Purification, Properties and Specificity of the Restriction Endonuclease from *Bacillus stearothermophilus*

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The restriction endonuclease *Bst*I was purified from 70 kg of *Bacillus stearothermophilus*. The final product is at least 97% pure as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; this major protein species co-migrates with the enzyme activity on native polyacrylamide-gel electrophoresis and isoelectric focusing. Pure restriction endonuclease *Bst*I has a subunit mol.wt. of 26000 and is probably a loosely associated dimer. The enzyme shows maximum activity at pH values between 7 and 9.5, and in the presence of 0.5–2 mM-Mg²⁺. NaCl inhibits the restriction enzyme activity. Restriction endonuclease *Bst*I cleaves DNA in a position identical with that cleaved by endonuclease *Bam*HI (for *Bacillus amyloliquefaciens*), i.e.:

In the presence of high concentrations of enzyme, DNA cleavage occurs at secondary sites. This side-specificity is enhanced by the addition of glycerol. Preliminary studies indicate that these sites are of the type:

The class-II restriction endonucleases, which cleave DNA at defined nucleotide sequences, are extensively used in the structural analysis of DNA molecules, and for the construction of recombinant DNA species *in vitro* [for a review see Roberts (1975)]. However, the detailed biochemical investigation of these enzymes has not kept pace with their widespread use. This situation is unfortunate, since restriction endonucleases constitute a potentially useful model system for the study of site-specific protein–DNA interactions. So far, *Eco*RI† is the only restriction enzyme to have been purified to homogeneity and investigated in detail at a mechanistic level (Greene *et al.*, 1975; Modrich & Zabel, 1976).

Although the slow progress in the study of restriction enzymes may in part be due to their great

usefulness as research tools (where purification to homogeneity is rarely required), it is also attributable to the low content of these enzymes in the bacteria from which they are derived. Thus, to obtain sufficient homogeneous enzyme for detailed structural and mechanistic study, it is necessary either to use large-scale purification procedures or to use artificial methods to amplify the synthesis of the desired enzyme.

We have chosen the first approach in our purification of the restriction endonuclease *Bst*I from *Bacillus stearothermophilus*. This allowed us to take advantage of the methods that have already been developed for the large-scale fractionation of extracts from this organism (Atkinson, 1973).

Restriction and modification systems were first detected in strains of *B. stearothermophilus* by Welker and his co-workers (Lees & Welker, 1973; Catterall *et al.*, 1976). The class-II endonuclease (*Bst*I) from strain NCA 1503 has been purified on a small scale and some of its properties have been investigated (Catterall & Welker, 1977). Comparison of the cleavage products obtained with several DNA

Abbreviations used: SDS, sodium dodecyl sulphate; DNAase, deoxyribonuclease.

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† The nomenclature of Smith & Nathans (1973) is used, except that the prefix 'endo R' (restriction endonuclease) has been omitted throughout.

molecules showed *Bst*I to be an isoschizomer of *Bam*HI (Catterall & Welker, 1977), which is known to recognize the sequence

cleaving the DNA in the positions indicated by the arrows (Roberts *et al.*, 1977). The position of cleavage by *Bst*I has not so far been investigated.

The present paper describes the large-scale purification of *Bst*I from 70 kg of cells, together with some properties of the homogeneous enzyme. We have demonstrated that *Bst*I cleaves DNA at the same position as *Bam*HI, and have also investigated a secondary activity of *Bst*I, which is analogous to the *Eco*RI* (Smith & Nathans, 1973) activity observed by Polisky *et al.* (1975).

Materials and Methods

Materials

B. stearothermophilus strain NCA 1503 (N.C.I.B. 8924) was grown in 3000-litre fermenters in this Department on a sucrose/yeast extract/salts medium (Sargeant *et al.*, 1971). The wet cell paste was harvested 1 h after the end of exponential phase and was stored at -20°C until required.

DNA from lysis-defective mutants of phage λ was prepared as described by Haseltine & Müller-Hill (1972). Two strains were used in this work, λ_{627} and λ_{prbt} . Phage λ_{627} , a gift from Dr. N. E. Murray (University of Edinburgh), is a *gam*⁺ S7 variant of $\lambda(srI\lambda 1-2)^{\nabla} srI\lambda 3^{+} gam210 ci857 srI\lambda 4^{\circ} ninR srI\lambda 5^{\circ}$ described by Borck *et al.* (1976), and gives an identical *Bst*I fragmentation pattern. The positions of the three *Bst*I cleavage sites can be predicted by using the *Bam*HI cleavage 'map' of Haggerty & Schleif (1976). Strain λ_{prbt} , a transducing phage derived from strain λ_{627} and carrying the genes of the *Klebsiella aerogenes* ribitol operon (M. S. Neuberger, unpublished work), has an additional *Bst*I cleavage site in the bacterial substitution. However, the resulting extra restriction fragment is too small to be detected on the gel systems used in the present work, so that the observed *Bst*I fragmentation pattern of strain λ_{prbt} is indistinguishable from that of strain λ_{627} .

DNA from the plasmid pBR 313 (Bolivar *et al.*, 1977), prepared by centrifugation in CsCl/ethidium bromide (Clewell, 1972), and DNA from *Escherichia coli* K12 strain 1EA of Rigby *et al.* (1976), prepared by the method of Marmur (1961), were kindly provided by Dr. M. S. Neuberger (Imperial College, London). SV40-virus DNA was a gift from Dr. P. W. J. Rigby (Imperial College, London). All DNA

solutions were stored in 10 mM-Tris/acetate, pH 8.2, containing 1 mM-EDTA.

