PURIFICATION AND PROPERTIES OF A β-LACTAMASE FROM *PROTEUS PENNERI*

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A cephalosporin-hydrolyzing enzyme from strains of *Proteus penneri* resistant to β-lactam antibiotics was purified and characterized. The enzyme gave a single protein band on SDS-polyacrylamide gel electrophoresis with a molecular weight of 30,000. This cephalosporinase has an isoelectric point of 6.8, a pH optimum of 6.5 and a temperature optimum of 45°C.

The enzyme hydrolyzed cephaloridine, cephalothin, cefuroxime, and cefotaxime more rapidly than penicillins. The relative rate, with cephaloridine as 100, were: cephalothin, 50; cefuroxime, 93; cefotaxime, 48; ceftriaxone, 23; cefoperazone, 11; benzylpenicillin, 3; ampicillin, 9; and carbenicillin, <1.

Cephamycins had low affinities for the enzyme. However, clavulanic acid and sulbactam, with high affinites for the enzyme, were inhibitors of this enzyme.

Proteus penneri has been recognized recently as a new member of the species Proteeae[1]. It is indole, esculin, and salicin negative, and chloramphenicol-resistant, and has been called *Proteus vulgaris* indole-negative or *P. vulgaris* biogroup 1. In a recent report, *P. penneri* strains were found to be more resistant to newer semi-synthetic penicillins and quinolones than *P. vulgaris*[2,3,4]. Production of β-lactamase has been considered to be one of the important biochemical mechanisms of resistance to β-lactam antibiotics in bacteria[5,6]. YOTSUJI *et al.*[7] and MATSUBARA *et al.*[8] reported on a β-lactamase from *P. vulgaris*, but no study as yet has reported on a β-lactamase from *P. penneri*. It is the purpose of this study to isolate and characterize a β-lactamase from this newly described species and to compare it with that from *P. vulgaris*.

Materials and Methods

Five clinical strains of *P. penneri* were obtained from Dr. H. C. NEU of Columbia University, NY, and from American Type Culture Collection. All five strains were comfirmed as *P. penneri* by using the method described by HICKMAN *et al.*[1] and all strains produced β-lactamase as tested by nitrocefin. Two β-lactamase-producing clinical isolates of *P. vulgaris* were also included in this study. Antimicrobial agents were kindly provided by the respective manufacturers. Minimal inhibitory concentrations were determined by the agar dilution method using a final inoculum of 2×10^5 cfu/ml.

Crude sonicated supernatants of the various cell cultures were prepared as described by NEU[9]. The β-lactamase production was induced by the addition of a concentration of benzylpenicillin equal to one-half the MIC value against the particular strain to a 1: 100 diluted overnight culture and incubated for 6 hours. The supernatant was then exhaustively dialyzed against 20 mM triethanolamine (0.5 M KCl; pH 7.0), and was concentrated to approximately 1: 200 of the original culture volume using an Amicon Ultra-filtration Cell fitted with a PM-10 membrane. The crude concentrate was loaded on an aminophenylboronic acid-Sepharose 4B affinity column[10]. After washing with the same buffer, the β-lactamase was eluted with 0.5 M borate/0.5 KCl, pH 7.0. The borate fractions showing β-lactamase activity as detected by nitrocefin were pooled and dialyzed against 10 mM phosphate buffer, pH 7.0. Approximately 75% of the loaded activity was recovered from the borate column, with the

Table 1. Antimicrobial activities of β-lactam against β-lactamase-producing *P. penneri* and *P. vulgaris*.

Compound	MIC (μg/ml)	
	P. penneri	*P. vulgaris*
Benzylpenicillin	>256	>256
Ampicillin	>256	>256
Carbenicillin	>256	128
Ticarcillin	>256	256
Piperacillin	4~>256	1~4
Cephaloridine	>256	>256
Cephalothin	>256	>256
Cefuroxime	>256	>256
Cefoxitin	4~32	4~8
Cefmetazole	4~64	2~8
Cefsulodin	>256	>256
Cefotetan	0.5~4	<0.25~0.5
Latamoxef	<0.25~0.5	<0.25
Cefoperazone	4~128	4
Cefotaxime	4~32	0.125~0.5
Ceftriaxone	4~64	0.25~1
Ceftazidime	0.06~0.5	0.125
Aztreonam	<0.25~1	<0.25~1

* Five strains of *P. penneri* and two strains of *P. vulgaris* were test.

