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Isoelectric Focusing of *Bacteroides melaninogenicus* Group β-Lactamases

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β-Lactamases extracted by sonication from the *Bacteroides melaninogenicus* group organisms (*B. asaccharolyticus*, *B. melaninogenicus*, *B. bivius*, and *B. oralis*) were found to be in the form of complexes with molecular weights of ≥40 × 10^6, and this resulted in failure to characterize them by isoelectric focusing. Purification by gel filtration in the presence of deoxycholate resulted in β-lactamase preparations from *B. bivius* with pI's of 5.7. A β-lactamase preparation extracted by osmotic shock from *B. bivius* also had a pI of 5.7. Osmotic shock preparations from *B. asaccharolyticus*, *B. melaninogenicus*, and *B. oralis* had two bands of equal intensity with pI's of 4.2 and 4.35.

In the past few years considerable work has been done on the β-lactamases from fragilis-like *Bacteroides* species (7, 14; R. Timewell, E. Taylor, and I. Phillips, J. Antimicrob. Chemother., in press) and from *B. fragilis* in particular (10, 11). There has been much less work done on the β-lactamases from the melaninogenicus group of organisms (*B. asaccharolyticus*, *B. melaninogenicus*, *B. bivius*, and *B. oralis*), and this probably reflects the greater difficulty of isolating and identifying these organisms and in obtaining measurable amounts of β-lactamase from them. Until recently, studies within this group had been confined to substrate profile and inhibitor sensitivity investigations, by which criteria several workers have shown that melaninogenicus group β-lactamases are different from those of the fragilis group (9, 12; Timewell et al., in press). Although the fragilis-like group of *Bacteroides* have been shown to produce β-lactamases with a variety of isoelectric points (2, 7, 14; Timewell et al., in press), there is little similar information on β-lactamases of the melaninogenicus group.

As part of a study of the most common clinical isolates of *Bacteroides*, we attempted to focus the β-lactamases produced by organisms in the melaninogenicus group and found that, by the usual enzyme preparation and isoelectric focusing (IEF) techniques, no measurable isoelectric point was obtainable (Timewell et al., in press). The object of the present study was to investigate these enzymes further.

MATERIALS AND METHODS

Organisms. The organisms used were isolated from clinical specimens in St. Thomas's Hospital and were identified as to species according to the criteria given elsewhere (6). They were all penicillin resistant on disk testing (2 U) and included four isolates of *B. bivius*, four of *B. asaccharolyticus*, three of *B. melaninogenicus* subsp. *intermedius*, two of *B. melaninogenicus* subsp. *melaninogenicus*, and three of *B. oralis*. All 16 isolates had penicillin minimal inhibitory concentrations in the range of 2 to 32 μg/ml. The organisms were stored in 10% glycerol broth on glass beads at −70°C (4).

Preparation of high-activity β-lactamase extracts. (i) Sonication. From a 24-h plate culture, five blood agar plates were inoculated by spreading and incubated anaerobically at 37°C for 20 to 24 h. *B. asaccharolyticus* and *B. melaninogenicus* isolates were grown on lysed blood agar for 48 h. All of the resulting growth was then removed, washed twice in 0.01 M phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (Sigma Chemical Co., Poole, England) (1), and finally resuspended in 2 ml of the same buffer. The cells were then sonicated in an MSE Ultrasonicator (total of 2 min at 6 μm peak to peak) in an ice bath, and cell debris was removed by centrifugation at 20,000 × *g* at 4°C for 45 min. The extract was tested for β-lactamase activity with the chromogenic cephalosporin nitrocefin (formerly 87/312; Glaxo Research Ltd., Greenford, England). On the basis of previous experience, we only used preparations that turned nitrocefin red in 30 s to 1 min, as these gave good IEF results.

(ii) Osmotic shock. Organisms from five blood agar plates (lysed blood agar for *B. asaccharolyticus* and *B. melaninogenicus* isolates) as described above were washed three times in 0.1 M phosphate buffer (pH 7.0). The cells were then osmotically shocked by the method described by Heppel (5) except that 30% sucrose in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2) was used at the shocking stage. The supernatant containing the enzyme was then concentrated in an Amicon concentrator (Amicon Corp., Lexington, Mass.), 0.1 M phosphate buffer (pH 7.0) containing 10 mM dithiothreitol was added to 10% of the total volume, and the preparation was stored at −20°C.

(iii) Freeze-press fracture. A heavy suspension of cells was prepared and washed as in section (i) above. The cells were then fractured by the X-press technique originally described by Edebo (3). After two passages of the cells through the X-press, 100% disintegration of the organisms was observed microscopically. The cell debris was then centrifuged, and the supernatant was taken as the β-lactamase extract.

