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ORIGINAL ARTICLE

Purification and characterization of ß-lactamase from *Neisseria gonorrhoeae* from clinical samples

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ABSTRACT. ß-lactamase was isolated from *Neisseria gonorrhoeae*, obtained from male patients with gonococcic urethritis. Biochemical properties of the enzyme were studied. The enzyme was purified 38-fold by ammonium sulphate precipitation and using Sephadex G75 and DEAE-cellulose columns. The purified extract exhibited a single band by polyacrylamide gel electrophoresis. Maximum enzyme activity was obtained at 37°C and pH 7.0-7.2 in 50 mM phosphate buffer. Addition of Ni^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and p-chloromercurybenzoate to the reaction buffer partially inhibited ß-lactamase activity, whereas Hg^{2+} and EDTA produced complete inhibition. The molecular weight was estimated to be 35,000 Da and the pI of the enzyme was 5.4.

Key words. *Neisseria gonorrhoeae;* ß-lactamase.

RESUMEN. Una ß-lactamasa fue obtenida de *Neisseria gonorrhoeae*, aislada de pacientes masculinos cun uretritis gonocócica. Las propiedades bioquímicas de la enzima fueron estudiadas. La enzima fue purificada 38 veces usando precipitación con sulfato de amonio, y columnas de Sephadex G-75 y DEAE-celulosa. El extracto purificado exhibió una sola banda por electroforesis en gel de poliacrilamida. La máxima actividad enzimática fue obtenida a 37°C y pH 7.0-7.2 en regulador de fosfatos 50 mM. La adición de Ni^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and p-chloromercurybenzoate al regulador de la reacción inhibió parcialmente la actividad ß-lactamasa, mientras que el Hg^{2+} y el EDTA produjeron una inhibición completa. El peso molecular de la enzima se estimó en 35,000 Da y el pI fue de 5.4.

Palabras clave. *Neisseria gonorrhoeae;* ß-lactamasa.

INTRODUCTION

Since the first report of Abraham and Chain1 on the production of ß-lactamase by Escherichia *coli, it* has been considered that the enzyme plays a significant role in the resistance of organisms to ß-lactam antibiotics.[11,18,23] There are numerous reports on the synthesis of ß-lactamase by Gram-positive and Gram-negative bacteria, including *Neisseria gonorrhoeae.* Initially, penicillin became the drug of choice for treatment of infections due to this microorganism. Remarkably small doses were used to cure gonorrhoea, reflecting the high susceptibility of the gonococcus to penicillin. In the next decades a gradual increase in gonococcal resistance to penicillin was documented in the United States and elsewhere, resulting in an increase in the penicillin dose.

The emergence of penicillinase-producing *N.gonorrhoeae* (PPNG) in 1976 has led to widespread high-level penicillin resistance.[3,4,22,28] These strains harbour various types of resistant plasmids and have been discovered at several international locations.[6,24]

In this study we describe the purification, properties and characterization of ß-lactamase obtained from N.gonorrhoeae isolates from urethral exudates from male patients suffering from gonococcic urethritis in Tucumán, Argentina.

MATERIAL AND METHODS

Microorganisms. Strains of *N. gonorrhoeae resistan*t to penicillin were isolated from urethral exudates from male patients with a clinical diagnosis of acute urethritis. They were kept in Trypticase Soy Broth with 25% glycerol (v/v) and stored at -70ºC.

Culture media. Thayer Martin (GC-Difco) medium, supplemented with Vitox (cod SR 90-Oxoid). SJ-GC medium[27] used for the growth of the microorganism was as follows.

Base medium (in grams per 100 ml of water): protease peptone Number 3 (Difco), 15; K_2HPO_4, 4; KH_2PO_4, 1; NaCl, 5; soluble potato starch (Sigma), 1.

Supplement A: cocarboxylase (Sigma), 0.01 g/l; glutamine, 5 g/l; glucose, 200 g/l.[13]

Supplement B: glucose, 400 g/l; casamino acids, 110 g/l; cysteine-hydrochloride, 4 g/l; $MgSO_4$, 60 g/l.

