Biochem. J. (1981) **193**, 75–82
Printed in Great Britain

The exocellular β-lactamase of *Streptomyces albus* G

Purification, properties and comparison with the exocellular DD-carboxypeptidase

Colette DUEZ, Jean-Marie FRÈRE, Daniel KLEIN, Martine NOËL and Jean-Marie GHUYSEN
Service de Microbiologie, Faculté de Médecine, Institut de Botanique, Université de Liège, Sart Tilman, B-4000 Liège, Belgium

and Lucien DELCAMBE and Louis DIERICKX
Centre National pour la Production et l'Etude de Substances d'Origine Microbienne, Boulevard de la Constitution 32, B-4020 Liège, Belgium

(*Received 2 May 1980/Accepted 6 August 1980*)

The exocellular β-lactamase of *Streptomyces albus* G has been purified to near protein homogeneity. It consists of one single polypeptide chain of mol.wt. 30000–31000, has a rather low isoelectric point (at pH 6.0) and contains less lysine (2.1%) and more half-cystine residues than most β-lactamases from other Gram-positive bacteria. Penicillins are much better substrates than Δ^3-cephalosporins; the catalytic-centre activity of good penicillin substrates is 333–500 s^{-1}. The exocellular, mol.wt. 17000 DD-carboxypeptidase of *S. albus* G [previously purified to protein homogeneity; Duez, Frère, Geurts, Ghuysen, Dierickx & Delcambe (1978) *Biochem. J.* **175**, 793–800] behaves as an exceedingly poor β-lactamase, hydrolysing benzylpenicillin into benzylpenicilloate 5×10^{-6}-fold less rapidly than does the exocellular β-lactamase. To all appearances, the β-lactamase has no bivalent cation requirement whereas, as shown elsewhere [Dideberg, Charlier, Dupont, Vermeire, Frère & Ghuysen (1980) *FEBS Lett.* **117**, 212–214, and Dideberg, Joris, Frère, Ghuysen, Weber, Robaye, Delbrouck & Roelands (1980) *FEBS Lett.* **117**, 215–218], the DD-carboxypeptidase possesses one essential Zn^{2+} ion per molecule. Peptide 'mapping' and immunological studies suggest that the two *Streptomyces* enzymes probably have very different structural and mechanistic properties.

Two families of enzymes specifically recognize the fused-ring system of β-lactam antibiotics. The β-lactamases effectively hydrolyse the endocyclic amide bond of the β-lactam ring, yielding biologically inactive products [for reviews, see Citri (1971) and Hamilton-Miller & Smith (1979)]. With the same antibiotics, the DD-carboxypeptidases form intermediates of rather high stability and, consequently, are inhibited by these compounds [for a review, see Ghuysen *et al.* (1979)]. The complexes formed between the DD-carboxypeptidases and the β-lactam antibiotics, however, are not stable indefinitely, and, in several cases, it has been shown that the degradation products arising from penicillin differ from those obtained after β-lactamase action. Thus, DD-carboxypeptidases exist which (slowly) fragment the penicillin molecule into *N*-acylglycine and *N*-formylpenicillamine (Frère *et al.*, 1975; Fuad *et al.*, 1976; Hammarström & Strominger, 1975).

Streptomyces albus G, like other Gram-positive bacteria, excretes a β-lactamase in the culture medium during growth (Johnson *et al.*, 1975). In addition, the same *Streptomyces* strain excretes a DD-carboxypeptidase which is inhibited only by high concentrations of cephalosporins and even higher concentrations of penicillins (Leyh-Bouille *et al.*, 1970; Frère *et al.*, 1978). The present paper deals with the final purification of the β-lactamase of *S. albus* G and describes experiments which were undertaken in order to study the possible relationships that might exist between this β-lactamase and the exocellular DD-carboxypeptidase produced by the same micro-organism.

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Bis, *NN'*-methylenebisacrylamide.

Materials and methods

Enzymes

The DD-carboxypeptidase of *S. albus* G was purified to protein homogeneity as described by

0306-3275/81/010075-08$01.50/1

Duez *et al.* (1978). One enzyme unit hydrolyses 1 μmol of the D-Ala-D-Ala-OH linkage of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala per min at maximum velocity and at 37°C. The released D-alanine was estimated by using the D-amino acid oxidase/peroxidase/*o*-dianisidine procedure (Frère *et al.*, 1976).

The β-lactamase of *S. albus* G had been partially purified (Johnson *et al.*, 1975). Further purification to near protein homogeneity has been achieved in the course of the present work. One unit of β-lactamase transforms 1 μmol of benzylpenicillin into benzylpenicilloate per min, at maximum velocity and at 30°C. The assays were performed by using the starch/iodine method. Routinely, however, the β-lactamase activity was also estimated by using as substrate the chromogenic cephalosporin nitrocefin (O'Callaghan *et al.*, 1972; a gift of Glaxo Chem. Co.). Enzyme samples (up to 50 μl) were supplemented with 350 μl of a 0.1 mM solution of nitrocefin in 50 mM-sodium phosphate, pH 7.0, and the mixture was incubated at 30°C; the enzyme activity was measured by monitoring the change of absorbance at 482 nm.