Bovine serum albumin, ovalbumin, cytochrome *c*, pyruvate kinase, aldolase, D-amino acid oxidase, myoglobin and lysozyme were used as molecular-weight markers and were obtained from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.). Ribitol dehydrogenase from *E. coli* C was a gift from Dr. I. Altosaar (Imperial College, London). The subunit mol.wt. (27000) is the same as that of the *K. aerogenes* enzyme (Taylor *et al.*, 1974).

Restriction endonuclease *Bam*HI was purchased from Miles Laboratories (Stoke Poges, Slough, Berks., U.K.). Bacterial alkaline phosphatase (grade BAP-C), bovine pancreatic DNAase (grade DPFF) and snake-venom phosphodiesterase were from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Polynucleotide kinase, prepared by the method of Richardson (1971) up to the hydroxyapatite step, was kindly provided by Dr. M. Szekeley (Imperial College, London); 1 unit is defined as the amount of enzyme required for a complete transfer of [γ -³²P]phosphate from 1 nmol of ATP to a dinucleotide in excess.

DEAE-cellulose (DE-23) and phosphocellulose (P-11) were obtained from Whatman (Maidstone, Kent, U.K.). DEAE-cellulose was precycled as described in the manufacturer's instructions. Phosphocellulose was prepared for use by successive washes with 0.5 M-H₃PO₄, 0.1 M-NaOH and 95% (v/v) ethanol. Sephadex G-50 and G-150 were from Pharmacia (Uppsala, Sweden). Ultrogel AcA 34 was purchased from LKB (Selsdon, Surrey, U.K.). Hydroxyapatite was prepared by the method of Atkinson *et al.* (1973). Agarose powder was obtained from Bio-Rad Laboratories (Watford, Herts., U.K.).

The deoxyribonucleotides dAMP, dGMP and dTMP were purchased from Sigma. Yeast RNA (free acid) was obtained from BDH (Poole, Dorset, U.K.). [γ -³²P]ATP (1000–2000 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Schleicher und Schüll cellulose acetate strips (5 cm × 55 cm) were obtained through Anderman and Co. (East Molesey, Surrey, U.K.). T.l.c. sheets having a mixed layer of cellulose and DEAE-cellulose (15:2, w/w) were purchased from Camlab (Cambridge, U.K.). The dyes used for electrophoretic markers (Xylene Cyanol FF, Orange G and Acid Fuchsin) were obtained from G. T. Gurr (London S.W.6, U.K.). Radioautography was carried out by using Kodirex film (Kodak, Hemel Hempstead, Herts., U.K.), which was developed in a Kodak Xomat industrial processor.

Flat-bed isoelectric focusing was carried out on the LKB Multiphor apparatus with LKB PAG-plates. Dimethyl suberimide was obtained from Sigma. Buffers were prepared from AnalaR-grade

reagents wherever possible, and were standardized at room temperature (20°C).

Methods

Enzyme assay. The assay mixture contained 100mM-Tris/HCl, pH7.5, 10mM-MgCl₂ and 0.1μg of DNA in a total volume of 25μl. Enzyme solution (1μl) was added, after dilution, when necessary, in a buffer containing 20mM-potassium phosphate, pH7.5, 10mM-2-mercaptoethanol, 10% (v/v) glycerol, 0.1mM-phenylmethanesulphonyl fluoride and 1mg of bovine serine albumin/ml (dilution buffer). After incubation at 37°C for 30min, the DNA fragments were examined by horizontal electrophoresis at 5–10V/cm in 0.4% (w/v) agarose gels containing ethidium bromide (2μg/ml) in the Tris/borate buffer system of Greene *et al.* (1974). Gels were photographed during irradiation with short-wave u.v. light (Ultraviolet Products, San Gabriel, CA, U.S.A.). A unit of enzyme activity was arbitrarily defined as the amount necessary for complete digestion of 0.1μg of phage λ₆₂₇ DNA under the assay conditions described above. A semi-quantitative estimate of the activity of an enzyme sample was obtained by assaying a series of 2-fold dilutions to determine the minimum concentration necessary for complete digestion of the DNA. Values obtained in this way are necessarily only correct to within a factor of 2.

To test the effect of varying reaction conditions on the enzyme activity, the apparent activity of a given enzyme sample was measured (as described above) under a range of different assay conditions (pH, metal-ion concentration etc.).

The activity of *Bst*I was also measured under the conditions described by Greene *et al.* (1974). The reaction mixture contained 100mM-Tris/HCl, pH7.5, 10mM-MgCl₂, 50mM-NaCl and 0.5μg of SV40-virus DNA in a total volume of 20μl, which was equilibrated at 37°C. The reaction was then initiated by the addition of 1μl of an enzyme solution, and terminated after 1 min with 5μl of 0.2M-EDTA (sodium salt), pH7.5. The reaction products obtained with a series of enzyme dilutions were examined by electrophoresis in a 0.7% agarose gel, as described above. A quantitative estimate of the extent of reaction was obtained by densitometry of a photographic negative of the gel.

The incubation conditions for other experiments are described in the legends to the relevant Figures.

Polyacrylamide-gel electrophoresis. SDS/polyacrylamide gel electrophoresis was based on the method of Laemmli (1970). In most cases, electrophoresis was carried out for 16h at 6mA on slab gels (15cm × 15cm × 0.2cm) containing 12% (w/v) acrylamide. The gels were stained with Coomassie Brilliant Blue R-250 and protein was quantified by scanning at 550nm with a Gilford model 240 spectrophotometer.

