifying ability simultaneously for all three enzymes reveals that the adsorbents behave in a rather different way in terms of binding the three enzymes. For ex-

Table 2
Purification of Restriction Endonucleases by Dye-Ligand Chromatography[a]

Enzyme	Sph I			EcoRI			EcoRV		
Cell paste	16 gr, 20 mL			2 gr, 5 mL			5 gr, 20 mL		
Crude extract	541 mg, 64 mL			101 mg, 16 mL			544 mg, 48 mL		
	Enzyme activity and protein bound and then eluted; SA = Specific Activity								
Dye-agarose Adsorbents	Activity, units	Protein, mg	SA, units/mg	Activity, units	Protein, mg	SA, units/mg	Activity, units	Protein, mg	SA, units/mg
Yellow H-5G	—	1.9	—	—	1.2	—	—	1.4	—
Brown H-2G	—	1.1	—	—	.5	—	—	1.2	—
Orange H-GR	**700**	**3.3**	**212**	2950	1.7	1735	10000	5.1	1960
Red HE-3B	—	2.6	—	580	.9	644	1850	3.2	578
Red HE-7B	850	6.6	128	2800	2.1	1333	6800	5.9	1152
Red H-8BN	650	5.0	130	3100	1.7	1823	5200	6.5	800
Red MX-5B	—	1.9	—	—	.9	—	—	.8	—
Blue HE-RD	—	4.9	—	—	1.6	—	11100	5.5	2018
Blue MX-3G	650	3.5	186	**2700**	**1.0**	**2700**	1850	3.9	474
Blue MX-4GD	550	4.2	131	2900	1.5	1933	**15300**	**5.7**	**2684**
Blue MX-7RX	—	.3	—	—	.1	—	—	.6	—
Navy H-ER	—	3.3	—	3000	1.9	1579	14700	6.6	2227
Green HE-4BD	—	6.2	—	3050	2.0	1525	9600	5.6	1714
Olive H-7G	600	6.2	97	1555	1.5	1033	10600	6.5	1630
Turquoise H-A	—	.5	—	—	.1	—	—	.1	—
Violet H-3R	—	1.4	—	—	.8	—	—	1.1	—

[a] The following bacterial extracts were used: *Streptomyces phaeochromogenes* (Sph I), *Escherichia coli* RY 13 (EcoRI), and *Escherichia coli* J62PLG74 (EcoRV). Column bed 1.3×2.3 cm. Total protein loaded on each column was as follows: Sph I, 33.8 mg; EcoRI, 6.3 mg; EcoRV, 34 mg. Numbers in bold characters indicate adsorbents that yielded enzymes with the highest specific activity.

ample, although Blue MX-3G yields high specific activity preparations of Sph I and EcoRI, it achieves the lowest specific activity for EcoRV (Table 2). On the other hand, Orange H-GR is very effective for purifying Sph I but displays moderate performance for EcoRI and EcoRV. Dyed-adsorbents such as Red HE-3B and Olive H-7G have afforded enzyme preparations of moderate to low specific activities, whereas the performance of the other gels again varied depending on the enzyme (Table 2). Such differences in the binding ability and selectivity of dye affinity adsorbents are valuable in establishing enzyme purification procedures. Consequently, screening a number of dyes in order to find the most promising one is a worthwhile process (*6,15,16*). In this context, for example, it has been reported that employment of Blue H-B and Red H-8BN during the first step of the purification protocols of Sph I and Sac I, respectively, was necessary for satisfactory results, whereas other dyes were not equally effective (*6*). Taking into account both specific activity and total activity recovered, Orange H-GR appear best suited for Sph I, Blue MX-3G for EcoRI, and Blue MX-4GD for EcoRV.

We have demonstrated the applicability of dye-ligand chromatography in the purification of restriction enzymes by purifying EcoRV from appropriate cell-free bacterial extracts. Our procedure, as described under "Materials and Methods," involves a three-step sequential dye-chromatography in the order of Blue HE-RD, Blue MX-4GD, and Navy H-ER. These dyes were chosen from Table 2 as exhibiting the best performance in terms of specific activity for EcoRV. The EcoRV activity adsorbed on each column was subsequently eluted, in all three occasions, with a linear gradient of KCl. Figure 2 shows the elution profile of the enzyme from the third and final dye column, Navy H-ER-Sepharose. Fractions 33-39 containing the purified enzyme were pooled and dialyzed against the appropriate storage buffer. The whole chromatographic protocol, as summarized in Table 3, affords EcoRV of specific activity 262,500 U/mg. When, the final enzyme preparation was subjected to the overdigestion test, up to 60 U EcoRV did not produce any unspecific cleavage products after 16 h

Table 3
Summary of the Purification of EcoRV by Dye-Ligand Chromatography[a]

	Total volume, mL	Total activity, units	Total protein, mg	Specific activity, units/mg	Volume activity, units/μL	Yield %
Crude extract	42	—[b]	496	—[b]	—[b]	—
Blue HE-RD-agarose	10	500000	100	5000	50	100
Blue MX-4GD-agarose	4.5	360000	2.8	128750	80	72
Navy H-ER-agarose	10.5	157500	.6	262500	15	31

[a] The bacterial extract used was from Escherichie coli J62 PLG74.
[b] Enzyme activity is not detectable because of the presence of contaminating nonspecific nucleases.

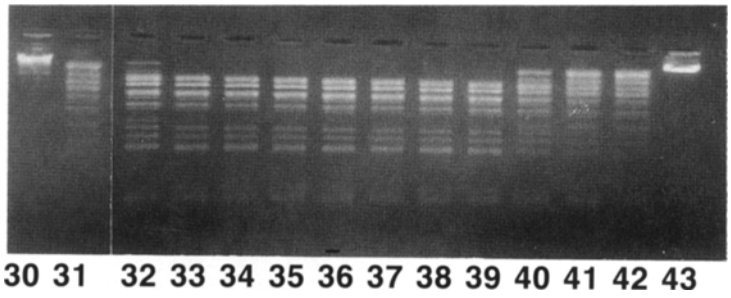

Fig. 2. Purification of EcoRV restriction endonuclease on Navy H-ER-agarose column. This is the picture obtained from the third column of the three-step procedure of dye-chromatography of EcoRV. The dialyzed sample obtained from the previous column (4.5 mL, 2.8 mg protein, 360,000 U) was loaded on a Navy H-ER-agarose column (1.3×3.8 cm) previously equilibrated in Tris-HCl buffer, 20 m*M*, pH 7.4 containing 10 m*M* β-mercaptoethanol and 5% (v/v) glycerol. The column was washed with irrigating buffer until no adsorption at 280 nm was present in the washings and then developed with a linear gradient of KCl (50 mL; 0–1*M*) in the same buffer at a flow rate of 7 mL/h. Fractions (1.5 mL) assayed for EcoRV and those with activity (Nos. 33–39), corresponded to *ca* .6*M* KCl in the gradient, were pooled (10.5 mL, .6 mg protein, 157,500 U) and both dialyzed and stored in the storage buffer.

incubation with 1 μg λDNA at 37°C, i.e., after 960-fold overdigestion. In the "cut-ligate-recut" test after more than 100-fold overdigestion of λDNA with EcoRV greater than 90% of the DNA fragments could be ligated and recut with this enzyme. The purification protocol presented here can be used routinely to prepare EcoRV for commercial purposes. Furthermore, appropriate combinations of dye-adsorbent may provide protocols useful in the resolution and purification of other restriction endonucleases in an economical way.

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