Rapid, Single-Step Purification of Restriction Endonucleases on Cibacron Blue F3GA–Agarose†

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ABSTRACT: After sonication and high-speed centrifugation, crude extracts of *B. amyloliquefaciens, P. alcalifaciens, X. holicola,* and *B. globiggi* were adsorbed on the dye Cibacron blue F3GA covalently cross-linked to agarose. The restriction endonucleases *Bam*HI, *Pal*I, *Xho*I, and *Bgl*I together with *Bgl*II were isolated by elution of the dye column with linear gradients to 0.5 M NaCl. The enzymes so purified were free of contaminating nucleic acids and other nucleases and were sufficiently concentrated for direct, specific DNA hydrolysis.

Enzymes containing the dinucleotide fold and those which possess nucleotide binding sites have a specific affinity for blue dextran, an adsorbent containing a covalently bound polyaromatic dye (Thompson et al., 1975; Wilson, 1976). After dissociation of nonspecifically bound proteins by denaturing agents or buffers of increased ionic strength, these specific interactions can be reversed by a substrate or cofactor for the enzymes, enabling a high degree of enzyme purification by a single procedure.

Because of their enzymatic specificity, type II restriction endonucleases might be expected to bind to this dye. Interest in these enzymes is due to their use for physical mapping of chromosomes, nucleotide sequence analysis of DNA, isolation of genes, and restructuring of DNA molecules. At present, isolation of type II restriction enzymes requires, after cell breakage and high-speed centrifugation, at least two more steps: removal of nucleic acids and column chromatography of the enzymes (Roberts, 1976; Smith and Wilcox, 1970). An additional problem in preparation is that these nucleases readily lose activity upon dialysis or during protein concentration procedures (Smith, 1974), possibly because of their di- or tetrameric nature (Smith and Wilcox, 1970; Gromkova and Goodgal, 1972). We show here that dye chromatography of type II restriction enzymes combines nucleic acid removal and protein purification in a single rapid step. Conditions for binding and elution were determined with four different commercially purified enzymes. The procedure was then applied to four crude bacterial extracts containing these enzymes. In each case, the restriction enzymes known to be present (Roberts, 1976) were obtained in a form sufficiently pure for specific DNA hydrolysis. The dye-binding procedure also simplifies assays of type II enzyme activity in large numbers of crude samples. As an example, the procedure was used to follow the production of *Pal*I and *Bam*HI during growth of *P. alcalifaciens* and *B. amyloliquefaciens*, respectively.

Experimental Section

The blue dye Cibacron blue F3GA, covalently attached to 4% cross-linked agarose, was obtained from Pierce Chemical Co., Rockford, Ill. In this paper, mention of the blue dye or adsorbent refers to the dye-substituted agarose. λ DNA was from Miles and calf thymus DNA was from Sigma. Col E1 DNA was hydrolyzed with the restriction endonuclease *Hae*III as described (Eshaghpour and Crothers, 1978). The trinucleotides ApGpAp and TpCpCp were isolated from micrococcal nuclease digests of calf thymus DNA (Rushizky et al., 1972). Purified restriction enzymes *Alu*I, *Xho*I, *Bam*HI, and *Hae*III were obtained from New England Biolabs, Beverly, Mass.

Growth of Cells. Inocula were received from Drs. R. diLauro for *B. globiggi*, R. W. Hartley for *B. amyloliquefaciens*, and from the American Type Culture Collection (Rockville, Md.): ATCC 13461 for *X. holicola* and ATCC 9866 for *P. alcalifaciens*. Cells were grown at 37 °C on 8 g of bactopeptone and 5 g of yeast extract/L in fermentors with stirring (100–400 rpm) and forced aeration (10–50 L of air/min), to the middle or end of log phase, harvested in a Sharples AS-16P centrifuge at a flow rate of about 1 L/min, and stored frozen at −20 °C. The medium for *B. amyloliquefaciens* contained, in addition to the above, 6 g of KH_2PO_4, 3 g of $K_2HPO_4 \cdot 3H_2O$, and 5 g of sucrose/L.

To determine the amount of enzyme activity at various stages of growth, *P. alcalifaciens* or *B. amyloliquefaciens* was grown in a 14-L fermentor. Aliquots of 1, 0.5, or 0.2 L were withdrawn at suitable time intervals, and cells were collected at 4 °C by centrifugation for 10 min at 9000*g* and frozen.