Electrophoresis under native conditions was carried out in a similar manner, except that SDS was omitted and gels containing 5% (w/v) acrylamide were used. When it was desired ultimately to locate the enzyme activity, the gel was pre-run for 30min at 20mA. Duplicate samples were then applied to the gel, which was run at 6mA for 16h at 4°C. The gel was cut to separate the duplicate lanes. One half was stained with Coomassie Brilliant Blue R-250, and the other sample track was sliced into segments (3mm), which were placed in dilution buffer (100μl). After 3h or more at 4°C the fractions were assayed as described above.

Isoelectric focusing. Flat-bed isoelectric focusing, in the pH range 3.5–9.5, was carried out for 2.5h at maximum settings of 1000V, 50mA and 40W. The pH gradient was determined by cutting a blank sample track into segments (5mm × 5mm), each of which was placed in de-aerated water (1 ml). The pH of each sample was measured after 1h at room temperature. When it was necessary to locate both protein and restriction-enzyme activity, duplicate samples were applied. One sample track was sliced into segments (5mm × 5mm), each of which was extracted into dilution buffer (200μl) for 16h at 4°C before assay. The remainder of the gel was stained with a solution containing Coomassie Brilliant Blue R-250 (0.1%, w/v), sulphosalicylic acid (3.2%, w/v) and trichloroacetic acid (10.7%, w/v) in 700ml of methanol/water (1:2, v/v) by the procedure of Vesterberg (1972).

Assay of protein concentration. Protein was measured by the method of Lowry *et al.* (1951), with bovine serine albumin as a standard. Samples were dialysed to remove 2-mercaptoethanol before analysis.

Protein cross-linking. Cross-linking with dimethyl suberimide (Davies & Stark, 1970) was carried out for 3h at room temperature in 50mM-N-ethylmorpholine/acetate, pH8, containing 50μg of pure *Bst*I and 2mg of dimethyl suberimide/ml. Protein concentrations in the range 0.1–1.0mg/ml were tested. The reaction mixtures were freeze-dried and the cross-linked products examined by SDS/polyacrylamide-gel electrophoresis (as above), on 10% (w/v) acrylamide gels, in the presence of molecular-weight markers.

Preparation of endonuclease *Bst*I

All operations were carried out at 4°C unless otherwise noted; 10mM-2-mercaptoethanol and 0.1mM-phenylmethanesulphonyl fluoride were added to all buffers.

Cell disruption and DEAE-cellulose batch fractionation. Washed cell paste (70kg) was suspended in 0.1M-imidazole/phosphate buffer, pH8.0 (200 litres), and ruptured by two passes through a Manton-

Gaulin homogenizer (model K.3) operated at 50 MPa (8000 lbf/in²). The resulting suspension was treated for 5 min with a rotary homogenizer (Silverson model DX high-speed mixer emulsifier) to decrease viscosity caused by DNA, before removal of cell debris in a continuous-flow centrifuge (Westfalia model SAMR 6). The supernatant (180 litres) was titrated to pH 6.8 with 20% (v/v) phosphoric acid, and diluted with water containing 10 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride to a conductivity not exceeding 4 mS. To this material (300 litres) was added DEAE-cellulose (Whatman DE-23; 40 kg wet wt.) previously equilibrated with 30 mM-potassium phosphate, pH 6.8. After equilibration for 2 h at 4°C, with intermittent stirring, the non-bound material was separated from the resin by 'basket' centrifugation at room temperature by using a cloth bag inside a domestic spin-dryer. The non-bound fraction (284 litres) was used as the starting material in the preparation of the restriction enzyme (fraction I). The resin-bound material was further processed to yield other enzymes of interest (Atkinson, 1973).

Phosphocellulose batch treatment. To the fraction not bound to DEAE-cellulose (fraction I) was added phosphocellulose (Whatman P-11; 22 kg wet wt.), equilibrated with 20 mM-potassium phosphate, pH 7.5. The mixture was stirred at 4°C for 16 h. After removal of the non-bound material by centrifugation (as above), the resin was washed once with 20 mM-potassium phosphate, pH 7.5 (50 litres). The restriction enzyme was then eluted with the same buffer containing 400 mM-NaCl (2 × 50 litres), and these combined fractions were passed through a Sharples tubular-bowl centrifuge (model A5.6) to remove resin fines. The clarified material (100 litres, fraction II) was concentrated to 13 litres by ultrafiltration in a hollow-fibre unit (Amicon DC-50) fitted with four SM 10 cartridges (10000 daltons cut-off).

Ammonium sulphate precipitation. Solid (NH₄)₂SO₄ was added to fraction II to 50% saturation. After removal of the precipitate by centrifugation (13000g, 20 min), the supernatant was treated with (NH₄)₂SO₄ to 70% saturation. The pellet was recovered by centrifugation and resuspended in 20 mM-potassium phosphate, pH 7.5 (355 ml, fraction III).

Phosphocellulose column chromatography. The 50–70%-satd.-(NH₄)₂SO₄ fraction (III) was dialysed for 40 h against 20 mM-potassium phosphate, pH 7.5 (3 × 4 litres), and centrifuged (16000g, 15 min) to remove a slight precipitate. The clarified supernatant was loaded on to a column (5 cm × 28 cm) of phosphocellulose (Whatman P-11) equilibrated with the same buffer. The column was eluted at 100 ml/h with a linear gradient (10 litres) of 0–1 M-NaCl in 20 mM-potassium phosphate, pH 7.5. Fractions containing *Bst*I, which is eluted between 0.4 and 0.5 M-NaCl, were pooled (790 ml, fraction IV) and concentrated to

22.5 ml by ultrafiltration at 0.2 MPa (30 lbf/in²) (Amicon PM 10 membrane). Centrifugation (12000g, 20 min) was necessary to remove a precipitate, which was shown by SDS/polyacrylamide-gel electrophoresis not to be the restriction enzyme.

