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Purification by Affinity Chromatography and Properties of a β-Lactamase Isolated from *Neisseria gonorrhoeae*

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β-Lactamase activity was detected in cell-free preparations obtained from ultrasonic treatment of *Neisseria gonorrhoeae* after growth in liquid medium. Crude preparations of β-lactamase were subjected to affinity chromatography, using several β-lactam antibiotics as ligands bound to agarose supports. Affinity gels produced by coupling 7-aminocephalosporanic acid or 6-aminopenicillanic acid by their amino groups to carboxyl-terminal agarose via a five- to eight-carbon spacer arm proved to be effective chromatography media. β-Lactamase preparations subjected to chromatography using these gels were purified 200-fold, with approximately 80% recovery of active material. Purified preparations were judged homogeneous by their behavior during electrophoresis on polyacrylamide in both the presence and absence of sodium dodecyl sulfate. Characterization of the purified enzyme established a molecular weight of approximately 25,000 and an isoelectric point of 5.4. Analyses of substrate specificity, effect of inhibitors, pH, and kinetic parameters were performed. The evidence suggests that the β-lactamase produced in *N. gonorrhoeae* closely resembles the character of class IIIa (TEM-type) β-lactamases.

Although *Neisseria gonorrhoeae* has been traditionally regarded as susceptible to β-lactam antibiotics, resistance due to the production of a β-lactamase has recently been observed among strains isolated from diverse geographic locations (2, 17). This resistance to β-lactam antibiotics has been shown to be plasmid mediated (7), and R-factors responsible for specifying β-lactamase production have been investigated (19). It has been suggested that the plasmids in *N. gonorrhoeae* responsible for β-lactamase production are very similar to plasmids specifying a TEM-type β-lactamase in *Haemophilus influenzae* (22) in terms of size, Tn*A* segment homology, and guanine plus cytosine mole-fraction content (19). In addition, it has been suggested that although the R-plasmids detected in different strains of *N. gonorrhoeae* from different geographical locations may represent independent events, a common ancestral origin is probable (7). To gain insight into the nature and origin of the R-plasmids in *N. gonorrhoeae*, it is necessary to examine the molecular properties of the β-lactamase produced. Methods of purification of β-lactamases, using ion-exchange and permeation chromatography (20, 26), require multiple steps and are not suitable for use with small samples. Affinity chromatography on various supports utilizing cephalosporin C (4), ampicillin (11), and methicillin (3) as ligands have been investigated, but these methodologies have not been applied to the purification of *N. gonorrhoeae* β-lactamase. This report deals with the establishment of a suitable means for purifying small quantities of β-lactamase from *N. gonorrhoeae* and the partial characterization of the purified enzyme.

MATERIALS AND METHODS

Organisms. Strains of β-lactamase-producing *N. gonorrhoeae* were a gift of Clyde Thornsberry, Center for Disease Control, Atlanta, Ga. Cultures were maintained frozen (−70°C) in Trypticase soy broth–skim milk–rabbit serum (1 ml:0.75 ml:0.2 ml).

Chemicals. Cyanogen bromide (CNBR)-activated Sepharose 4B, AH-Sepharose 4B, QAE-Sephadex, diethylaminoethyl-Sephadex, and Sephadex G-100 were products of Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Affi-Gel 201, ampholytes, and chromatography columns were obtained from Bio-Rad Laboratories, Rockville Center, N.Y. 1-Ethyl-(3-dimethylaminopropyl)-carbodiimide, CNBR, tris(hydroxymethyl)aminomethane (Tris) base, and 6-aminopenicillanic acid (6-APA) were purchased from Sigma Chemical Co., St. Louis, Mo. Chromogenic cephalosporin 87/312 was a gift of Glaxo Research Ltd., London, England.

Growth of microorganisms. Growth from chocolate agar plates incubated for 18 to 24 h in 5% CO_2 at 35°C was inoculated into 50 ml of liquid contained in 250-ml Erlenmeyer flasks. The medium was based on the following modifications of the medium described by Jones and Tally (9), per liter: glucose, 5.0 g; peptone no. 3 (Difco), 15.0; K_2HPO_4, 4.0 g; KH_2PO_4, 1.0 g;

$NaHCO_3$, 0.5 g; supplement B (Difco), 10 ml. The medium was sterilized by filtration. Flasks were incubated at 35°C on a rotary shaker operated at 80 rpm. After 20 h of incubation, bacteria were harvested by centrifugation at 5,000 rpm at 20°C.