Purification of β-lactamase preparations. (i) Preliminary investigation of β-lactamase extracts. Crude β-lactamase extracts were mixed with deoxyribonuclease (DNase), ribonuclease (RNase) (0.5 mg/ml, final concentrations), 2% Triton X-100, 1 M urea, 1% trypsin, 0.1% sodium periodate, and 1% sodium deoxycholate. The DNase, RNase, and trypsin mixtures were incubated at 37°C for 30 min, and the other mixtures were incubated at room temperature for 10 to 15 min.

(ii) Gel filtration of β-lactamase extracts. Preliminary attempts to purify the crude β-lactamase preparations involved treatment of the enzyme as above and elution through a column of Sepharose 2B (Pharmacia, Hounslow, England) with either 0.005% sodium dodecyl sulfate (SDS), 2 M urea, 0.05% octyl-β-D-glucopyranoside, 2% Triton X-100, or 1.5, 1.0, or 0.5% sodium deoxycholate. Fractionation was carried out by standard procedures with an LKB fraction collector and ultraviolet light (UV) flow monitor, with the UV absorption (254 nm) of each fraction recorded.

β-Lactamases were purified before IEF as follows. To 0.9 ml of crude β-lactamase preparation was added 0.1 ml of 20% sodium deoxycholate (BDH Chemicals Ltd., Poole, England). This was loaded on to a column of Bio-Gel A-5m (Bio-Rad Laboratories Ltd., Watford, England; 23 by 1.5 cm) and was eluted with 0.5% sodium deoxycholate in glycine-ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0) at 4°C. Fractions of 1-ml volume were collected, and β-lactamase activity was measured by mixing a 50-μl sample with 50 μl of nitrocefin (0.5 mg/ml) and measuring the time taken for the substrate to turn red. The fractions with the lowest molecular weight that showed β-lactamase activity were pooled and dialyzed overnight at 4°C in 0.01 M phosphate buffer (pH 7.0) (see Results). Dialyzed fractions were then filtered (Millipore Corp., Bedford, Mass.; 0.2-μm pore size) and stored at −20°C for isoelectric focusing. The glycine-EDTA buffer consisted of glycine (3.75 g/liter) and EDTA (0.375 g/liter) adjusted to pH 9.0 with 6 M sodium hydroxide.

Protein standards were eluted through the column under the same conditions to determine the approximate molecular weight of the treated β-lactamase.

IEF. IEF was carried out with a polyacrylamide gel of specification C = 3%, T = 5% as described by Matthew et al. (8) except that the acrylamide was polymerized by ammonium persulfate (0.15 mg/ml) and *N,N,N',N'*-tetramethylethylenediamine (0.325 mg/ml). Samples (40 μl) were loaded on the gel on Paratex strips (Paratex III/80; Lohmann KG, Fahl, Germany) approximately 15 mm from the cathode; 1 M sodium hydroxide and 0.04 M aspartic acid were the cathode and anode electrolytes, respectively. Focusing was carried out in an LKB Multiphor apparatus (LKB Instruments Ltd., Croydon, England) with the gel on a water-cooled heat-exchange plate, using a constant voltage of 300 V applied for 6 h.

After removal of the electrode strips, the pH gradient was measured with a microsurface electrode (Pye Unicam Ltd., Cambridge, England), and the β-lactamase bands were identified by flooding the gel with nitrocefin (0.5 mg/ml).

RESULTS

Preliminary purification investigations of β-lactamases. Figure 1 represents the UV absorption and β-lactamase activity profile after a crude untreated *B. bivius* or *B. asaccharolyticus* extract, prepared by sonication, was filtered through a Sepharose 2B column. The enzyme activity peak corresponded broadly with the first UV absorption peak. The molecular weight range of Sepharose 2B is 7×10^4 to 4×10^7, and the void volume of this column was 2.5 ml (4.5 fractions). Therefore, the approximate molecular weight of the material in fraction 6 was $\geq 40 \times 10^6$.

The UV absorption pattern and the enzyme activity peak were the same for SDS-, urea-, or octyl-β-D-glucopyranoside-treated *B. bivius* extracts as for untreated extracts (Fig. 1). However, after Triton X-100 treatment, two small UV absorption peaks were evident, and the β-lactamase activity was present in each with a blur of activity between the peaks.