Sephadex G-150 gel filtration. The concentrated phosphocellulose pool was loaded on a column (5 cm × 88 cm) of Sephadex G-150, and eluted at 45 ml/h with 20 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl. The main *Bst*I peak was obtained immediately after the void volume; less than 10% of the material penetrated the column beads and was eluted in a position corresponding to the dimer.

Hydroxyapatite column chromatography. The pooled fractions (194 ml, fraction V) from the Sephadex G-150 column were loaded, without dialysis, on a column (4.5 cm diameter × 3 cm) of hydroxyapatite in 20 mM-potassium phosphate, pH 7.5. After washing with starting buffer to remove non-bound material, bound protein was eluted at 150 ml/h with a linear gradient (400 ml) of 20–500 mM-potassium phosphate, pH 7.5. *Bst*I was eluted at 0.2–0.3 M-potassium phosphate, and enzyme activity coincided with the main *A*₂₈₀ peak.

Storage. The hydroxyapatite pool (37 ml, fraction VI) was concentrated 2-fold by ultrafiltration, and stored at –20°C in 20 mM-potassium phosphate, pH 7.5, containing 50% (v/v) glycerol.

Sequence analysis of the site of cleavage by endonuclease *Bst*I restriction digests. The reaction mixtures (110 µl) contained 10 mM-MgCl₂, 30 µg of *E. coli* DNA and either 20 units of *Bam*HI [units of *Bam*HI activity are defined in Wilson & Young (1975)] or 130 units (defined above) of *Bst*I. To check that no *Bst*I* digestion had occurred in the digest with *Bst*I, a control was set up containing an equal concentration of phage-λ DNA and the products were examined by agarose-gel electrophoresis.

For digestion under *Bst*I* conditions, the reaction mixture (125 µl) contained 0.1 M-Tris/HCl, pH 9, 10 mM-MgCl₂, 20 µg of phage-λ*prbt* DNA and 6500 units of *Bst*I. All digests were carried out for 1 h at 37°C.

5'-Terminal labelling of DNA fragments. The methods used were based on standard procedures (Murray, 1973). To each restriction digest was added 200 µl of a buffer containing 0.1 M-Tris/HCl, pH 9, and 10 mM-MgCl₂, followed by 17.5 µg of bacterial alkaline phosphatase. After a further 1 h incubation at 37°C, the DNA fragments were isolated by phenol extraction and ethanol precipitation. The DNA fragments were labelled for 2 h at 37°C in a reaction mixture (40 µl) containing 0.1 M-Tris/HCl, pH 7.5, 10 mM-MgCl₂, 20 mM-2-mercaptoethanol, 100 µCi of [γ -³²P]-ATP (1000–2000 Ci/µmol) and 4 units of polynucleotide kinase. The reaction was terminated by the addition of 0.2 M-EDTA (sodium salt), pH 7.5 (10 µl), and subsequent heating for 10 min at 60°C. Excess ATP

was removed by dialysis for at least 4 h against 0.4 M-NaCl/1 mM-EDTA (2×350 ml), followed by gel filtration on a column (0.8 cm $\times$ 29 cm) of Sephadex G-50 in 10 mM-Tris/HCl, pH 7.9, containing 0.1 M-NaCl and 1 mM-EDTA. DNA-containing fractions were located by applying samples (5 μ l) to 0.4% (w/v) agarose containing ethidium bromide (2 μ g/ml) and irradiating with short-wave u.v. light. Radioactivity was monitored by Čerenkov counting (Clausen, 1968). The labelled DNA fragments were isolated by ethanol precipitation and divided into four equal portions for further digestion.

Identification of the 5'-terminal nucleotide. Labelled DNA fragments, prepared as above, were digested for 16 h at 37°C in a reaction mixture (10 μ l) containing 10 mM-sodium acetate, pH 5.1, 10 mM-MgCl₂ and 1 μ g of bovine pancreatic DNAase. 0.1 M-Tris/HCl, pH 9 (2 μ l), and 1 μ g of venom phosphodiesterase were added and incubation was continued for a further 2.5 h. The mononucleotide products were analysed by electrophoresis on Whatman 540 paper in pyridine/acetate, pH 3.5, containing 1 mM-EDTA (Barrell, 1971), by using unlabelled deoxyribonucleotides (100 μ g of each) as u.v. markers. Radioactive deoxyribonucleotides were located by radioautography and quantified by dividing the relevant region into 1.5 cm squares, which were counted for radioactivity in a Nuclear–Chicago gas-flow counter.

Analysis of partial digests. Labelled DNA fragments, prepared as above, were digested for 1 h at 37°C in a reaction mixture (5 μ l) containing 0.1 M-sodium acetate, pH 5.1, 10 mM-MgCl₂ and 5 μ g of bovine pancreatic DNAase. The digestion products were analysed by electrophoresis on cellulose acetate at pH 3.5 in the presence of 7 M-urea, followed by homochromatography (Brownlee & Sanger, 1969) on layers of mixed cellulose and DEAE-cellulose (15:2, w/w) in homomix VI of Jay *et al.* (1974). Radioactive oligonucleotides were located by radioautography.

Results and Discussion

Purification of *Bst*I

The restriction enzyme *Bst*I was isolated from 70 kg of *B. stearothermophilus*, as described in the Materials and Methods section. This preparation is routinely used for the isolation of about 20 other enzymes of interest, which are separated from the restriction endonuclease in the initial DEAE-cellulose batch treatment (Atkinson, 1973). (When the restriction enzyme is one of the desired products of this multi-enzyme preparation, it is essential not to add DNAase at the initial cell-disruption stage. This enzyme is normally added to decrease the viscosity caused by released DNA, but can become an unwelcome and persistent contaminant in the restriction-enzyme preparation.)