Production of crude β-lactamase. Harvested cells were washed twice in 0.85% NaCl and suspended in 4.0 to 8.0 ml of 0.01 M phosphate buffer, pH 6.8. The suspension was cooled to 4°C and disrupted with the aid of a Branson Sonifier (model 200) equipped with a microtip. The instrument was operated at 20,000 Hz and was used in a pulsed 40% duty cycle at a power setting of 100 W. Disruption was carried out for 6 to 10 min with continuous cooling. The resulting sonic extract was subjected to centrifugation at 20,000 rpm for 30 min at 4°C. The supernatant fraction was stored at −20°C and constituted the crude β-lactamase preparation.

Enzyme assays. β-Lactamase was routinely assayed by the spectrophotometric method of Samuni (21). In some cases, iodiometric (15) or colorimetric (8) assays were carried out.

Enzyme units. One unit of β-lactamase activity was defined as that amount of enzyme which would hydrolyze 1.0 μmol of β-lactam antibiotic per min at 25°C and pH 6.8. The specific activity was expressed in terms of units per milligram of protein. Protein was measured spectrophotometrically (24) or by the method of Lowry et al. (13), using bovine serum albumin as the standard.

Preparation of affinity columns. Affinity chromatography columns were prepared as follows.

(i) Affi-Gel 201-β-lactam antibiotic columns. Affi-Gel 201, a carboxyl-terminal, cross-linked beaded agarose with an eight-atom spacer arm, was coupled to the appropriate β-lactam antibiotic containing a reactive amino group adjacent to the β-lactam ring through a carbodiimide condensation (12). The final concentrations of 1-ethyl-(3-dimethylaminopropyl)-carbodiimide and β-lactam ligand were 20 and 10 mg/ml of gel, respectively. The mixture was placed on a reciprocating shaker at 100 rpm for 18 h at 25°C. Ethanolamine (50 mg/ml of gel) and 1-ethyl-(3-dimethylaminopropyl)-carbodiimide (50 mg/ml of gel) were added, the pH was corrected to 4.5, and the mixture was incubated for an additional 6 to 10 h at 25°C. The resulting gel was washed by filtration, using distilled water (4.0 liters)–1 M NaCl (1.0 liter)–distilled water (2.0 liters), and equilibrated with appropriate buffer.

(ii) AH-Sepharose 4B-β-lactam antibiotic columns. Columns of β-lactam antibiotic bound to AH-Sepharose 4B (amino-terminal six-carbon spacer arm) were prepared in a manner similar to that described for Affi-Gel 201 columns, except that β-lactam antibiotics containing a reactive carboxyl group were subjected to coupling and acetate was used to block excess functional groups (12).

(iii) CNBR-activated Sepharose 4B-β-lactam antibiotic columns. Cephalosporin C, 7-aminocephalosporanic acid (7-ACA), 6-APA, or other β-lactam antibiotics were coupled to CNBR-activated Sepharose as previously described (4). The final concentration of β-lactam in the coupling mixture was 5.0 mg/ml. The coupled gel was washed with 0.1 M Tris-hydrochloride buffer (pH 8.0) and suspended in Tris buffer for 2 h at room temperature.

Table 1 lists the affinity absorbents prepared and investigated. The degree of substitution of the gel by the ligands was estimated by reaction of the gel with 2,4,6-trinitrobenzene sulfonate, differential absorbance at 240 to 260 nm, or by reaction with starch–iodine after treatment with strong alkali.

Gel electrophoresis. Disc electrophoresis was carried out as described by Davis (6). Sodium dodecyl sulfate-gel electrophoresis and staining methods were performed as described previously (16). Fluorescent sodium dodecyl sulfate-gel electrophoresis was accomplished by using the methods of Talbot and Yphantis (23).

Isoelectric focusing. Gel isoelectric focusing was performed as described by Wrigley (25). Column electrofocusing in a sucrose gradient was carried out by using the methods of Kint (10).

RESULTS

Affinity chromatography. Samples of crude β-lactamase preparations (usually 3.0 to 7.0 ml; 10 to 25 mg of protein) were applied to affinity gel columns (1.5 by 10 cm) at 4°C. After the sample had entered the gel bed, the gel surface was washed with equilibrating buffer, and primary elution was initiated. Columns were eluted at a flow rate of 0.5 ml/min, and fractions of 4.0 ml were collected and assayed for protein and enzymatic activity. Active fractions were pooled and concentrated with the aid of an

TABLE 1. *Supports, ligands, and coupling methods used to prepare affinity matrices*