To 100 ml of the base medium, 2 ml of supplement A and 5 ml of supplement B were added; final pH was adjusted to 7.2.

Penicillin resistance. Minimum inhibitory concentrations were determined by a standard twofold serial dilution method.[10]

ß-lactamase production was tested by the cephalosporin chromogenic method, using Nitrocefin (Sefinare, Glaxo Research LTD, Greenford, Middlesex, England) with Haemophylus influenzae ATCC 10211 as a negative control; E. coli ATCC 35218 was used as a positive control.

ß-lactamase assaying. Enzyme activity was determined by UV spectrophotometry,19,31 measuring the change in cephaloridine concentration. Hydrolysis of the ß-lactamic ring was measured through a decreasing optical density of the antibiotic solution. The reaction was initiated by addition of 10 µl of the enzyme extracts to 2 ml of 0.1 mM ce-

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phaloridine in 50 mM phosphate buffer (Na_2HPO_4 – NaH_2PO_4), pH 7 (preincubated at 37ºC). Absorbance at 255 nm was read every minute for three min. One enzyme unit was defined as the amount of ß-lactamase necessary to hydrolyse 1 µmol of cephaloridine per min under the assay conditions. Specific activity is expressed as enzyme units per mg of protein.

Protein content analysis. Protein concentration was measured by the method of Lowry et al[16] with bovine serum albumin (BSA) as a standard.

I**solation and Purification of ß-lactamase.**

Preparation of cell free enzyme extract. Gonococcal strains were activated by three successive transfers to SJ-GC medium supplemented with ampicillin. 500 ml of this medium were inoculated and incubated on a shaker at 37ºC. At the end of the exponential phase cells were harvested by centrifugation at 10,000 rpm for 10 min (4ºC). Cells were washed twice with 100 mM sodium phosphate buffer, pH 7, containing 50 mM NaCl (buffer A), centrifuged at 10,000 rpm, resuspended in the same buffer (20% w/v) and then sonicated for 5 min to cause cell rupture (Ultrasonicator Sonifier B-12). Afterwards cell debris was separated by centrifugation at 10,000 rpm for 15 min (4ºC).

Protein precipitation with ammonium sulphate. The supernatant was precipitated with solid ammonium sulphate (NH4)2SO4 between 30% and 75% saturation and was then centrifuged at 30,000 g for 30 min (4ºC). The pellet was resuspended in buffer A and dialysed against the same buffer for 12 h at 4ºC.

Gel filtration. The dialysed extract was fractionated on a Sephadex G75 column (33 x 2.4 cm). Equilibration and elution were carried out with buffer A. Elution was carried out at a flow rate of 35 ml/h and aliquots of 3.5 ml were collected. Fractions with ß-lactamase activity were pooled and concentrated by lyophilization.[7] The preparation was dialysed against buffer A.

Ionic exchange chromatography. The concentrated fraction was applied onto a DEAE-cellulose column (30.5 x 2 cm), equilibrated with buffer A. The enzyme was eluted with a linear gradient of NaCl (0.1 M - 0.5 M) prepared in buffer A. Fractions of 1 ml were collected at a flow rate of 42 ml/h and fractions with ß-lactamase activity were pooled. The purified preparation was stored at -20°C until used.

Polyacrylamide Gel Electrophoresis (PAGE). Gel electrophoresis of the native protein was carried out with 7.5% (w/v) polyacrylamide at pH 8.8 according to Ornstein and Davies.20 Gels were stained a) with 0.1% Coomassie Brilliant Blue R 25012 and destained with 50% methanol - 7.5% acetic acid, b) by argentic impregnation and c) using a specific substrate for ß-lactamase activity (Nitrocefin-Glaxo). SDS-PAGE was performed according to Laemmli.[14] The following molecular mass markers were used: lysosyme (Mw 14,300), trypsinogen (Mw 24,000), pepsin (Mw 34,700), ovalbumin (Mw 45,000) and BSA (Mw 66,000). Gels were stained with silver nitrate for protein detection.