The constraints of working on a large scale dictate the strategy of the early stages of the enzyme purification. As has been noted by Bruton *et al.* (1975), continuous-flow centrifugation is considerably less effective than centrifugation with a normal laboratory centrifuge. In the present work, the non-bound fraction remaining after the initial DEAE-cellulose treatment was too turbid for concentration with the hollow-fibre membranes. As its large volume ruled out (NH₄)₂SO₄ precipitation or column chromatography, a second batch adsorption and elution step was an essential preliminary to further processing. The phosphocellulose treatment was invaluable, not only giving considerable purification, but also in yielding an extract of sufficient clarity to be concentrated by ultrafiltration. The 22-fold decrease in volume achieved in this single purification stage meant that the following stages could be handled on a normal laboratory scale. The only disadvantage was that a portion of the enzyme (typically around 60%) persistently failed to bind to the phosphocellulose, resulting in low recovery at this stage. It seems likely that endogenous DNA in this extract competes with the resin for the enzyme binding sites. The presence of this DNA can be demonstrated on the agarose/ethidium bromide gels used in the enzyme assay.

Although these batch procedures are the only feasible method for the processing of very large volumes of extract, they are equally suitable for smaller volumes. This preparation may therefore be scaled-down for use with a few kilograms of cells or even less (with very small quantities, the separation of resin from extract may conveniently be achieved by filtration on a sintered-glass funnel). In fact, when this preparation was carried out on a smaller scale (5.5 kg of cells) the yield of enzyme activity in the initial stages was proportionately greater, since the time involved in centrifugation (at room temperature) was much less.

The progress of the purification is outlined in Table 1 and is further illustrated by SDS/polyacrylamide-gel electrophoresis of appropriate fractions (Fig. 1). The final pool exhibited one major protein band in this gel system, which was shown by densitometry to account for at least 97% of the protein. There was no detectable contamination by nucleic acid, as judged by the A_{280}/A_{260} ratio of 1.61.

The major protein component was identified as the restriction enzyme by the co-migration of enzyme activity with protein during polyacrylamide-gel electrophoresis under native conditions at pH 9.5 (Fig. 2), isoelectric focusing (Fig. 3) and gel filtration on Sephadex G-150, under conditions where the protein migrates near to the dimer position (results not shown).

When assayed with SV40-virus DNA under the standard conditions of Greene *et al.* (1974), 1 mg of pure *Bst*I catalysed the hydrolysis of 24 nmol of

Table 1. Purification of endonuclease *Bst*I from 70 kg of cells

Fraction	Step	Total protein (g)	$10^{-6} \times$ Total activity (units)	$10^{-3} \times$ Specific activity (units/mg)	Increase in specific activity (fold)	Yield (%)
I	DEAE-cellulose batch	412	140	0.345	1	100
II	Phosphocellulose batch	34	50	1.47	4.3	36
III	Ammonium sulphate	10	110	11.35	33	79
IV	Phosphocellulose column	0.355	51	142	412	36
V	Sephadex G-150	0.093	50	540	1565	36
VI	Hydroxyapatite	0.038	24	620	1797	17

Fig. 1. SDS/polyacrylamide-gel electrophoresis of fractions during the purification of *Bst*I

The following fractions were examined on a 12% (w/v) slab gel as described under 'Methods'. Lane 1, DEAE-cellulose non-bound fraction; lane 2, 400 mM-NaCl eluate from phosphocellulose; lanes 3 and 4, 50–70% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction; lane 5, pooled material from phosphocellulose column; lane 6, pooled material from Sephadex G-150 column; lane 7, pooled material from hydroxyapatite column. Lanes 1–3 represent approx. 100 units of endonuclease *Bst*I, and lanes 4–7 approx. 1000 units.

phosphodiester bonds/min. This compares with values in the range 20–50 nmol/min per mg variously reported for pure *Eco*RI (Greene *et al.*, 1974; Modrich & Zabel, 1976).

The small-scale isolation of homogeneous *Bst*I has been reported (Catterall & Welker, 1977). However, both the subunit mol.wt. (45000) and isoelectric

Fig. 2. Correspondence of *Bst*I activity and major protein species

Duplicate samples ($10 \mu\text{g}$) of pure *Bst*I were subjected to polyacrylamide-gel electrophoresis under native conditions as described in the Materials and Methods section. One sample track was stained and protein quantified by densitometry (b); the other was sliced into 3 mm segments, which were extracted and assayed for enzyme activity (a). Recovery was at least 20%. (Because of the small number of dilutions assayed for each sample, the activity plotted for the peak fractions is a minimum value.)

point (6.6–6.8) reported for this material differ substantially from those that we have observed for *Bst*I. It is possible that the product isolated by Catterall &

Fig. 3. Isoelectric focusing of pure *BstI*

After focusing of duplicate samples (20 μ g) of pure *BstI*, one sample lane was stained with Coomassie Blue (shaded areas); the other was sliced into 5 mm segments, which were extracted and assayed for enzyme activity. The horizontal arrow indicates those fractions yielding at least 200 units of *BstI*. The pH gradient (x) was determined as described under 'Methods'.

Welker (1977) contains a single major protein component, with the restriction enzyme as a minor contaminant. In this context, it may be significant that we have observed a predominant protein species at an early stage in our preparation (Fig. 1, lane 3); moreover, the subunit molecular weight and isoelectric point of this material are close to the values reported by Catterall & Welker (1977). The much higher yield and smaller number of purification stages required by these authors are also consistent with this interpretation.