Support	Ligand	Coupling method	Affinity matrix	μmol of ligand/ml of gel
Affi-Gel 201	Ampicillin	EDC[a]	AG-201-AMP	1.2–1.6
Affi-Gel 201	6-APA	EDC	AG-201-6APA	2.2–2.6
Affi-Gel 201	7-ACA	EDC	AG-201-7ACA	2.0–2.3
AH-Sepharose 4B	Ampicillin	EDC	AH-Seph-AMP	1.2–1.6
AH-Sepharose 4B	Methicillin	EDC	AH-Seph-METH	1.9–2.4
AH-Sepharose 4B	6-ACA	EDC	AH-Seph-6APA	1.5–1.8
CNBR-Sepharose 4B	Cephalosporin C	CNBR	CNBR-Seph-CC	2.1–2.6
CNBR-Sepharose 4B	7-ACA	CNBR	CNBR-Seph-7ACA	2.2–2.6
CNBR-Sepharose 4B	6-APA	CNBR	CNBR-Seph-6APA	1.8–2.4

[a] EDC, 1-Ethyl-(3-dimethylaminopropyl)-carbodiimide.

Amicon ultrafiltration cell (model 10-PA) equipped with a PM-10 membrane. Each affinity column was subjected to two elution schemes. Elution method A consisted of the use of 0.05 M Tris-phosphate buffer (pH 6.8) for equilibration and primary elution, followed by the same buffer containing 0.8 M NaCl as secondary eluent. Elution method B made use of 0.1 M acetate buffer (pH 4.4) for equilibration and primary elution, followed by 0.1 M phosphate buffer (pH 6.8) as secondary eluent.

The results of purification of *N. gonorrhoeae* β-lactamase by various affinity supports are summarized in Table 2. Substitution of AH-Sepharose 4B by β-lactam antibiotics containing a reactive carboxyl group resulted in affinity gels which retained enzyme activity as well as nonspecific protein. In most cases β-lactamase activity was partitioned between passage through the column and absorption, with an approximately 60 to 70% recovery. The use of elution method B in comparison to elution method A resulted in a lesser amount of nonspecific protein binding to the AH-Sepharose 4B-substituted columns. The elution patterns obtained using various β-lactam antibiotics bound to AH-Sepharose 4B were very similar, and differences due to the nature of the affinity ligand were not observed.

Coupling of 6-APA to Sepharose 4B via its free amino group without a spacer arm (CNBR-Seph-6APA) resulted in an affinity support which demonstrated poor purification qualities (Fig. 1A). In contrast, the use of an eight-carbon spacer arm and coupling via the 6-amino group of 6-APA (AG-201-6APA) provided an affinity support which behaved similarly to a cephalosporin C-substituted agarose (CNBR-Seph-CC). The 6-APA-substituted gel demonstrated a capacity to bind nonspecific protein, using elution method A (Fig. 1B). The use of elution method B dramatically decreased nonspecific binding as well as β-lactamase recovery (Fig. 1C). Cephalosporin C coupled to Sepharose 4B via its L-aminoadipyl side chain amino group (CNBR-Seph-CC) provided a fairly effective affinity support when elution method B was used for chromatography. The use of elution method A resulted in β-lactamase activity being retarded, but not retained, by the column. Affinity gels prepared using 7-ACA with (AG-201-7ACA) and without (CNBR-Seph-7ACA) an eight-carbon spacer arm behaved in a manner similar to gels prepared using 6-APA. The use of 7-ACA linked to Affi-Gel 201 via its free amino group as affinity support and chromatography using elution method B demonstrated the highest purification capability of the gels tested (Table 2). Approximately 200-fold purification with over 80% recovery of activity was routinely achieved. The cephalosporin-substituted gels were found to be substantially more stable and resistant to enzymatic and spontaneous hydrolysis than penicillin-substituted gels.

Polyacrylamide electrophoresis. During gel electrophoresis material purified by affinity chromatography using AG-201-7ACA, AG-201-6APA, and, in some cases, CNBR-Seph-CC as affinity supoorts was shown to migrate as a single band of active protein. In contrast, material purified by the remainder of the affinity supports tested demonstrated multiple protein species. Purified β-lactamase isolated by the use of an AG-201-7ACA affinity column migrated as a single entity during electrophoresis in the presence of sodium dodecyl sulfate coelectrophoresed with a chymotrypsinogen marker protein, corresponding to a molecular weight of approximately 25,000.

Molecular-weight estimation. The molecular weight of the β-lactamase purified using a AG-201-7ACA column was estimated by gel permeation chromatography (1). The apparent molecular weight was estimated to be 25,000.