β-Lactamase purification using deoxycholate. Figure 2 shows the UV absorption and

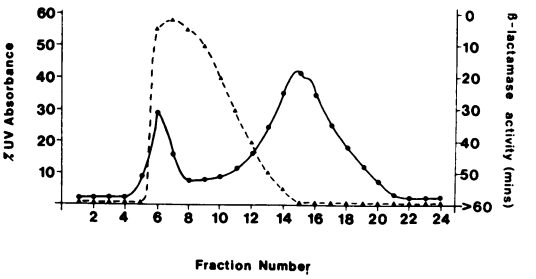

FIG. 1. *Sepharose 2B column chromatography of an untreated β-lactamase prepared by sonication of B. bivius. Symbols: (●) UV absorbance (254 nm); (▲) β-lactamase activity (time taken for β-lactamase to turn nitrocefin red).*

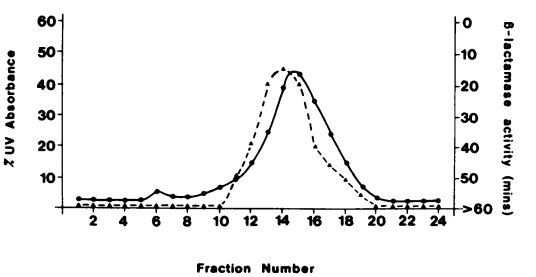

FIG. 2. *Sepharose 2B column chromatography of a deoxycholate-treated β-lactamase prepared by sonication of B. bivius. Symbols are as in Fig. 1.*

β-lactamase activity of fractions obtained from the gel filtration of the same crude sonicated extracts as in Fig. 1, but treated with sodium deoxycholate. The void volume peak of the untreated extract (Fig. 1) had almost disappeared, and the β-lactamase activity peak had shifted to correspond with the second UV absorption peak around fractions 14 and 15.

The maximum β-lactamase activity was noticeably reduced after deoxycholate treatment (untreated fraction 7 turned nitrocefin red in 2 min, whereas treated fraction 14 took 15 min) but was still high enough to be easily measurable. The *B. asaccharolyticus* extract showed very reduced β-lactamase activity after deoxycholate treatment.

A smaller-pore-size gel (Bio-Gel A-5m; molecular weight filtration range 10^4 to 5×10^6) was found to give better purification of the treated β-lactamase, with the enzyme activity in a fairly discrete peak around fractions 24 to 28. However, fractions 27 and 28 were taken for dialysis and subsequent IEF since earlier fractions were found to still show some streaking on focusing. Therefore, after the initial investigations, the Bio-Gel column was used for subsequent fractionations.

The β-lactamases from three isolates of *B. bivius* have so far been treated in this way without major loss of activity, but the β-lactamase of one other isolate was found to be deoxycholate sensitive (>90% loss of enzyme activity). The *B. asaccharolyticus* β-lactamase exhibited approximately 50% loss of enzyme activity after mixing with deoxycholate.

Molecular weight determination. Calibration of the Bio-Gel A-5m column with protein standards, under the same conditions as for the β-lactamase preparations, indicated that the molecular weights of the deoxycholate-treated *B. bivius* and *B. asaccharolyticus* β-lactamases were 30,000 to 40,000.

β-Lactamase activity in other extracts. The four isolates of *B. bivius* were each subjected to osmotic shock, but only one released a measurable amount of β-lactamase. This preparation was not active enough for successful gel filtration. A total of nine isolates of *B. asaccharolyticus/melaninogenicus* were examined for liberation of β-lactamase by osmotic shock. All nine provided various amounts of β-lactamase, and seven shockates were sufficiently active for IEF. Two of the three isolates of *B. oralis* investigated released measurable amounts of β-lactamase by osmotic shock.

The X-press-prepared β-lactamase, when filtered through the Sepharose 2B column, produced results the same as those from untreated extracts prepared by sonication (Fig. 1).

IEF. Table 1 summarizes IEF results. Attempts to focus (i) untreated extract, (ii) extracts incubated with DNase, RNase, trypsin, Triton X-100, urea, and sodium periodate, (iii) first-peak fractions from untreated extract gel filtration, (iv) SDS-, urea-, octyl-β-D-glucopyranoside-, or Triton X-100-treated gel filtration fractions, and (v) X-press-prepared extract all resulted in formation of a streak of activity across the gel from the loading site (approximately pH 9.0) to approximately pH 5.0 with no measurable bands.

From these results and those of the column chromatography investigations, it seemed most likely that, by a process of exclusion, the substance to which the enzyme was attached was carbohydrate, although we were unable to test this hypothesis directly by the techniques available to us.

Large amounts of activity were also found at the loading site both when Paratex strips were used and when samples were loaded directly on to the gel. Inclusion of 1% Triton X-100 in the gel before polymerization resulted in no measurable bands at all in tests or controls.

The β-lactamases from the *B. bivius* isolates that were treated with deoxycholate and purified and the β-lactamase from the osmotically shocked isolate all successfully focused with a pI of 5.7. The β-lactamases from the *B. asaccharolyticus/melaninogenicus* and *B. oralis* isolates prepared by osmotic shock focused with pI's of 4.2 and 4.35 (double line of equal activity). The *B. bivius* isolate that yielded β-lactamase by osmotic shock was included with the deoxycholate-treated preparations, and the pI's of the enzymes obtained by these two different routes were the same.