Table 2. β-Lactamase activity of *P. penneri* and *P. vulgaris*.

Strain	Enzyme activity* (mM/minute/mg protein)	
	Induced	Constitutive
P. penneri Wy 1001	2,460	79
P. penneri Wy 1005	3,140	201
P. penneri Wy 1003	2,950	30
P. penneri 33759	4,830	432
P. vulgaris Wy 2001	1,910	23
P. vulgaris 589	3,300	32

* Activity was calculated on the basis of the hydrolysis of a fixed saturating concentration of cephaloridine.

borate wash accounting for almost 55% of the recovered activity.

Isoelectric focusing of the purified enzyme was carried out on an Ultraphor Electrofocusing Unit (LKB, Model 2217) using precasted Ampholine polyacrylamide gel (PAG) plates (pH 3.5~9.5) and pI standards from Pharmacia. Protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad Labs.). Molecular weights were determined by SDS-polyacrylamide gel electrophoresis with known molecular weight markers (Pharmacia). β-Lactamase activity was determined spectrophotometrically by measuring the decrease of absorbance of antibiotics tested with a spectrophotometer (Beckman Model 125) equipped with a cell holder with thermostat. The pH and temperature optima of the purified enzymes were determined based on initial rates of the hydrolysis of a fixed saturating concentration of cephaloridine. The Michaelis-Menten parameters were calculated using a computerized non-linear regression analysis without the preincubation of enzyme and inhibitors.

Results and Discussion

The susceptibility of five strains of *P. penneri* and two strains of *P. vulgaris* against various β-lactam antibiotics is shown in Table 1. All *P. penneri* as well as *P. vulgaris* strains were highly resistant to β-lactams except for the cephamycin antibiotics, aztreonam, and ceftazidime against which both *Proteus* species were sensitive. In general, *P. penneri* strains were more resistant to β-lactam antibiotics, including some of the newly developed cephalosporins tested. This is in agreement with the antimicrobial susceptibilities to β-lactam antibiotics reported by PENNER *et al.*[11], HAWKEY *et al.*[3] and FUKSA *et al.*[2].

All *P. penneri* strains produced minimal amounts of β-lactamase as indicated by the nitrocefin test. The enzymes were inducible by the addition of sub-inhibitory concentration of benzylpenicillin (Table 2). The enzymes were purified over 100-fold from crude sonicated extracts of *P. penneri* Wy 1001, Wy 1005, and *P. vulgaris* Wy 2001. All of the purified enzymes showed a single protein band on polyacrylamide gel electrophoresis. The molecular weights of the enzymes were estimated to be 30,000 for both *P. penneri* strains and 43,000 for *P. vulgaris* (Fig. 1). The pI values determined by electrofocusing were 6.8 and 6.25 for *P. penneri* and *P. vulgaris*, respectively (Fig. 2). The pH and tempera-

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified β-lactamases from *P. penneri* Wy 1001, *P. vulgaris* Wy 2001, and crude sonicated extracts of β-lactamase from *P. penneri*.
Molecular weight standards (M) range from 14,400 to 94,000.