TPCK-trypsin (trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one) was purchased from Worthington (Freehold, NJ, U.S.A.) and Pronase (protease from *S. griseus*) from Sigma (St. Louis, MO, U.S.A.).

Standard proteins

The following proteins were used: bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 43000), carbonic anhydrase (mol.wt. 29000), chymotrypsinogen (mol.wt. 25700), myoglobin (mol.wt. 17000), lysozyme (mol.wt. 14500) and insulin (mol.wt. 5700).

Protein determination

Routinely, proteins were estimated by measuring the A_{280} of the solutions. For more accurate estimations, they were estimated by total hydrolysis with 6 M-HCl followed by reaction of the free amino groups with dinitrofluorobenzene as described in Duez *et al.* (1978).

Preparation of Sepharose–cephalosporin C

The Sepharose–cephalosporin C affinity adsorbent was prepared by reaction between CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) and cephalosporin C (a gift of Dr. D. Boyd, Eli Lilly and Co., Indianapolis, IN, U.S.A.) as described by Le Goffic *et al.* (1975). The residual activated sites were saturated by treatment with a solution of bovine serum albumin (1 mg/ml), and the gel was thoroughly washed with water.

Polyacrylamide-gel electrophoresis and electrofocusing

Electrophoreses under non-denaturing conditions at pH 8.45, and in the presence of 0.1% SDS at pH 8.5, were performed as described by Frère *et al.* (1973) and Duez *et al.* (1978), and electrofocusing (pH 3–10) as described by Wrigley (1969).

Amino acid analyses

Samples were hydrolysed at 110°C for 24 h under vacuum in 1 ml of azeotropic HCl. Cysteine was estimated as cysteic acid after performic oxidation (Hirs, 1956). Tryptophan was determined after hydrolysis by mercaptoethanesulfonic acid (96 h at 105°C).

Spectra

U.v. spectra were recorded manually by using a Zeiss MIVQIII spectrophotometer. Fluorescence emission was measured at 90° to the excitation beam (285 nm) with an Aminco–Bowman recording spectrofluorimeter.

Peptide 'maps'

Peptide 'maps' were developed on cellulose t.l.c. plates as described by Duez *et al.* (1978).

Thin layer chromatography and paper electrophoresis

T.l.c. on silica gel G was performed as described by Frère *et al.* (1975), with solvent I: [butan-1-ol/water/acetic acid/ethanol (10:4:3:3, by vol.)] and solvent II [chloroform/ethanol/acetic acid (44:5:1, by vol.)]. High-voltage paper electrophoreses were performed on Whatman 3MM paper strips at 60 $V \cdot cm^{-1}$ in collidine/acetic acid/water buffer (17:7:2500, by vol.), pH 6.5.

Growth of S. albus G

S. albus G was grown in 500-litre tanks as described by Duez *et al.* (1978). After clarification of the culture fluid by centrifugation, the β-lactamase activity in the culture fluid was 0.1 unit $\cdot ml^{-1}$ or 0.012 unit $\cdot$ (mg of protein)$^{-1}$.

Preparation of antiserum against the DD-*carboxypeptidase*

The purified native enzyme (100 μg) was dissolved in 1 ml of 50 mM-sodium phosphate buffer, pH 7.0, and the solution was supplemented with 1 ml of Freund's complete adjuvant. A reduced and alkylated protein was also, tentatively, used as antigen; reduction of disulphide bridges, carried out with dithiothreitol in 8 M-urea, was followed by alkylation with iodoacetate (Crestfield *et al.*, 1963). Immunization of New Zealand white rabbits and collection of the antisera was performed as described by Vaitukaitis *et al.* (1971). Immuno-

diffusion experiments in 1.2% (w/v) agar (Noble–Difco) containing 0.01 M-potassium phosphate, pH 7.2, 0.15 M-NaCl and 0.25% NaN_3 were performed as described by Ouchterlony (1958).

Radioactivity measurements

[^{14}C]Benzylpenicillin (57 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. A Packard Tri-Carb 2425 liquid-scintillation spectrometer and a Packard radiochromatogram scanner model 7201 were used for the radioactivity measurements.

Results

Purification of the β-lactamase

Step 1. The culture fluid (500 litres) was adsorbed on 4 kg of Amberlite CG50 (H^+ form), the enzyme was eluted and the solution concentrated and dialysed as described by Duez *et al.* (1978). At this stage, both the DD-carboxypeptidase and the β-lactamase were recovered together. All subsequent steps were carried out at 4°C. The concentrated solution was added to 2 kg of DEAE–cellulose (previously equilibrated against 10 mM-Tris/HCl, pH 8.0, containing 2 mM-$MgCl_2$) and the mixture was stirred for 30 min. The ion exchanger with the β-lactamase specifically adsorbed on it was separated by filtration and washed once with 5 litres of the Tris/$MgCl_2$ buffer. Since the DD-carboxypeptidase remained in the filtrate, the method provided an easy and efficient separation of the two enzymes. The washed DEAE-cellulose was then extracted three times with 5 litres of 0.1 M-Tris/HCl, pH 7.7, containing 0.2 M-NaCl and 10% (v/v) ethylene glycol.