Molecular-weight studies

SDS/polyacrylamide-gel electrophoresis of homogeneous *BstI* gave a value of 26 000 for the molecular weight of the enzyme subunit (Fig. 4). However, gel-filtration studies of the native enzyme did not yield an unequivocal result. Pure *BstI* was eluted from Ultrogel AcA 34 in a position corresponding to that of the monomer, whereas on Sephadex G-150 the elution position was closer to that expected for the dimer (Fig. 5). This suggests that the association between subunits may be weak and unusually susceptible to influence by experimental conditions.

During the purification of *BstI*, the enzyme was obtained at the void volume after gel filtration of the concentrated phosphocellulose pool (2.5×10^6 units/ml) on Sephadex G-150. This indicates an apparent mol.wt. in excess of 200 000. Dilution of this material caused the peak of enzyme activity to broaden and move to successively larger elution volumes, until, with the most dilute samples (6000 units/ml); the

Fig. 4. Determination of the subunit molecular weight of *BstI*

Electrophoresis was carried out on 12% (w/v) polyacrylamide gels in the presence of 0.1% (w/v) SDS. The following molecular-weight standards were used: pyruvate kinase (57 000), aldolase (40 000), D-amino acid oxidase (37 000), ribitol dehydrogenase (27 000), myoglobin (17 200) and lysozyme (14 300). The vertical arrow indicates the mobility of *BstI*.

restriction activity was found at the monomer position. However, no high-molecular-weight aggregates were observed when using the homogeneous enzyme, whose behaviour on gel filtration was different from that of the less-pure fraction, even when compared under identical conditions of enzyme concentration. It therefore seems likely that aggregation of *BstI* in the less-pure fraction is caused by trace amounts of nucleic acid or by a protein contaminant. Certainly this effect does not seem to be analogous to the aggregation observed for *EcoRI* (Modrich & Zabel, 1976).

The absence of high-molecular-weight aggregates from the homogeneous *BstI* preparation was confirmed by intramolecular cross-linking with dimethyl suberimide. The results were identical over the range of enzyme concentrations tested (0.1–1.0 mg/ml), and demonstrated the presence of the dimer but not of any higher oligomers (Fig. 6). It is tempting to speculate that the *BstI* dimer may be the catalytically active species, in view of the twofold symmetry of the DNA-recognition sequence, and that the unusually weak interaction between the subunits may play some significant role in the enzyme reaction.

Fig. 5. Gel filtration of pure *BstI*

Columns of Sephadex G-150 (1.6 cm $\times$ 80 cm) (●) and Ultrogel AcA 34 (1 cm $\times$ 75 cm) (○) were eluted at 10 ml/h with 20 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl, 10 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride. In each case 6.2×10^5 units of *BstI* were loaded in a volume of 1 ml. Protein markers were: 1, bovine serum albumin dimer (136 000); 2, aldolase (160 000); 3, ribitol dehydrogenase (108 000); 4, bovine serum albumin (68 000); 5, ovalbumin (43 000); 6, cytochrome *c* (11 700). The void volume (V_0) was determined with Blue Dextran. The vertical arrows mark the elution positions of *BstI* on the two columns.

Fig. 6. Protein cross-linking of *BstI*

Pure *BstI* (0.2 mg/ml) was treated with dimethyl suberimidate as described in the Materials and Methods section. The cross-linked products were separated by electrophoresis on a 10% (w/v) polyacrylamide gel in the presence of SDS, and located by densitometry of a stained gel. The following protein markers were used: bovine serum albumin (68 000), pyruvate kinase (57 000), ovalbumin (43 000), D-amino acid oxidase (37 000) and ribitol dehydrogenase (27 000).

Influence of reaction conditions on *BstI* activity

As shown in Fig. 7, *BstI* has a broad pH optimum between 7 and 9.5. The maximum enzyme activity is observed at Mg^{2+} concentrations in the range 0.5–2 mM (Fig. 8). In the absence of Mg^{2+} no activity can be detected. Mn^{2+} can replace Mg^{2+} , though it is about 10-fold less effective, whereas Ca^{2+} is ineffective.

Fig. 9 shows that the addition of even small amounts of NaCl causes a decrease in *BstI* activity, in contrast with the stimulatory effect of moderate concentrations of NaCl on some other restriction endonucleases (DeFilippes, 1974; Bron *et al.*, 1975).

As expected for an enzyme isolated from a thermophilic organism, *BstI* is active at temperatures up to at least 70°C. Higher temperatures were not tested, since the results of Catterall & Welker (1977) suggest that there would be significant losses in enzyme activity over the time period of the assay. In our assay system, the maximum activity was observed at 37°C (Fig. 10). This contrasts with the results of Catterall & Welker (1977), in which maximum enzyme activity was obtained at 65°C. However, in view of the substantial differences in the assay systems used, this discrepancy may not be surprising.

Fig. 7. pH-dependence of *BstI* activity

Each reaction mixture (25 μ l) contained 10 mM- $MgCl_2$, 0.1 μ g of phage- λ_{627} DNA and 0.1 M-buffer. The buffers used were succinic acid/NaOH, pH 5.5 and 6.0, imidazole/HCl, pH 6.5 and 7.0, Tris/HCl, pH 7.5, 8.0 and 8.5, and glycine/NaOH, pH 9.0 and 9.5. Incubation was carried out for 30 min at 37°C. The activity at each pH was determined by assaying a series of enzyme dilutions (see under 'Methods'), and is expressed relative to the activity under standard assay conditions (pH 7.5).

Fig. 8. Mg^{2+} -dependence of *Bst*I activity. Each reaction mixture (25 μ l) contained 0.1 M-Tris/HCl, pH 7.5, 0.1 μ g of phage- λ prbt DNA and various concentrations of $MgCl_2$. The activity at each concentration was determined as described in the legend to Fig. 7, and is expressed relative to the activity at 10 mM- $MgCl_2$. (The EDTA present in the DNA stock solutions would contribute a concentration of only 20 μ M-EDTA to the reaction mixture.)