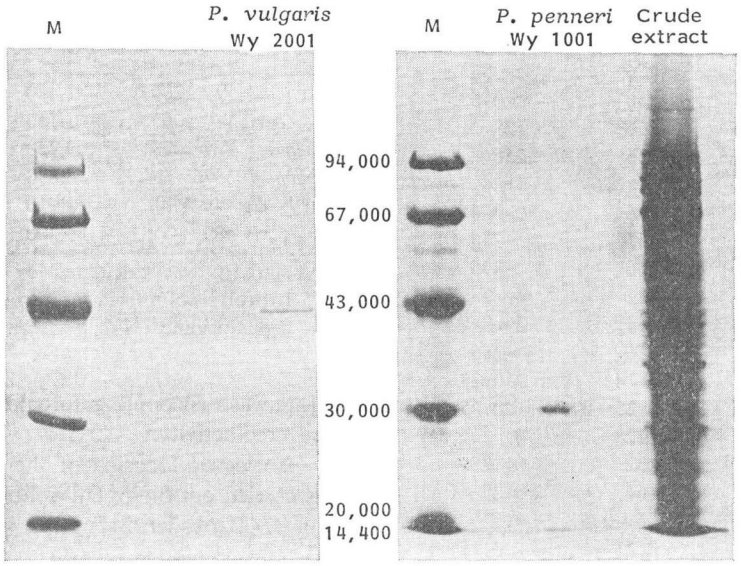
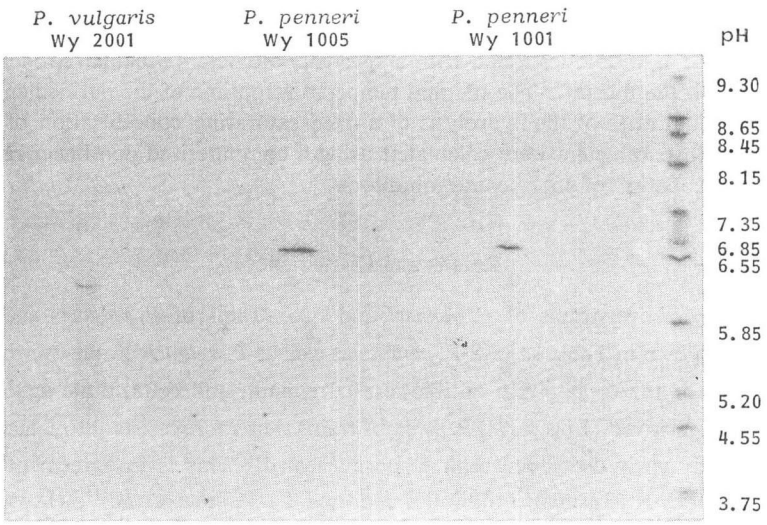


Fig. 2. Isoelectric focusing gel showing band patterns of β-lactamases from *P. penneri* Wy 1001, Wy 1005, and *P. vulgaris* Wy 2001.



ture optima were pH 6.5 and 45°C for *P. penneri*, and pH 7.5 and 45°C for *P. vulgaris*.

The kinetic constants of the purified enzyme from *P. penneri* Wy 1001 and the MIC values to various β-lactam antibiotics were determined (Table 3). The enzyme showed much higher hydrolyzing activity against cephaloridine, cefuroxime, and cefotaxime than penicillins. As inhibitors all four cephamycin antibiotics were resistant to the enzyme hydrolysis. Cefoperazone and ceftriaxone were less susceptible, but ceftazidime and aztreonam were not susceptible to the enzyme. Both clavulanic acid and sulbactam were inhibitors of this enzyme.

Table 3. Kinetics of hydrolysis of β-lactam antibiotics by β-lactamase from *P. penneri* Wy 1001**.

Compound	Vmax*	*Km* (mM)	*Ki* (μM)	MIC (μg/ml)
Benzylpenicillin	3.4	0.017		>256
Ampicillin	8.5	0.079		>256
Carbenicillin	<1	0.035		128
Piperacillin	5.0	0.038		8
Cephaloridine	100	0.368		>256
Cephalothin	50.3	0.071		>256
Cefuroxime	92.9	0.343		>256
Cefoxitin			4.24	8
Cefmetazole			33.46	8
Cefotetan			33.65	1
Latamoxef			33.4	0.25
Cefsulodin	5.8	0.525		>256
Cefoperazone	11.1	0.013		4
Cefotaxime	48	0.328		4
Ceftriaxone	23.4	0.114		4
Ceftazidime	<1	0.199		0.06
Aztreonam	<1	5.43		0.06
Clavulanic acid			1.21	
Sulbactam			2.35	

* Relative rates of hydrolysis are expressed as the percentage of cephaloridine.
** The specific activity equals to 0.315 units/mg protein.

Our data showed that the cephalosporinase produced by *P. penneri* has a substrate profile different from those of *P. rettgeri*[7], *Morganella morganii*[12], and *P. mirabilis*[13]. It hydrolyzed a variety of cephalosporins, including cefuroxime and cefotaxime in a manner similar to the β-lactamase produced by *P. vulgaris*[8,14]. The fact that this enzyme also hydrolyzed ceftriaxone suggests that cephalosporins of iminomethoxy type are susceptible to this enzyme hydrolysis. However, physico-chemical properties of these two enzymes were quite different as demonstrated by the differences in their molecular weights, pI values as well as pH optima. This study showed that in addition to the differences in biochemical reaction between *P. penneri* and *P. vulgaris*. *P. penneri* produced a unique cephalosporinase which may account for its higher resistance to β-lactam antibiotics.

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