Step 2. The enzyme preparation (15 litres) obtained after Step 1 was concentrated to 1100 ml with a Millipore (Millipore Benelux S.A., Brussels, Belgium) Pellicon Cassette device (using PTGC 00005 membranes; mol.wt. cut-off 10000) and the concentrated preparation was submitted to $(NH_4)_2SO_4$ fractionation. The β-lactamase precipitated between 60 and 90% saturation. This fraction was redissolved in 25 mM-sodium phosphate buffer, pH 7.0, containing 10% (v/v) ethylene glycol and 5% (v/v) glycerol. The solution was dialysed against the same buffer.

Step 3. The enzyme after Step 2 was adsorbed onto a 250 ml column (4 cm × 20 cm) of DEAE–Sephacel (Pharmacia, Uppsala, Sweden; previously equilibrated with the above described phosphate/ethylene glycol/glycerol buffer, pH 7.0) and eluted with a convex gradient of NaCl in the same buffer (mixing flask at constant volume, 800 ml of buffer; added solution, 0.08 M-NaCl in the same buffer). The enzyme was eluted at a NaCl concentration of about 0.055 M. The active fractions (260 ml) were pooled, concentrated by ultrafiltration to 20 ml and dialysed against 0.1 M-Tris/HCl buffer, pH 7.7, containing 10% (v/v) ethylene glycol and 5% (v/v) glycerol.

Step 4. The enzyme after Step 3 was adsorbed onto a 75 ml column (2.5 cm × 15 cm) of DEAE–Sephadex (Pharmacia, Uppsala, Sweden; previously equilibrated against the same Tris/HCl/ethylene glycol/glycerol buffer as above) and eluted with a convex gradient of NaCl (mixing flask at constant volume, 800 ml of buffer; added solution, 0.08 M-NaCl in the same buffer). The enzyme was eluted at a NaCl concentration of about 0.035 M. The active fractions (200 ml) were pooled, concentrated to 6 ml and dialysed against 25 mM-sodium phosphate buffer, pH 7.0, containing 10% (v/v) ethylene glycol and 5% (v/v) glycerol.

Step 5. Polyacrylamide-gel electrophoresis at pH 8.45 (for conditions, see the legend to Fig. 1*a*) of the enzyme preparation obtained after Step 4 revealed the presence of one major protein band, which was found to be associated with the enzymic activity, and several minor protein bands of similar mobility. Attempts to eliminate these contaminants by semi-preparative gel electrophoresis at the same pH failed. The enzyme preparation, as obtained after Step 4, was then divided into six identical samples and each 1 ml sample was separately applied to a small (0.6 cm × 5 cm) column of Sepharose–cephalosporin C [previously equilibrated against 25 mM-sodium phosphate buffer, pH 7.0, containing 10% (v/v) ethylene glycol and 5% (v/v) glycerol]. The columns were washed with the same buffer. The contaminating proteins were eluted in the void volume, whereas the β-lactamase was markedly retarded. The active fractions from the six chromatographies were pooled, concentrated to 1 ml and the concentrated preparation was submitted to an additional chromatography under the same conditions. The fractions that exhibited a constant specific activity were pooled, concentrated to 2 ml and dialysed against 25 mM-sodium phosphate buffer, pH 7.0, containing 10% (v/v) ethylene glycol and 10% (v/v) glycerol. The specific activity, measured with benzylpenicillin as substrate, was 34-fold higher than that previously reported (Johnson *et al.*, 1973, 1975). Table 1 summarizes the purification procedure.

Electrophoretic properties and molecular weight of the β-lactamase

Non-denaturing gel electrophoresis at pH 8.45 on a 7% (w/v) polyacrylamide gel, followed by Coomassie Blue staining, revealed one single protein band (Fig. 1*a*). In turn, electrofocusing, performed in the pH range 3–10, revealed one major protein band with an isoelectric point at pH 6.0–6.05 (Fig. 1*b*). Two minor contaminants were also observed,

Table 1. *Purification of the exocellular β-lactamase from S. albus G*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Volume (litre)	Enrichment (-fold)
Culture supernatant	4×10^6	5×10^4	0.012	100	500	1
1	6.2×10^4	2×10^4	0.32	40	15	27
2	3.4×10^3	0.6×10^4	1.8	12	0.2	150
3	39	0.42×10^4	110	8.4	0.1	9.25×10^3
4	5	0.2×10^4	400	4	0.006	33×10^3
5	1.55	1.43×10^3	924	2.8	0.002	77×10^3