Characterization. The substrate profile of purified β-lactamase from *N. gonorrhoeae* is as follows. Rates of hydrolysis, relative to benzyl-

TABLE 2. *Purification of N. gonorrhoeae β-lactamase using various affinity matrices*

Affinity matrix	Applied to affinity support			Concentrated secondary eluate			Recovery (%)	Purity (fold)
	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Total protein (mg)	Total activity (U)	Sp act (U/mg)		
AG-201-7ACA	21.4	4.96	0.22	0.09	3.93	43.6	79	189
AG-201-6APA	21.9	6.62	0.30	0.18	5.83	32.5	80	108
AG-201-AMP	18.7	4.22	0.23	3.11	2.13	0.7	50	3
AH-Seph-AMP	11.4	3.02	0.26	8.70	3.10	0.4	67	2
AH-Seph-METH	15.1	3.91	0.26	1.02	0.71	0.7	18	3
AH-Seph-6APA	16.7	3.92	0.23	1.26	1.08	0.8	28	4
CNBR-Seph-CC	22.8	5.10	0.27	0.11	4.08	36.1	88	131
CNBR-Seph-4ACA	21.4	4.96	0.23	1.19	3.93	3.3	79	14
CNBR-Seph-6APA	12.9	3.60	0.28	1.47	1.40	3.8	39	14

penicillin, were (in percent): benzylpenicillin, 100; ampicillin, 130; methicillin, 4; oxacillin, 4; carbenicillin, 45; amoxicillin, 14; 6-APA, 47; cephaloridine, 112; and cephalosporin C, 10. Assays contained approximately 0.4 U of activity. The inhibition profile was as follows: cloxacillin, susceptible; *p*-chloromercuribenzoate, resistant; NaCl, resistant. Concentrations of inhibitors were 10^{-3} M. The apparent K_m values were 33 μmol for penicillin G and 38 μmol for ampicillin as substrate. V_{max} values were 434.7 and 565.0

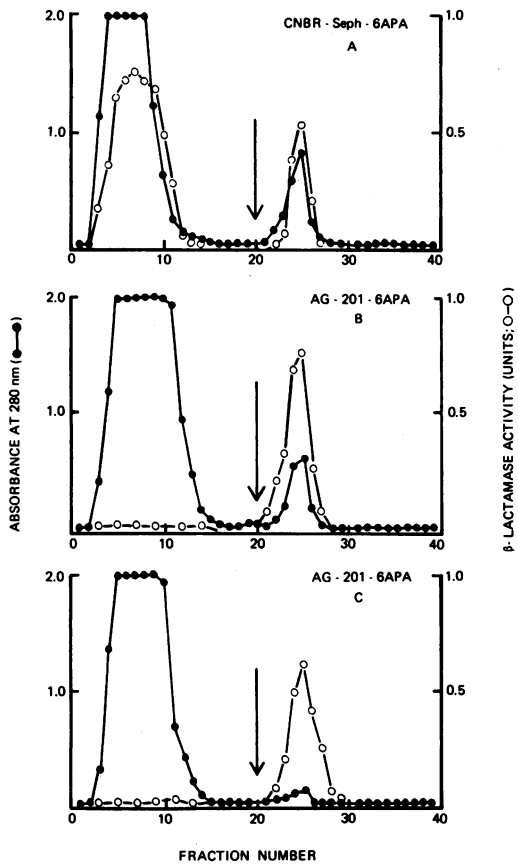

FIG. 1. *Affinity chromtography elution profiles of N. gonorrhoeae β-lactamase preparations, using 6-APA as ligand. Fractions of 4.0 ml were collected at a flow rate of 0.5 ml/min and monitored for protein (●) and activity (○). Primary elution was carried out using 0.05 M Tris-phosphate buffer (pH 6.8) followed by the secondary elution in the same buffer containing 0.8 M NaCl. The arrow indicates the buffer change fraction. Curve A was generated using 6-APA coupled to Sepharose via a CNBR reaction (CNBR-Seph-6APA), curve B using 6-APA coupled to Affi-Gel 201 via a carbodiimide reaction (AG-201-6APA), and curve C using 6-APA as in curve B except that elution scheme B was carried out.*

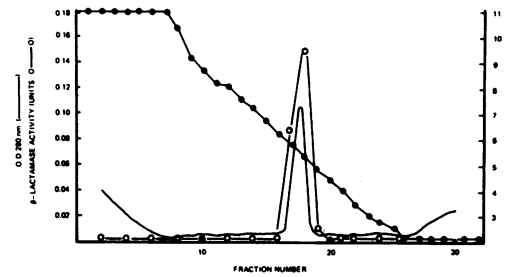

FIG. 2. *Isoelectric focusing of purified β-lactamase from N. gonorrhoeae. Electrofocusing was performed in a sucrose gradient, using pH 3 to 10 ampholytes, for 18 h. Fractions of 0.5 ml were collected and monitored for protein (—), enzyme activity (○), and pH (●).*

μmol/min per mg for penicillin G and ampicillin, respectively. The effect of pH on enzymatic activity was investigated. β-Lactamase activity was detected between pH 4.0 and 8.5, reaching a maximum value at pH 6.8 to 7.2.