Isoelectric focusing. Electric focusing was performed on a sucrose ampholyte gradient (pH 3.0 to 10.0). The experiment was carried out at a temperature below 4ºC on an ampholyte electric focusing column (LKB 8100) for 48 h with a final gradient of 300 U. The contents of the column were cut out into 3 ml fractions and each fraction was assayed for ß-lactamase activity and pH.[17]

Determination of optimal pH, temperature and thermal stability. The effect of pH on ß-lactamase activity was determined in 0.2 M phosphate buffer in a pH range from 5.8 to 8.0.

The effect of temperature on enzyme activity was analysed by incubating the assay mixture for 15 min at temperatures ranging from 15ºC to 55ºC.

Thermal stability was tested by incubating the enzyme extract at temperatures ranging from 15ºC to 60ºC for 1 to 25 min. The substrate was then added and enzyme activity was measured at 255 nm.

Stability of the enzyme after storage. Enzyme suspensions were stored at 25ºC, 4ºC, -30ºC and -70ºC for 1, 3 and 5 months. Residual enzyme activity was determined at the end of each period.

Effect of metal ions, inhibitors and other substances on enzyme activity. Stock solutions 1M of $CaCl_2$, $HgCl_2$, $CuSO_4$, $FeCl_3$, $CdCl_2$, $NiSO_4$, $FeSO_4$, $MnCl_2$, $ZnSO_4$ and $MgCl_2$ were prepared in 0.2 M phosphate buffer, pH 5.8, and added separately to the reaction mixture at a final concentration of 10^{-5} to 10^{-2} M. Inhibitors such as *p*-chloromercurybenzoate (PCMB) and EDTA were also assayed at these final concentrations. Residual enzyme activity was assayed and is expressed as the percentage of the activity determined by UV spectrometry.

Reproducibility. All results presented in this paper are means of three replicate assays.

RESULTS AND DISCUSSION

Purification of ß-lactamase. Purification data are summarized in Table 1. After the Sephadex G75 and DEAE-cellulose columns, the enzyme was 29.44 and 37.97 times purified respectively and yielded an electrophoretically pure ß-lactamase (Fig. 1, 2).

C**haracterization of the purified ß-lactamase.** The estimated native relative molecular mass was 35,000 according to gel filtration chromatography (Fig. 3). Silver stained SDS-PAGE of the purified enzyme revealed a single band with a Mr of 35,000. This demonstrates that the ß-lactamase of *N. gonorrhoeae* has a monomeric structure.

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Table 1. Purification of ß-lactamase from *N. gonorrhoeae.*

Purification step	Total protein (mg/ml)	Enzyme Units (Units/mg)	Specific activity (Units/mg protein)	Purification (folds)	Total (Enzyme units)	Yield (%)
Crude Extract	4.8	1.75	0.36	1	70	100
30%-75% $(NH_4)_2SO_4$ saturation	7.75	6.83	0.88	2.44	40.98	58.54
Sephadex G75	0.08	0.85	10.60	29.44	17	24.28
DEAE-Cellulose	0.03	0.41	13.67	37.97	12.3	17.57