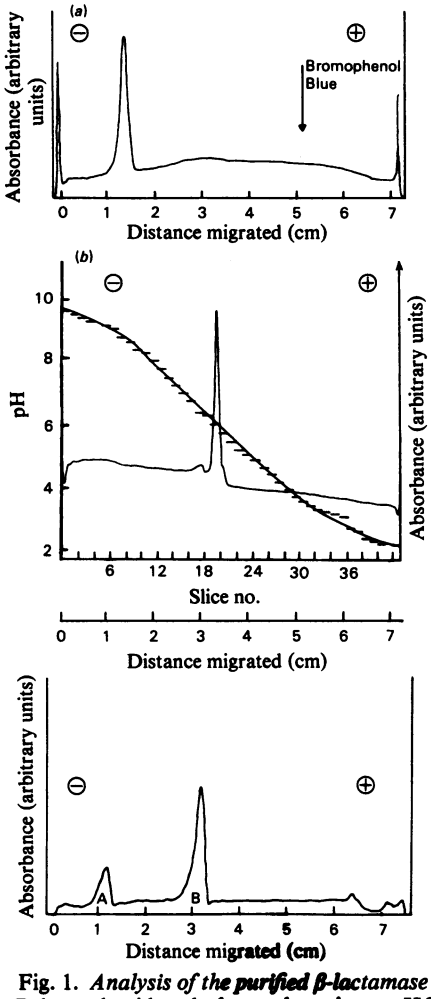

Fig. 1. *Analysis of the purified β-lactamase*
(*a*) Polyacrylamide-gel electrophoresis at pH 8.45; 25 μg of enzyme was used. The gel contained 7.5% (w/v) acrylamide and 0.2% (w/v) Bis. The gel was stained with Coomassie Brilliant Blue and scanned at 265 nm. (*b*) Isoelectric focusing between pH 3–10; 30 μg of enzyme was used. The gels contained 7.5% (w/v) acrylamide and 0.2% (w/v) Bis and were made in duplicate. After the focusing, one gel was dialysed, stained with Coomassie Brilliant Blue and scanned at 265 nm. A second gel was sliced, each slice was eluted with 1 ml of water and the pH was measured. (*c*) Polyacrylamide-gel electrophoresis in the presence of SDS; 25 μg of β-lactamase was added to 10 μg of bovine serum albumin and the mixture was boiled for 2 min in 25 mM-sodium phosphate, pH 7.5, containing 1% SDS and 1% β-mercaptoethanol. The gels contained 7.5% (w/v) acrylamide, 0.2% (w/v) Bis and 0.1% SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue and scanned at 265 nm. A, bovine serum albumin; B, β-lactamase.

representing a maximum of 8% of the total protein. Finally, polyacrylamide-gel electrophoresis in the presence of SDS also revealed one single protein band (Fig. 1*c*). Comparison of the velocity of migration with that of standard proteins indicated a mol.wt. of 30 500.

Analytical chromatography on Sephadex G-75 in 25 mM-sodium phosphate, pH 7.0, containing 10% (v/v) glycerol and 10% (v/v) ethylene glycol yielded one symmetrical protein peak and all the fractions exhibited the same specific activity. This latter technique indicated a mol.wt. of 23 500, which was considerably smaller than that obtained by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. The molecular weight was then measured by a third method (Thorun & Maurer, 1971) where the migrations of the protein to be analysed and of four standard proteins used as references (myoglobin, carbonic anhydrase, ovalbumin and bovine serum albumin) were determined in gels prepared with various concentrations (from 5 to 11.3%, w/v) of polyacrylamide (without SDS). This method indicated a mol.wt. of 31 500 ± 2000. Hence, the apparent low molecular weight obtained by chromatography on Sephadex G-75 was probably due to non-specific interactions between the gel matrix and the enzyme. The value 30 000–31 000, obtained by two widely different electrophoresis methods, has been accepted as representing the true molecular weight of the enzyme.

Absorption and fluorescence spectra of the β-lactamase

The u.v. absorption spectrum did not present any

particularlity ($\lambda_{max.}$ 280nm, $\lambda_{min.}$ 250nm, A_{280}/A_{260} 1.6). The $A^{1\%}_{1cm}$ at 280nm was 7.0. The fluorescence emission spectrum ($\lambda_{exc.}$ 285nm) exhibited a maximum at 355nm. The spectra were determined with protein solutions (0.62mg·ml^{-1}) made in 25mM-sodium phosphate, pH7.0, containing 10% (v/v) ethylene glycol and 10% (v/v) glycerol.

Amino acid composition of the β-lactamase

Table 2 gives the amino acid composition of the β-lactamase and compares it with that of the DD-carboxypeptidase. Although the β-lactamase has a lower isoelectric point, the ratio (Lys + Arg)/(Glx + Asx) is higher for the β-lactamase (0.6) than for the DD-carboxypeptidase (0.4), indicating that a high proportion of the glutamic and aspartic residues are amidated in the DD-carboxypeptidase. In contrast with other β-lactamases of Gram-positive bacteria, the β-lactamase of *S. albus* G contains 4 half-cystine or cysteine residues and a low percentage of lysine.