Measurement of Endonuclease Activity. Enzymes were assayed in a solution, containing 0.02 M Tris-HCl[1] (pH 7.6), 0.01 M $MgCl_2$, and 0.01 M β-mercaptoethanol (buffer A), and 2 μg of λ DNA in a volume of 50 μL. Enzyme dilutions of 2–10 μL in buffer A were added, and reactions were incubated at 37 °C for 15–60 min. After digestion, 5 μL of a solution containing 50% sucrose and 0.2% bromophenol blue was added. Agarose slab-gel electrophoresis of DNA digests (Sharp et al., 1973; Sugden et al., 1975) was performed on 1.4% gels in an EC 490 cell (EC Apparatus Corp., St. Petersburg, Florida) with a Teflon slot former, producing wells of 1-mm thickness and 1-cm width. The size of the slab was 0.3 × 17 × 24 cm. Electrophoresis was for 2.5 h at 250 V and 25 °C in Peacock's buffer (Dingman and Peacock, 1971). The gels were stained with ethidium bromide (0.5 mg/L) and photographed with ultraviolet illumination on Polaroid 55 P/N film using a red filter. One unit is defined as the amount of enzyme required to hydrolyze 1 μg of λ DNA to completion in 1 h at 37 °C.

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[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DEAE, diethylaminoethyl.

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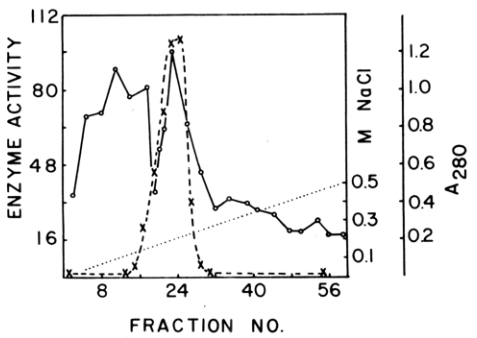

FIGURE 1: Column chromatography of an extract of *P. alcalifaciens* on Cibacron blue F3GA. The high-speed supernatant from 6 g of frozen cells of *P. alcalifaciens* was adsorbed on a 0.9 × 9 cm column of the absorbant, and nuclease activity (see below) was eluted by a linear gradient from 0 to 0.5 M NaCl in buffer A: A_{280} as a measure of protein content (—), enzyme activity in units/mL ($\times 10^{-3}$) (- - -), NaCl gradient (• • •).

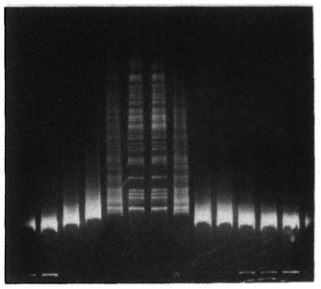

FIGURE 2: Agarose gel electrophoresis of λ DNA digested with *Pal*I fractions obtained by dye chromatography (Figure 1). The assay solution contained λ DNA aliquots digested by suitable dilutions of column fractions for 15 min–1 h. Every third tube starting at no. 5 is shown, from left to right. The enzyme peak is centered at no. 20, 23 (lanes 6 and 7 from left).

Purification of Restriction Endonucleases. All procedures were carried out at 4–8 °C. In a representative experiment, frozen cells of *P. alcalifaciens* (6 g) were suspended in 0.5 volume of buffer A, sonicated for six 15-s intervals, and centrifuged at 60 000*g* for 2 h. The high-speed supernatant (9 mL) was diluted 1:5 with buffer A and loaded at 15 mL/h on a 0.9 × 9 cm column of Cibacron blue equilibrated with buffer A. After washing with 3 bed volumes of buffer A, the column was eluted with a 100-mL gradient from 0.0 to 0.5 M NaCl in buffer A. Fractions (1.6 mL) were assayed for nuclease activity, and the peak fractions were pooled and stored in 50% glycerol at −20 °C. Protein determinations (Lowry et al., 1951) were carried out on the pooled enzyme fractions as well as on the high-speed supernatants from which they were derived, using bovine serum albumin as a standard. Commercially purified restriction nucleases (100–200 units) were adsorbed on Cibacron blue columns after 1:10 dilution with buffer A. A bed volume of 1 mL was routinely used. After washing the columns as above, they were eluted with 0.5 M NaCl in buffer A. Bovine serum albumin (5 mg/mL) or calf thymus DNA (125 μg/mL) in buffer A was tested as eluant in place of 0.5 M NaCl.