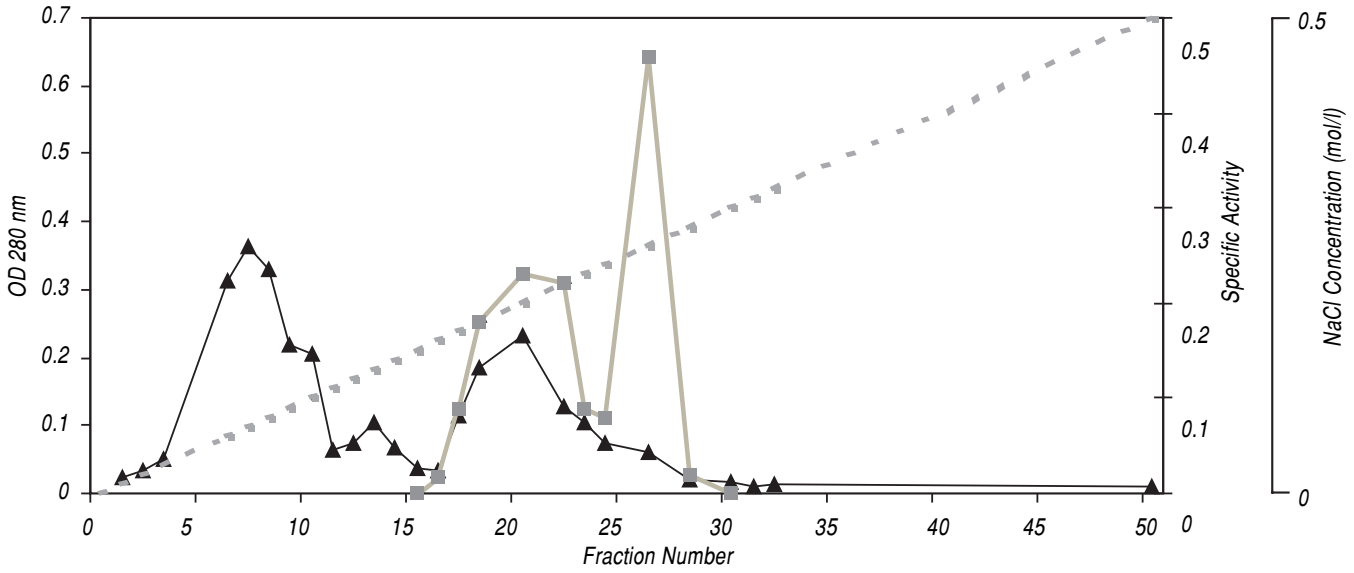

Figure 1. *Elution profile of ß-lactamase of* N. gonorrhoeae o*n a DEAE-cellulose column. 0.1-0.5 M NaCl gradient (— — — —). Protein content was measured spectrophotometrically at 280 nm (▲). Specific activity was determined at 255 nm (■).*

These results are similar to those obtained for ß-lactamase isolated from *Pseudomonas cepacia:* Mw 22,000,8 *Legionella gormanii:* Mw 25,000,7 *Bacteroides fragilis:* Mw 28,000[33] and *Fusobacterium nucleatum*: Mw 26,000.[29]

The isoelectric point obtained was 5.4, which suggests that this ß-lactamase isolated from *N. gonorrhoeae* belongs to the TEM-1 type.[30] Percival et al[21] and Phillips[22] found similar results in *Neisseria* strains.

Optimal activity was found at 37ºC and pH 7.0 - 7.2, using cephaloridine as substrate (Fig. 4), with a marked decrease at slightly higher temperatures (Fig. 5). At pH values higher than 7.2 a decrease of the enzyme activity was observed, having lost 50% at pH 8. These results agree with those reported by Livermore and Corkill.[15] These authors demonstrated in *E. coli* 976 (ß-lactamase TEM-1) that rising the pH from 6.5 to 8.0 hydrolysis of cephalosporin diminished. The optimum temperature range for this enzyme is similar to that obtained for other microorganisms.[26] ß-lactamases are thermolabile proteins which inactivate rapidly by heat.

Study of the thermal denaturation of the enzyme showed a residual activity of 82% after 25 min at 37ºC and 0% after 1 min at 60ºC or 5 min at 55ºC (Fig. 6). This behaviour is similar to that of ß-lactamases from other microorganisms such as *Bacillus cereus*, which lost activity completely after 10 min at 50ºC.[5]

The optimum temperature to preserve enzyme stability is -70°C. After storage for two months at this temperature it conserved 100% of its activity. After storage for five months 22% of the activity was lost.

At -30°C residual activity was 21% after storage for 30 days and at 4°C, 50% of the activity was lost after 5 days. At 25°C enzyme activity was completely lost after storage for 25 days.