Table 2. *Amino acid composition of the β-lactamase and DD-carboxypeptidase of S. albus G*

For the β-lactamase, the amount of enzyme hydrolysed was 35μg. For the carboxypeptidase, the values given by Duez *et al.* (1978) have been slightly corrected (B. Joris, J. M. Frère & J. M. Ghuysen, unpublished work).

	β-Lactamase (mol.wt. 30500)		DD-Carboxypeptidase (mol.wt. 17850)	
Residue	Residues/molecule	% in number	Residues/molecule	% in number
Lys	6	2.1	4	2.3
His	1	0.35	6	3.5
Arg	27	9.4	8	4.7
Trp	3*	1.05	4	2.3
Asx	33	11.5	18	10.5
Thr	24	8.4	10	5.9
Ser	14	4.9	11	6.4
Glx	22	7.7	12	7.0
Pro	13	4.5	7	4.1
Gly	36	12.5	25	14.6
Ala	34	11.8	24	14.0
½Cys	4†	1.4	4	2.3
Val	17	5.9	8	4.7
Met	3	1.05	3	1.8
Ile	5	1.4	6	3.5
Leu	35	12.2	9	5.3
Tyr	5	1.4	5	2.9
Phe	5	1.4	7	4.1
Total	287	99.9	171	101

* Determined in an independent experiment after hydrolysis by mercaptoethanesulphonic acid.

† Determined in an independent experiment after performic acid oxidation.

Specific activities and catalytic-centre activities of the β-lactamase

The K_m value for benzylpenicillin was identical with that found by Johnson *et al.* (1973) but the maximum velocity was 34-fold higher. Consequently, the data of Table 3 are those reported previously (Johnson *et al.*, 1973) except that the maximum velocity values were multiplied by the same factor (34). The catalytic-centre activity for benzylpenicillin is 470s^{-1}. For ampicillin, the best substrate assayed so far, this value is 570s^{-1}.

β-Lactamase activity of the DD-carboxypeptidase

In addition to the efficient β-lactamase described above, the culture filtrates of *S. albus* G contain a DD-carboxypeptidase which also behaves as a penicillin-destroying enzyme, although of very low efficiency. The properties of this DD-carboxypeptidase, which is known to be highly resistant to benzylpenicillin (Frère *et al.*, 1978), have been further investigated. The DD-carboxypeptidase (63nmol) and 10mM-[^{14}C]benzylpenicillin (sp. radioactivity 2.5mCi/mmol) were incubated together in 300μl of 10mM-Hepes buffer, pH8.0, containing 3mM-$MgCl_2$. After 4h at 37°C, the enzyme activity was inhibited by 56%. The protein (both active and inactivated) was separated from the excess of [^{14}C]benzylpenicillin by chromatography on a column (16ml, 1cm × 20cm) of Sephadex G-25 in the same buffer. Measurements of radioactivity and residual enzyme activity indicated that 1.02mol of penicillin was bound per mol of inactivated enzyme. The kinetics of enzyme reactivation and of release of the radioactive label by further incubation at 37°C in the Hepes/$MgCl_2$

Table 3. *Kinetic parameters for the hydrolysis of β-lactams by the β-lactamase*

β-Lactam	K_m (mM)	$V_{max.}$ [μmol·min^{-1}·(mg of enzyme)$^{-1}$]	$k_{cat.}$ (s^{-1})	$10^{-3} \times \frac{k_{cat.}}{K_m}$ (M^{-1}·s^{-1})
Benzylpenicillin	0.74	924	470	635
6-Aminopenicillanic acid	0.6	693	350	587
Penicillin V	0.63	739	375	596
Ampicillin	0.9	1122	570	513
Carbenicillin	1	92	47	47
Oxacillin	0.33	171	87	263
Cloxacillin	0.25	16	8	32
Methicillin	0.77	14	7	9.2
Cephalosporin C	3.33	51	26	7.8
Cephaloglycine	3.83	69	35	9.2
Cephalexin	4.55	11	6	1.2
Cephalothin	1.33	23	12	8.8

Table 4. *Identification of the β-lactamase activity of the* DD-*carboxypeptidase*

Compound and treatment	R_F in solvent I	R_F in solvent II	Electrophoretic mobility at pH 6.5 ($cm \cdot h^{-1}$)
Benzylpenicillin	0.76	0.60	16
Benzylpenicillin incubated with Tris buffer	0.68	0.20	12.5
Compound released from the benzylpenicillin–enzyme complex by incubation in Tris/HCl buffer	0.72	0.19	12
Benzylpenicilloic acid	0.56	0.03	24
Compound released from the benzylpenicillin–enzyme complex by incubation in Hepes buffer	0.52	0.02	23
Phenylacetylglycine	0.76	0.40	20

buffer indicated a half-life of about 180 min for the complex formed between the enzyme and penicillin, or a first-order rate constant of about $0.64 \times 10^{-4} s^{-1}$ for complex breakdown. The released ^{14}C-labelled compound behaved as penicilloate in the three analytical systems used (see Table 4). It was clearly different from phenylacetylglycine, which is known to be produced by degradation of the benzylpenicilloyl–enzyme complexes formed with other DD-carboxypeptidases (see the Introduction).