Fig. 9. Effect of NaCl on *Bst*I activity. Each reaction mixture (25 μ l) contained 0.1 M-Tris/HCl, pH 7.5, 10 mM- $MgCl_2$, 0.1 μ g of phage- λ prbt DNA and various concentrations of NaCl. The activity at each concentration was determined as described in the legend to Fig. 7 and is expressed relative to the activity in the absence of NaCl.

DNA cleavage at secondary sites

When high concentrations of *Bst*I are used, cleavage of DNA occurs at sequences other than the primary *Bst*I sites (Fig. 11, lanes 1–7). The progress of this reaction has been followed by using the plasmid

Fig. 10. Temperature-dependence of *Bst*I activity. Reactions were carried out in the standard assay mixture, which was incubated for 30 min at various temperatures. The activity at each temperature is expressed relative to the activity at 37°C.

pBR 313, whose small size should result in a comparatively simple fragmentation pattern (Fig. 12). With an enzyme/DNA ratio of 3:1 (w/w), cleavage at the primary *Bst*I site occurred within 1 min. However, cleavage at secondary sites was considerably slower, and may not even have been complete after 2 days' reaction. This secondary enzyme activity is referred to as *Bst*I*, since it appears to be analogous to the *Eco*RI* activity observed by Polisky *et al.* (1975).

*Bst*I* activity is greater at high pH (Fig. 11, lanes 8–11) and decreases as the Mg^{2+} concentration is lowered below 10 mM (results not shown). The decrease in *Bst*I* activity observed on addition of NaCl (Fig. 11, lanes 12–16) is probably merely a consequence of the overall inhibition of enzyme activity by NaCl, discussed above. In contrast, there is a noticeable increase in *Bst*I* activity on addition of glycerol to the reaction mixture (Fig. 11, lanes 17–20), although glycerol has no effect on the rate of *Bst*I catalysis. The relaxation of specificity in the presence of glycerol suggests a role for hydrophobic interactions in the process of recognition, but not catalysis.

The implicit assumption that both the *Bst*I and *Bst*I* activities are properties of the same protein molecule is, we consider, justified by the fact that these two activities have failed to separate even after a lengthy purification procedure. Moreover, the analogous side-specificities of *Eco*RI and *Bsu*R, which

Fig. 11. Digestion of phage- λ prb1 DNA with high concentrations of *Bst*I

Lanes (1)–(7) show the effect of enzyme concentration. Each reaction mixture (13 μ l) contained 0.1 M-Tris/HCl, pH 9, 10 mM-MgCl₂, 2 μ g of phage- λ prb1 DNA and the following amounts of *Bst*I: (1), 1280 units; (2), 640 units; (3), 256 units; (4), 128 units; (5), 64 units; (6), 25.6 units; (7), 12.8 units. Lanes (8)–(11) show the effect of pH. Each reaction mixture (13 μ l) contained 10 mM-MgCl₂, 1 μ g of phage- λ prb1 DNA, 256 units of *Bst*I and 0.1 M-Tris/HCl at the following pH values: (8), pH 7.5; (9), pH 8.1; (10), pH 8.6; (11), pH 9.1. To eliminate the effects of changes in ionic strength, the buffers for lanes (9)–(11) contained sufficient added NaCl to equalize their conductivities with that of the pH 7.5 buffer. Lanes (12)–(16) show the effect of added NaCl. Each reaction mixture (11 μ l) contained 0.1 M-Tris/HCl, pH 9, 10 mM-MgCl₂, 1 μ g of phage- λ prb1 DNA, 256 units of *Bst*I and the following NaCl concentrations: (12), none; (13), 50 mM-NaCl; (14), 100 mM-NaCl; (15), 150 mM-NaCl; (16), 200 mM-NaCl. Lanes (17)–(20) show the effect of added glycerol. Each reaction mixture (11 μ l) contained 0.1 M-Tris/HCl, pH 9, 10 mM-MgCl₂, 1 μ g of phage- λ prb1 DNA, 128 units of *Bst*I and the following concentrations (v/v) of glycerol: (17), none; (18), 5%; (19), 10%; (20), 15%. All incubations were carried out for 1 h at 37°C. After quenching of the reaction by addition of SDS to 1% (w/v), the digestion products were examined by electrophoresis on a 0.7% (w/v) agarose gel, as described under 'Methods'.

Fig. 12. Time course of *BstI** digestion

The reaction mixture (0.5ml) contained 0.1M-Tris/HCl, pH9, 10mM-MgCl₂, 16μg of plasmid-pBR313 DNA and 25600 units of *BstI*. Samples (40μl) were removed at timed intervals during incubation at 37°C, and quenched by addition of SDS to 1% (w/v). The reaction products were examined by electrophoresis on a 1.2% (w/v) agarose gel.

are observed under experimental conditions similar to those described above, have both been shown to be intrinsic properties of the respective restriction endonucleases (Polisky *et al.*, 1975; Heininger *et al.*, 1977). The analogy between the *EcoRI*, *BsuR* and *BstI* systems is reinforced by nucleic acid-sequence data (see below), which demonstrate a similar relationship between primary and secondary sites for all three enzymes.