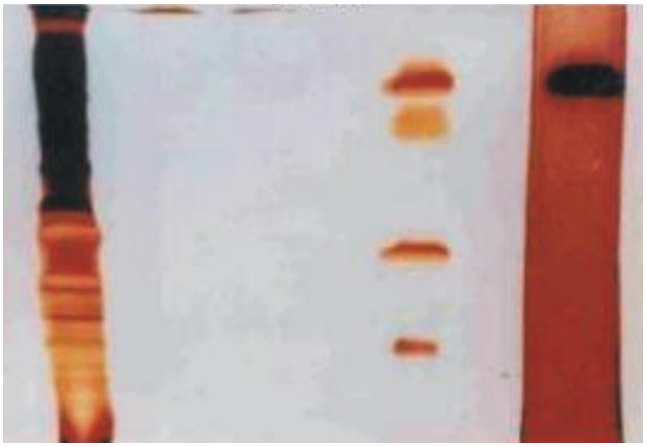

Figure 2. *Polyacrylamide gel electrophoresis (PAGE) (5%) of samples of purification steps of ß-lactamase of* N. gonorrhoeae. *A, 30%-75% (NH4)2SO4 fraction; B, peak fraction eluted from Sephadex G75 chromatography column; and C, peak fraction eluted from DEAE-cellulose chromatography column. For experimental details see text.*

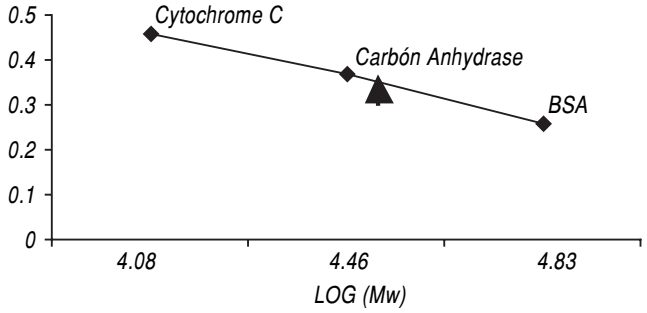

Figure 3. *Calculation of molecular weight (Mw) of ß-lactamase of* N. gonorrhoeae, *using a Sephadex G100 column. The following standard protein samples with known Mw were used: cytochrome C (12,400), carbon anhydrase (29,000) and bovine serum albumin (BSA) (Mw 66,000). For experimental details see text.*

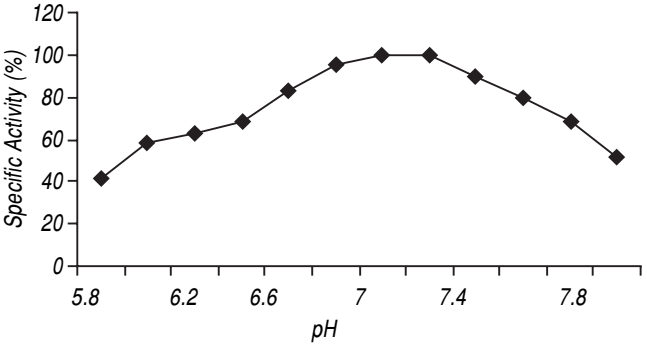

Figure 4. *Effect of pH on the specific activity (U/mg of enzyme) of ß-lactamase of* N. gonorrhoeae.

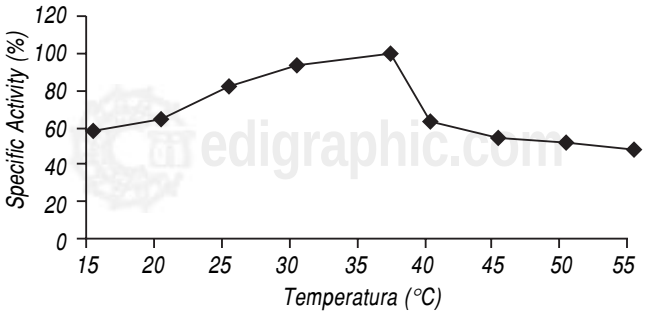

Figure 5. *Effect of temperature on the specific activity (U/mg of enzyme) of ß-lactamase of* N. gonorrhoeae. *Temperatures assayed: 15, 20, 25, 30, 37, 40, 45, 50 and 55°C.*

Table 2. Effect of ions and reducing agents on the activity of ß-lactamase from *N. gonorrhoeae*.