Isoelectric focusing. Purified β-lactamase obtained from affinity chromatography using an A6-201-7ACA affinity column was subjected to isoelectric focusing in a sucrose gradient (Fig. 2) and in polyacrylamide gels. Based on these analyses, a value of 5.4 was estimated as the isoelectric point of the enzyme.

DISCUSSION

The emergence of plasmid-mediated β-lactam antibiotic resistance in *N. gonorrhoeae* has established the need for the investigation of the nature of the β-lactamase produced. Affinity chromatography systems were investigated for their ability to purify small quantities of β-lactamase from crude mixtures. In many cases, the free amino group of the bound β-lactam antibiotic induced ion-exchange properties in the gels which could be reduced, but not eliminated, by buffer systems of appropriate pH values or by blocking excess functional groups. Coupling of ampicillin or methicillin via carboxyl groups produced gels which demonstrated various degrees of hydrophobic interactions during chromatography, again depending upon the buffer systems used. Compared to gels prepared by coupling ligand carboxyl to gel amino groups, gels synthesized by coupling ligand amino groups to carboxyl-terminal agarose with or without an eight-carbon spacer arm demonstrated an increased ability to specifically absorb β-lactamase activity. Gels without spacer (CNBR-Seph-6APA) were shown to absorb β-lactamase poorly. Gels incorporating a spacer arm demonstrated a higher degree of purification of crude enzyme.

The evidence suggests that the β-lactam substrate is probably inaccessible to the binding site of the enzyme without a spacer arm and that the specific nature of the 6-amino-substituted side chain plays a small part in affinity absorption. Penicillin-coupled gels demonstrated a rapid loss of binding capacity due to apparent on-column hydrolysis. The use of cephalosporin C bound to Sepharose as an affinity support proved to be an efficient gel but was considered inferior to 7-ACA bound to a spacer gel by virtue of the interference in coupling and binding of the α-aminoadipyl side chain carboxyl group. The use of 7-ACA coupled to a carboxyl-terminal eight-carbon spacer arm agarose as affinity medium and a buffer system consisting of 0.05 M Tris-phosphate buffer (pH 6.8) as primary eluent, followed by the same buffer containing 0.8 M NaCl as secondary eluent, was shown to be the most efficient affinity system investigated. The column was stable over a period of several weeks, and a purification of approximately 200-fold with an 80% recovery of activity was routinely achieved. Carbodiimide coupling of 7-ACA and the absence of an aminodipyl side chain carboxyl group increase the amount of ligand that can be bound to the gel, provide a system capable of engaging several coupling methods and spacer arm lengths, and eliminate the interference of nonessential carboxyl groups.

Preparations of β-lactamase purified by affinity chromatography using AG-201-7APA, AG-201-6APA, and, in some cases, CNBR-Seph-CC gel columns were shown to migrate as a single entity during polyacrylamide disc electrophoresis. In many cases, the same preparation, when subjected to electrophoresis in the presence of sodium dodecyl sulfate, revealed two smaller proteins of very low molecular weight. A subunit structure of β-lactamase enzymes with molecular weights in the range of 20,000 to 27,000 has not been reported, although the dimeric nature of a 41,000-molecular-weight, R-factor-mediated β-lactamase has been described (5). It has also been reported (4) that upon affinity chromatography contaminating protein consistently remained with a purified plasma membrane β-lactamase from *Bacillus licheniformis*. In a similar manner, the microheterogeneity observed during isoelectric focusing of β-lactamases (14) may be a result of various impurities complexed with native β-lactamase active protein. The association of β-lactamase with membranes and cell envelope may provide the opportunity for the acquisition of heterogeneous material by the enzyme. Purified β-lactamase from *N. gonorrhoeae* migrated as a major band with an occasional minor band during isoelectric focusing. An isoelectric point of 5.4 was estimated and compared favorably with the isoelectric points of several class IIIa β-lactamases (14, 18), including those isolated from *H. influenzae* (23). This behavior is comparable to that of a TEM-1-type enzyme (14). Analysis of active material purified by cephalosporin–agarose affinity chromatography demonstrated a pattern of several properties and characteristics similar, if not identical, to the R-factor-mediated β-lactamases of class IIIa of Richmond and Sykes (18).

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