Exonuclease activity in *Pal*I was tested for by incubation, for 3 h at 37 °C, of 8000 units of nuclease with 1 mg of TpCpCp or ApGpGp in buffer A, followed by chromatography on Whatman No. 3 paper with *n*-PrOH/H_2O/concd NH_4OH, 55:10:35 (v/v).

Results

The blue dye is known not to adsorb DNA and the enzymatic specificity of the type II restriction endonucleases suggested that they might bind to this matrix, providing a single-step purification of these enzymes; this possibility was tested with four purified nucleases. *Alu*I, *Bam*HI, *Xho*I, and *Hae*III, purified to specific activities of 20 000–30 000 units of enzyme per mg of protein (2500–5000 units/mL), did indeed adsorb to the blue dye. NaCl, at a concentration of 0.5 M, did elute all these enzymes will full recovery of enzyme activity as determined by agarose gel electrophoresis of λ DNA digested with aliquots of the fractions before and after dye chromatography. Several other possible eluants for the activities were tried. The enzymes were not eluted from the dye by serum albumin at 5 mg/mL, by calf thymus DNA at 125 μg/mL, for *Pal*I by *Hae*III digests of Col E1 DNA at 70 μg/mL in buffer A, or by the much higher nucleic acid concentrations present in bacterial sonicates.

Chromatography on the blue dye adsorbent was next utilized to attempt a single-step separation of type II endonucleases from both DNA and contaminating nucleases. High-speed supernatants were loaded onto columns and DNA was removed by washing with buffer A. Elution was then performed using a linear gradient of NaCl. Chromatography of an extract from *P. alcalifaciens* on the blue dye yielded a single peak of nuclease activity eluting at 0.1–0.2 M NaCl (Figure 1). Since the band patterns produced from λ DNA by the restriction nucleases discussed here are known, aliquots of column fractions were tested as shown for *Pal*I in Figure 2; activities were identified by comparison with purified enzymes obtained commercially. In the case of *Pal*I, the isoschizomer *Hae*III was used, producing identical band patterns to those shown in Figure 2. With extracts from *B. globiggi, B. amyloliquefaciens, X. holicola,* and *P. alcalifaciens* (Roberts, 1976), *Bgl*I and *Bgl*II, *Bam*HI, *Xho*I, and *Pal*I, respectively, were obtained in a form sufficiently pure for specific hydrolysis of DNA molecules. The dye chromatography procedure did not distinguish between the two restriction nucleases present in *B. globiggi,* yielding a mixture of both enzymes. These two enzymes were identified by comparison with *Bgl*I and *Bgl*II (from N. E. Biolabs) mixtures and could also be separated by additional procedures (Roberts et al., 1976; Bickle et al., 1977).

Xanthomonas contains two enzymes, *Xho*I and *Xho*II, but only *Xho*I was found. The two enzymes, if present, are readily distinguished by the number of cleavages produced in λ DNA, namely, one for *Xho*I and more than 20 for *Xho*II (Roberts, 1976; Gingeras et al., 1978). The absence of *Xho*II was not due to inactivation during dye chromatography, for a workup of the high-speed supernatant by standard procedures (Roberts et al., 1976) did not reveal *Xho*II either. *Xho*II was therefore presumed not to be present in detectable amounts in the cells.

Dye chromatography of the high-speed supernatants of the four strains of bacteria investigated resulted in about a 30-fold decrease of protein content (Table I). Restriction endonuclease activity in the high-speed supernatants could not be determined without further purification, i.e., removal of nucleic acids and nonspecific nucleases. For example, both the sonicates of *P. alcalifaciens* and *B. amyloliquefaciens* contained an endonuclease, eluted from dye columns by washing with buffer A, which hydrolyzed DNA to small fragments that moved out of the agarose gel ahead of the Bromphenol blue marker, thus preventing an assay of the starting material. For these reasons, nuclease activity present after dye chromatography is listed in terms of units of enzyme activity recovered per gram of frozen cells and not in terms of specific activity.

After purification by dye chromatography, *Pal*I was found to be free of exonuclease activity, evidenced by lack of hy-

TABLE I: Purification of Restriction Endonucleases on Cibacron Blue F3GA.

	mg of protein/g of frozen cells, after:		units of nuclease/ g of frozen cells[a]
	high-speed centrif	blue dye chromatog	
*Pal*I	35.60	1.0	128 000
	43.80	1.0	190 000
*Bgl*I plus *Bgl*II	21.7	0.7	700
	25.0	0.81	990
*Xho*I	28.8	0.8	4 800
	26.2	0.75	4 180
*Bam*HI	43.24	1.20	7 800
	38.21	1.18	10 000

[a] After dye chromatography.