In another experiment, the isolated radioactive complex formed between the enzyme and [^{14}C]-benzylpenicillin was boiled for 1 min (which caused formation of a precipitate) and the suspension was supplemented with 10% (w/v) Pronase. After clarification (approx. 30 min), analysis of the preparation by high-voltage electrophoresis revealed the presence of one single radioactive compound which migrated as [^{14}C]benzylpenicilloate. Finally, in a third experiment, formation and spontaneous degradation of the [^{14}C]benzylpenicilloyl–enzyme complex was carried out in 10 mM-Tris/HCl, pH 8.0. Both enzyme recovery and release of the radioactive label were complete in less than 180 min. Irrespective of the analytical system used, the reaction product behaved as that obtained by incubating penicillin in the Tris buffer alone, showing that the penicilloyl–enzyme complex was susceptible to nucleophilic attack by the base form of the Tris/HCl buffer.

Possible relationships between β-lactamase and DD-*carboxypeptidase*

In spite of a tremendous difference in their efficacies as penicillin-degrading enzymes, both β-lactamase and DD-carboxypeptidase of *S. albus* G degrade benzylpenicillin into benzylpenicilloate. Relationships might thus exist between the two enzymes.

Immunological studies. Neither the control serum, nor the antiserum prepared against the native DD-carboxypeptidase, had any effect on the activities of the DD-carboxypeptidase (as measured on Ac_2-L-Lys-D-Ala-D-Ala) or the β-lactamase (as measured on benzylpenicillin, cloxacillin or nitrocefin). In these experiments, the enzyme samples were preincubated for 90 min at 37°C with various dilutions of the antiserum ranging between 1:2 and 1:100 and then the enzyme activities were estimated. By immunodiffusion, the native anti-(DD-carboxypeptidase) serum gave rise to clear precipitation lines with the corresponding DD-carboxypeptidase (with 5 and 10 μl of a $1 mg \cdot ml^{-1}$ solution) but not with the β-lactamase (with 4.8 and 13 μl of a $1 mg \cdot ml^{-1}$ solution). Finally, mixtures containing both the DD-carboxypeptidase (25 μg) and the β-lactamase (4 μg) in 10 mM-Tris/HCl buffer, pH 8.0, were incubated for 90 min at 37°C with either a 1:20 dilution of the control serum or a 1:20 dilution of the native anti-(DD-carboxypeptidase) serum. Each enzyme preparation was then filtered separately on the same column (0.85 cm × 12 cm) of Sephadex G-75 previously equilibrated against either the 1:20 dilution of control serum or the 1:20 dilution of antiserum. The elution volume of the DD-carboxypeptidase observed in the presence of the antiserum was markedly decreased when compared with that observed in the presence of the control serum. In contrast, the elution volume of the β-lactamase was the same in the presence of either the control serum or the anti-(DD-carboxypeptidase) serum. Finally, the antiserum prepared against the reduced and alkylated DD-carboxypeptidase had no detectable effect, as tested by immunodiffusion, on the reduced and alkylated enzymes.

Peptide 'maps'. Both DD-carboxypeptidase (216 μg) and β-lactamase (250 μg) were dialysed against 0.2 M-NH_4HCO_3, pH 8.0. After boiling for 2 min, the samples were supplemented with trypsin (5 μg) and incubated at 37°C for 8 h. Fresh trypsin (5 μg) was added after 2, 4 and 6 h during this incubation. Peptide mapping of the degraded DD-carboxypeptidase and β-lactamase yielded 13 and 17 peptides respectively, but only two of them migrated in similar positions. Two extensive 'smears' were also observed on the β-lactamase map, probably indicating incomplete degradation.

Effects of EDTA. Incubation of the β-lactamase for 24 h in 25 mM-sodium phosphate, pH 7.5, containing 10% (v/v) ethylene glycol, 10% (v/v) glycerol and 1 mM-EDTA, did not affect the activity of the enzyme. As shown elsewhere (Dideberg *et al.*, 1980*a*,*b*), the DD-carboxypeptidase of *S. albus* G possesses one Zn^{2+} ion per molecule and this Zn^{2+}

cofactor is required for enzyme activity. High concentrations of EDTA inhibit the activity of the DD-carboxypeptidase.