Attempts to focus crude *B. bivius* β-lactamase preparations treated with 1% deoxycholate resulted in a streak of activity between pH 9 and 5 as above, but with increased activity around pH 5.5. The deoxycholate in this preparation formed a large, white, opaque, rocket-shaped precipitate in the polyacrylamide gel from around pH 5 to the anode which prevented any higher concentrations from being used. Therefore, deoxycholate treatment of the crude β-lactamase preparation without the subsequent gel filtration purification stage was not satisfactory for accurate measurement of an isoelectric point.

DISCUSSION

The preliminary investigations of the β-lactamases from the melaninogenicus group organisms, prepared by sonication, indicated that the enzyme was tightly bound to some material which was fairly heterogeneous in molecular weight and overall molecular charge, thus the

TABLE 1. *Isoelectric focusing results of B. melaninogenicus group β-lactamases after various purification procedures*

Organism	β-Lactamase prepn	Purification of prepn with:	Sample used for IEF	Result of IEF
B. bivius	Sonication		Crude extract + DNase	Streak
	Sonication		Crude extract + RNase	Streak
	Sonication		Crude extract + trypsin	Streak
	Sonication		Crude extract + Triton X-100	Streak
	Sonication		Crude extract + urea	Streak
	Sonication		Crude extract + sodium periodate	Streak
	Sonication	SDS	Column fractions 6–8[a]	Streak
	Sonication	Urea	Column fractions 6–8	Streak
	Sonication	Octyl-β-D-glucopyranoside	Column fractions 6–8	Streak
	Sonication	Triton X-100	Column fractions 6–8	Streak and blur around pH 5.5
	Sonication	Deoxycholate	Column fractions 27–28[b]	pI 5.7
	Osmotic shock		Concentrated shockate	pI 5.7
	Freeze-press fracture		Crude X-press extract	Streak
B. asaccharolyticus	Sonication	Deoxycholate	Column fractions 27–28[b]	Enzyme deoxycholate sensitive
	Osmotic shock		Concentrated shockate	pI 4.2, 4.35
B. melaninogenicus subsp. *intermedius*	Osmotic shock		Concentrated shockate	pI 4.2, 4.35
subsp. *melaninogenicus*	Osmotic shock		Concentrated shockate	pI 4.2, 4.35
B. oralis	Osmotic shock		Concentrated shockate	pI 4.2, 4.35

[a] See Fig. 1.
[b] See Results.

streak on focusing crude extracts. This material seemed not to be deoxyribonucleic or ribonucleic acid fragments or protein, but by a process of exclusion was probably some form of lipopolysaccharide. However, it seems unlikely that the β-lactamase was attached to the material within the intact bacterial cell since release of periplasmic space contents by osmotic shock (11, 13) liberated the enzyme in pure "focusable" form.

We have shown the molecular weight of the enzyme complex to be greater than 40×10^6. Therefore, since the enzyme is possibly attached to lipopolysaccharide, it may be that the hydrophobic ends of these molecules associate with the β-lactamase molecules forming micelles; this would explain the high molecular weight. Treatment with deoxycholate (a detergent) would therefore break up the micelles, and fractionation in a deoxycholate solution would allow the separation of the complex material from the β-lactamase.

Purification of *B. bivius* β-lactamase with deoxycholate freed the enzyme for IEF and showed its molecular weight to be 30,000 to 40,000, which is approximately the molecular weight that other *Bacteroides* β-lactamases have been found to have (1, 11). Also, one isolate of *B. bivius* was used to prepare β-lactamase in two ways (osmotic shock and sonication followed by purification with deoxycholate), and both preparations gave identical isoelectric points. These facts suggest that the β-lactamases we used for IEF in this part of the study were uncontaminated.

The only report of IEF of melaninogenicus group β-lactamases, by Sherrill and McCarthy (13), indicated that they were all the same. However, in the same paper, the authors were unable to distinguish fragilis-like β-lactamases by IEF, which disagrees with our previous findings and those of others (2, 7). Those results do agree with the findings of Tally et al. (14), although their pI's for fragilis-like β-lactamases were different from those of Sherrill and McCarthy.

The *B. bivius* β-lactamases had a completely different isoelectric point from the rest of the

β-lactamases in the melaninogenicus group, and the *B. oralis* enzyme, although having the same isoelectric point as the *B. asaccharolyticus/melaninogenicus* enzymes, had a completely different substrate profile (Timewell et al., in press). These isoelectric points are also different from any seen so far in the fragilis group.

ACKNOWLEDGMENT

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