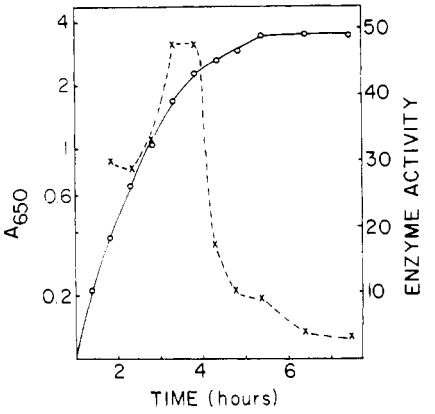

FIGURE 3: Growth of *P. alcalifaciens* and activity of *Pal*I. Growth of cells as measured by turbidity at 650 nm of suitable dilutions (—). *Pal*I nuclease activity (- - -) determined by agarose gel electrophoresis of λ DNA digests (units/L of suspension × 10^{-5}).

drolysis of TpCpCp or ApGpAp derived from calf thymus DNA. Fragments of λ DNA prepared with *Bam*HI purified by dye chromatography could be ligated with T4 DNA ligase (Weiss et al., 1968) (P. Leder and M. Nau, personal communication), again confirming the absence of contaminating nucleases in that restriction enzyme preparation.

The dye chromatography procedure was also used to allow assays of the content of *Pal*I and *Bam*HI in cultures during growth of *P. alcalifaciens* and *B. amyloliquefaciens*, respectively. The results obtained are shown in terms of growth (logarithmic scale) and enzyme activity per liter of medium (linear scale). For *Pal*I, an increase of nuclease activity with cell number was observed until late log phase, followed by a sharp decrease in enzyme activity (Figure 3). In contrast, for *Bam*HI, the highest amount of enzyme activity was obtained at the stationary phase of growth of *B. amyloliquefaciens* (Figure 4).

Discussion

It is not clear how Cibacron blue interacts with the restriction endonucleases described, especially since they are eluted by NaCl and not by the substrates tried here. Drocourt et al. (1978) observed that dye-bound polynucleotide phosphorylase was not eluted by nucleoside diphosphates but only by polymeric substrates, implying an interaction of the dye with enzymatic sites for the latter. There are three negatively charged groups on the dye that could mimic phosphate groups of

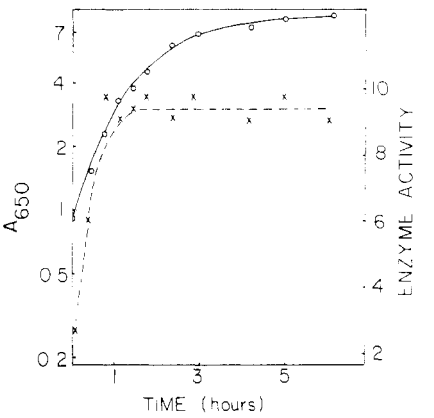

FIGURE 4: Growth of *B. amyloliquefaciens* and activity of *Bam*HI. *Bam*HI activity (- - -), growth of cells (—) as reflected by turbidity at 650 nm.

polynucleotides and, possibly, bind restriction enzymes in a cooperative fashion. This remains to be investigated.

Here, the important fact is that the restriction enzymes are bound to the dye while nucleic acids or their degradation products and contaminating nonspecific nucleases are not, thus facilitating a purification procedure shorter than those where nucleic acid removal is carried out in a separate step, on DEAE-cellulose (de Fillipes, 1974), agarose beads (Roberts et al., 1976), or by precipitation with streptomycin (Smith, 1974) or poly(ethylenimine) (Bickle et al., 1977). Also, the enzymes purified here from frozen cells were obtained in buffer A and 0.1–0.2 M NaCl solutions, in a sufficiently concentrated form for dilution and direct use. The procedure is readily scaled up using a ratio of 1 mL of packed Cibacron blue F3GA/g of frozen cells as described above.

Yields of restriction endonucleases obtained by other procedures are not always listed (Bickle et al., 1977) or are stated in different units (Smith, 1974). However, the yield of the enzymes fractionated on Cibacron blue gave recoveries similar to those reported for *Alu*I (Roberts et al., 1976) and *Xho*I (Gingeras et al., 1978).