Discussion

S. albus G. is a poor producer of extracellular β-lactamase; about 110 μg of enzyme is present per litre of culture supernatant, and this value may be overestimated because of the possible interference of other proteins in the nitrocefin assay. Nevertheless, this β-lactamase has been purified to near protein homogeneity. The low yield of purification is mainly due to losses during the first two steps, which involve large volumes of solutions containing very low concentrations of enzyme. The β-lactamase has an apparent mol.wt. of 30000–31000 and seems to consist of one single polypeptide chain. Filtration on Sephadex indicates a lower molecular weight but this observation is probably due to non-specific interactions with the molecular-sieve matrix. Maximum fluorescence emission is at 355 nm, a value that is close to that for free tryptophan. The enzyme contains a low proportion (2.5%) of sulphur-containing residues (as observed with other exocellular enzymes excreted by various actinomycetes; see Duez *et al.*, 1978). This value, however, is much higher, especially with regard to the number of half-cystine residues, than that found in β-lactamases from other Gram-positive bacteria. The proportion of polar residues, 45%, found in the β-lactamase of *S. albus* G is characteristic of a soluble protein. The enzyme has a much higher activity (as reflected by higher $V_{max.}$ and lower K_m values) towards the penicillins than towards the cephalosporins and may be regarded as an efficient penicillinase. With good penicillin substrates, the catalytic-centre activity (333–500 s^{-1}) is smaller than those of the β-lactamases I and II of *Bacillus cereus* and the β-lactamase of *Bacillus licheniformis* 749 C, similar to those of the β-lactamases of *B. licheniformis* 6346, *Staphylococcus aureus* A and C and *Escherichia coli* R-TEM; and higher than those of the β-lactamases of *Staph. aureus* B and *E. coli* GIlal (Citri, 1971; Davies *et al.*, 1974).

S. albus G also excretes an exocellular DD-carboxypeptidase during growth. This enzyme behaves as a β-lactamase in that it degrades benzylpenicillin to benzylpenicilloate but, in this respect, it has a very low efficiency. It was shown earlier (Frère *et al.*, 1978) that the reaction between the *S. albus* G DD-carboxypeptidase (E) and phenoxymethylpenicillin, caphalothin and cephalosporin C (I) proceeded according to the reaction:

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^{*} \xrightarrow{k_4} E + Ps$$

(where K = dissociation constant, k_3 and k_4 = first-order rate constants and Ps = degradation products) and that the first-order rate constants had very low values [$k_3 = (1–8) \times 10^{-4}\ s^{-1}$ and $k_4 = (0.8–3.3) \times 10^{-4}\ s^{-1}$, depending on the antibiotics]. The low k_3 values confer on the DD-carboxypeptidase its high resistance to the β-lactam antibiotics and both the low k_3 and low k_4 values confer on it its low efficiency as a β-lactamase. The k_3 value for benzylpenicillin was not determined directly. However, if the residual enzyme activity (44%) found after 4 h of reaction with benzylpenicillin (see the Results section) represents the steady-state level of the reaction, then an approximate value of $9 \times 10^{-5}\ s^{-1}$ can be attributed to the pseudo-first-order rate constant, k_a, for the formation of complex EI* [on the basis that the activity at the steady state is equal to $k_4/(k_a + k_4)$]. Since this k_a value is similar to that obtained previously with phenoxymethylpenicillin (Frère *et al.*, 1978), one can safely assume that the k_3 value is similar for both antibiotics ($9 \times 10^{-4}\ s^{-1}$). From this, and on the basis that $k_{cat.} = k_3k_4/(k_3 + k_4)$, $k_{cat.}$ values of 6.6×10^{-5} and $8.3 \times 10^{-5}\ s^{-1}$ can be estimated for benzylpenicillin and phenoxymethylpenicillin, respectively. It thus follows that, with these two penicillins, the catalytic-centre activity of the *S. albus* G DD-carboxypeptidase is about 5×10^6-fold lower than that observed with the corresponding β-lactamase. With cephalothin and cephalosporin C, the ratio $k_{cat.}$(β-lactamase)/$k_{cat.}$(DD-carboxypeptidase) is 3×10^5 and 5×10^5, respectively (Frère *et al.*, 1978). One should note, however, that the structure of the degradation product(s) arising from the Δ^3-cephalosporins are not known.

From the low k_3 values observed with the DD-carboxypeptidase, the question arises whether such low reaction rates have any specificity, since penicillin is known to react with amino and hydroxy groups of proteins, peptides and amino acids (Corran & Waley, 1975; Schneider & De Weck, 1968). The k_a value observed with the DD-carboxypeptidase, however, is at least 10-fold higher than those observed with lysozyme and insulin, and 1000–10000-fold higher than those observed with short peptides and free amino acids. Unfortunately, little information is available concerning the stability of these latter adducts.