	Inhibition (%) Concentration (mol/l)			
Effective Compound (mol/l)	10^{-6}	10^{-4}	10^{-3}	10^{-2}
PCMB	69	35	20	2
$HgCl_2$	0	0	0	0
EDTA	0	0	0	0
$NiSO_4$	97	62	36	18
$FeSO_4$	98	93	54	50
$FeCl_3$	89	62	43	24
$MnCl_2$	83	80	56	49
$CdCl_2$	97	71	47	24
$CaCl_2$	74	71	70	67
$CuSO_4$	93	63	55	42
$ZnSO_4$	15	11	8	6
$MgCl_2$	97	97	97	97

The effect of different salts at concentrations of 10^{-5} to 10^{-2} M on the ß-lactamase activity is shown in Table 2. ß-lactamase of N. gonorrhoe*ae w*as strongly inactivated by Hg^{2+}, Ni^{+}, Fe^{2}+, Fe^{3+}, Mn^{2+}, $Cd^{2+,}$ Cu^{2+} at a concentration of 10^{-2} M and to a lesser degree by Ca^{2+} and Zn^{2+} slightly increased the enzyme activity, and Mg^{2+} did not alter it. PCMB and EDTA behaved as strong inhibitors. Penicillinase produced by *P. cepacia* behaved similarly to that from the gonococcus as it was completely inhibited by PCMB.[8] *Bacillus* spp., *Proteus* and *L. gormanii* penicillinases were completely inhibited by EDTA, Hg^{2+} and Cu^{2+}.[7,9,25]

Properties of ß-lactamase of different bacteria vary greatly and therefore it is important to study them for each single microorganism.

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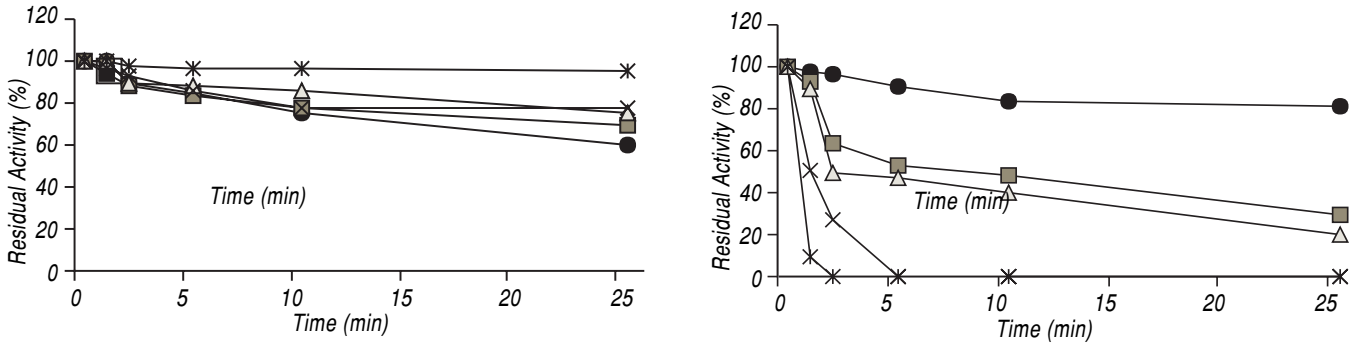

Figure 6. *Thermal denaturation of* N. gonorrhoeae *ß-lactamase. Enzyme extracts were incubated at the temperatures mentioned below for 1, 2, 5, 10 and 25 min. Residual activity was measured after substrate was added and the solution incubated for an additional 15 min at 37ºC. A, 15ºC (●); 20ºC (■); 25ºC (△); 30ºC (×); and 37ºC (✳); B, 40ºC (●); 45ºC (■); 50ºC (△); 55ºC (×); and 60ºC (✳).*

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