The protein purification obtained on Cibacron blue is comparable or better than that achieved by equivalent steps of other methods also designed to remove nucleic acids. Thus, agarose chromatography in the original H. O. Smith procedure lowered the protein content twofold during the isolation of *Hind*II (Smith and Wilcox, 1970), while dye chromatography lowered the protein content about 30-fold during the isolation of four other restriction enzymes (Table I). Clearly, a comparison of both steps depends on the recovery of enzyme activity, which was not determined in the dye procedure (see above) and actually increased after agarose filtration (Smith and Wilcox, 1970), probably due to the removal of inhibitors. The two steps also differ from each other in that agarose gel filtration in 1 M NaCl buffers requires subsequent enzyme concentration and is but one of several steps leading to the 200-fold purification of *Hind*II. Contaminating nucleases, if present, would be removed by the agarose step if their molecular weights differ from that of the restriction enzyme.

By contrast, enzyme purification on the dye requires no further concentration and yields a nuclease not as pure as *Hind*II but nevertheless suitable for specific DNA hydrolysis. Contaminating nonspecific nucleases, found in two of the four strains of bacteria investigated here, were not bound on the dye and could be removed by washing. With *P. alcalifaciens*, the contaminating nuclease was not removed from *Pal*I by agarose chromatography.

The dye procedure is well suited for the rapid determination of enzyme activity in multiple samples. Thus, differences observed between the production of *Bam*HI and *Pal*I (Figures 3 and 4) indicate that this parameter should be routinely evaluated for other enzymes and/or growth conditions.

In summary, since the Cibacron chromatography gave similar results with four crude and four purified restriction nucleases, we believe that it will also be applicable to the purification of other type II enzymes.

Acknowledgments

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Postproline Cleaving Enzyme: Kinetic Studies of Size and Stereospecificity of Its Active Site†

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ABSTRACT: Postproline cleaving enzyme [EC 3.4.21.–] has recently been purified from lamb kidney and tentatively identified as a serine endopeptidase with a high specificity for proline-containing peptides. The interaction of postproline cleaving enzyme with peptide substrates and competitive inhibitors has been studied in an effort to explore the size and stereospecificity of the active site of the protease. The substrates and inhibitors included proline-containing peptide amides, *p*-nitrophenyl esters, and free acids with increasing numbers of amino acid residues and residues of L and D configuration. Oligopeptides of alanine, which can also be recognized by the protease, were also tested as substrates. This series included Ala_3, Ala-D-Ala-Ala, Ala-Ala-D-Ala, Z-$(Ala)_3$, Ala_4 through Ala_6. The contribution of each of the three amino acid residues flanking the primary specificity site (S_1) of postproline enzyme to such kinetic parameters as K_m, k_{cat}, and k_{cat}/K_m in the case of substrates and K_i with inhibitors was determined. The results suggest that postproline cleaving enzyme has an extended substrate binding region in addition to the primary specificity site, S_1. It seems to be comprised of three sites located at the amino-terminal site (S_1, S_2, and S_3) and two sites at the carboxyl site from the catalytic point (S_1', S_2'). High stereospecificity was observed for subsites S_1, S_2, and S_1'.

Postproline cleaving enzyme [EC 3.4.21.–] was discovered by Walter et al. (1971) in human uterus. The enzyme has recently been purified from lamb kidney (Walter, 1976; Koida & Walter, 1976) and has tentatively been characterized as a serine protease using active site-directed, irreversible inhibitors (Yoshimoto et al., 1977).

It has been shown that the specificity of proteases is determined not only by the two amino acid residues flanking the scissile bond of a peptide substrate, but also by amino acid residues more distant from the point of hydrolysis (Schechter & Berger, 1967; Fruton, 1970; Shaw, 1970; Bauer et al., 1973a,b; Powers, 1977). Thus, it became apparent that the active site of a protease has a more or less extended substrate binding region with subsites for secondary interactions in addition to the primary specificity site, and each of these sites of the enzyme was postulated to interact with one amino acid residue of the peptide substrate (Schechter & Berger, 1967). These concepts have been confirmed by extensive kinetic studies using substrates with systematic structural modifications of the amino acid residues in locations close to and distant from the scissile bond (e.g., Morihara, 1974). Furthermore, X-ray crystallographic investigations of proteases, irreversibly

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