There exist at least two classes of β-lactamases. The *B. cereus* β-lactamase II is a Zn^{2+} enzyme (Sabath & Abraham, 1961). The *B. cereus* β-lactamase I and the *E. coli* R-TEM β-lactamase are serine enzymes (Knott-Hunziker *et al.*, 1979; Fisher *et al.*, 1980). The *S. albus* G β-lactamase seems not to possess any Zn^{2+}, nor any other bivalent cation that could be removed by EDTA. Whether or not it is a serine enzyme remains to be established. In contrast, the *S. albus* G DD-carboxypeptidase pos-

sesses one Zn^{2+} per molecule and this Zn^{2+} cofactor is required for both enzyme activity and penicillin binding (Dideberg *et al.*, 1980*a*,*b*). Finally, from the limited information so far available (peptide 'mapping' and immunological studies), the exocellular β-lactamase and DD-carboxypeptidase of *S. albus* G do not possess obvious structural similarities.

The work has been supported in part by the National Institutes of Health, U.S.A. (contract no. 2 R01 AI13364-04), the Fonds de la Recherche Scientifique Médicale, Bruxelles (contract no. 3.4501.79) and the Actions Concertées, Bruxelles (convention no. 79/84-I1).

References

Citri, N. (1971) *Enzymes, 3rd Edn.* **4**, 23–41

Corran, P. H. & Waley, S. G. (1975) *Biochem. J.* **149**, 357–364

Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627

Davies, R. B., Abraham, E. P. & Melling, J. (1974) *Biochem. J.* **143**, 115–127

Dideberg, O., Charlier, P., Dupont, L., Vermeiren, M., Frère, J. M. & Ghuysen, J. M. (1980*a*) *FEBS Lett.* **117**, 212–214

Dideberg, O., Joris, B., Frère, J. M., Ghuysen, J. M., Weber, G., Robaye, R., Delbrouck, J. M. & Roelands, I. (1980*b*) *FEBS Lett.* **117**, 215–218

Duez, C., Frère, J. M., Geurts, F., Ghuysen, J. M., Dierickx, L. & Delcambe, L. (1978) *Biochem. J.* **175**, 793–800

Fisher, J., Belasco, J. G., Charnas, C. L., Khosla, S. & Knowles, J. R. (1980) *Philos. Trans. R. Soc. London Ser. B* **289**, 309–319

Frère, J. M., Ghuysen, J. M., Perkins, H. R. & Nieto, M. (1973) *Biochem. J.* **135**, 463–468

Frère, J. M., Ghuysen, J. M., Degelaen, J., Loffet, A. & Perkins, H. R. (1975) *Nature (London)* **258**, 168–170

Frère, J. M., Leyh-Bouille, M., Ghuysen, J. M., Nieto, M. & Perkins, H. R. (1976) *Methods Enzymol.* **45B**, 610–636

Frère, J. M., Geurts, F. & Ghuysen, J. M. (1978) *Biochem. J.* **175**, 801–805

Fuad, N., Frère, J. M., Ghuysen, J. M., Duez, C. & Iwatsubo, M. (1976) *Biochem. J.* **155**, 623–629

Ghuysen, J. M., Frère, J. M., Leyh-Bouille, M., Coyette, J., Dusart, J. & Nguyen-Distèche, M. (1979) *Annu. Rev. Biochem.* **48**, 73–101

Hamilton-Miller, J. M. T. & Smith, J. T. (eds.) (1979) *β-Lactamases*, Academic Press, New York

Hammarström, S. & Strominger, J. L. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3463–3467

Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621

Johnson, K., Dusart, J., Campbell, J. N. & Ghuysen, J. M. (1973) *Antimicrob. Ag. Chemother.* **3**, 289–298

Johnson, K., Duez, C., Frère, J. M. & Ghuysen, J. M. (1975) *Methods Enzymol.* **43**, 687–698

Knott-Hunziker, V., Waley, S. G., Orlek, B. S. & Sammes, P. G. (1979) *FEBS Lett.* **99**, 59–61

Le Goffic, F., Andrillon-Spiegel, J. & Letarte, R. (1975) *Biochimie* **57**, 29–34

Leyh-Bouille, M., Ghuysen, J. M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H. & Kandler, O. (1970) *Biochemistry* **9**, 2955–2960

O'Callaghan, C., Morris, A., Kirby, S. A. & Shingler, A. H. (1972) *Antimicrob. Agents Chemother.* **1**, 283–288

Ouchterlony, O. (1958) *Prog. Allergy* **5**, 1–73

Sabath, L. D. & Abraham, E. P. (1966) *Biochem. J.* **98**, 11C–13C

Schneider, C. H. & De Weck, A. L. (1968) *Biochim. Biophys. Acta* **168**, 27–35

Thorun, W. & Maurer, H. R. (1971) in *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis* (Maurer, H. R., ed.), pp. 8–19, de Gruyter, Berlin

Vaitukaitis, J. L., Robbins, J., Mischlag, E. & Ross, G. T. (1971) *J. Clin. Endocrinol.* **33**, 988–994

Wrigley, C. W. (1969) *Shandon Instrum. Appl. Bull.* **29**, 1–10