It seems unlikely that these reactions at secondary sites on the DNA molecule play a significant physiological role. Murray & Murray (1974) have demonstrated that the five *EcoRI* sites alone are important in governing the restriction of phage λ *in vivo*. It is more likely therefore that side reactions of this type are an artifact of the reaction *in vitro* when carried out under unusual conditions and in the presence of high concentrations of enzyme. (It may be significant that these aberrant effects have been observed only for those restriction endonucleases that have been extensively purified, and are therefore available as

highly active, exonuclease-free preparations.) On a practical level, such phenomena emphasize the importance of careful control over the composition of restriction digests, and particularly the avoidance of unnecessarily high enzyme concentrations.

Sequence studies of the site of cleavage by endonuclease *BstI*

It has been reported by Catterall & Welker (1977) that *BstI* is an isoschizomer of *BamHI*, which is known to recognize the sequence

cleaving the DNA in the positions shown by the arrows (Roberts *et al.*, 1977). A knowledge of the exact position of cleavage by *BstI* within this sequence, and in particular whether *BstI* generates fragments with cohesive termini, is of importance for the use of this enzyme for the construction of recombinant DNA molecules *in vitro*. We have investigated the site of cleavage by *BstI* by analysis of the sequences present at the 5'-termini of restriction fragments produced by *BstI*, after terminal labelling with polynucleotide kinase and [γ -³²P]ATP.

Since a hexanucleotide sequence will occur relatively infrequently in a given length of DNA, we were concerned lest sequence analysis at the labelled restriction sites should be confused by spurious labelling of pre-existing termini. For this reason, total *E. coli* DNA was used in these experiments (in preference to phage λ DNA), as the endogenous termini of the former should provide a background of random sequence that can easily be distinguished from the unique sequence at the restriction sites. For investigation of the sites of cleavage under *BstI** conditions we were able to use phage-λ DNA, since the greater abundance of *BstI** sites should decrease the possibility of confusion with unique sequences derived from labelling at the phage-λ cohesive termini.

Labelled fragments were prepared from DNA digested by *BstI*, under normal and *BstI** conditions, and, for the purpose of comparison, by a commercial preparation of *BamHI*. The nucleotide present at the 5'-terminus was identified by complete digestion, yielding mononucleotides that were separated by high-voltage paper electrophoresis. Table 2 shows the radioactivity associated with each mononucleotide in the three DNA samples. Although the unexpected abundance of pT in the samples digested with *BamHI* and *BstI* suggests that the original assumption, that labelling at pre-existing termini would be statistically random, may not have been

Table 2. *Radioactivity in 5'-mononucleotides*
Radioactivity is expressed as a percentage of the total c.p.m. recovered from each experiment. These totals were 15800 c.p.m. (*Bam*HI), 20800 c.p.m. (*Bst*I) and 54900 c.p.m. (*Bst*I*).

5'-Mononucleotide	Digest	...	Radioactivity (%)		
			<i>Bam</i> HI	<i>Bst</i> I	<i>Bst</i> I*
C			10.0	11.5	1.9
A			11.0	12.4	3.2
G			49.1	57.3	90.7
T			29.9	18.8	4.2

valid, these results clearly indicate that pG is the 5'-terminal mononucleotide in all three cases.

Further sequence data from the 5'-terminus were obtained from two-dimensional 'fingerprints' of partial DNAase digests of the labelled DNA fragments. Comparison of the 'map' obtained for the *Bam*HI digest with the published data of Roberts *et al.* (1977), by using an identical two-dimensional fractionation, allowed us to identify the oligonucleotides as shown in Fig. 13(a). [The additional oligonucleotides derived from labelling of pre-existing termini obscure the trinucleotide pGAT, which appeared only weakly in the 'map' of Roberts *et al.* (1977).]

The 'fingerprint' of the labelled fragments from the *Bst*I digest (Fig. 13b) is identical with that obtained for *Bam*HI, clearly demonstrating that the position of cleavage by *Bst*I is identical with that by *Bam*HI, i.e.:

*Bst*I therefore provides an alternative to *Bam*HI in the construction of recombinant DNA molecules *in vitro*.

The 'fingerprint' of the DNA from the *Bst*I* digest (Fig. 13c) is similar to that of the *Bst*I digest up to the tetranucleotide. The presence of at least four pentanucleotides suggests that the transition to *Bst*I* activity involves loss of specificity for the outermost two base-pairs of the site for cleavage by *Bst*I, so that the cleavage site becomes:

However, the situation may be more complex, since, although pGATC is the predominant tetranucleotide, the presence of other spots in this region of the

Fig. 13. *Two-dimensional fractionation of pancreatic-DNAase digests of 5'-terminally labelled restriction fragments*

For details see the Materials and Methods section. The restriction fragments were derived from *Bam*HI (a), *Bst*I (b) and *Bst*I* (c) digests. B and Y mark the positions of the blue and yellow marker dyes respectively.

chromatogram implies that *Bst*I* digestion conditions result in some relaxation of specificity even within the central tetranucleotide of the site of cleavage by *Bst*I.

Thus, for the three restriction endonucleases studied, digestion under conditions of diminished specificity results in contraction of the recognition site from a hexanucleotide to the central tetranucleotide for *Bst*I (at least for the majority of cleavages) and *Eco*RI (Polisky *et al.*, 1975), or from a tetranucleotide to the central dinucleotide for *Bsu*R (Heininger *et al.*, 1977). The similarity of these observations suggests a common mode of recognition between these enzymes and their DNA substrate, such that nucleotides near the centre of the recognition sequence are of primary importance. Furthermore, since the local environment of each cleavage site is capable of modulating the rate of reaction at that site (Thomas & Davies, 1975), one

Fig. 13(b)

Fig. 13(c)

can envisage a whole series of recognition points of decreasing importance extending from the centre of the recognition site.

The similarity of the *EcoRI*, *BstI* and *BsuR* systems also suggests that many more restriction endonucleases will be found to exhibit this type of decrease in specificity.

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