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# Stereospecific γ-lactamase activity in a *Pseudomonas fluorescens* species

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**Twenty five environmental isolates enriched for their ability to grow on *N*-acetylphenylalanine as sole carbon source were investigated for their hydrolytic action on (+)γ-lactam (2-azabicyclo[2.2.1]hept-5-en-3-one). Strain CMC 3060, a mucoidal Gram-negative rod identified as a strain of *Pseudomonas fluorescens*, produced high levels of (+)lactamase, and was subsequently found to produce two distinct intracellular enantiomer-selective γ-lactamases, one for each isomer. The (+)lactamase was produced constitutively whereas the (–)lactamase was produced only in the presence of the substrate. The (+)lactamase was stable when stored as a frozen cell paste but unstable as a protein solution, losing activity during purification and storage. This enzyme was highly selective for the (+)lactam and showed no activity against a wide range of similar compounds. By use of rapid purification techniques and the inclusion of protease inhibitors and protein stabilisers, the (+)lactamase was purified to homogeneity by FPLC and found to be a monomer of molecular weight 61 000 Da.**

**Keywords:** *Pseudomonas*; stereospecific; γ-lactamase; purification; carbovir; biotransformation; nucleoside analogue precursor

## Introduction

In recent years great interest has been expressed in the field of enzyme-mediated racemate clarifications to yield chiral compounds [2,7,8,14]. Current chemistry allows for the production of asymmetric compounds in some instances, however these techniques are often complex, expensive and have limited applicability. Microbial processes using enzymes to hydrolyse one isomer have the advantage of being highly selective and rapid. Biotransformations by whole cells are useful in a number of instances, particularly when co-factor recycling is required or the enzyme is unstable. Reduced rates of production due to poor substrate transport and expensive downstream processing costs, however, limit their use. The use of purified microbial enzymes would solve these problems by enzyme immobilisation and also allow the use of flow-through reactors.

Carbocyclic purine and pyrimidine nucleoside analogues have in recent years generated large amounts of interest as antiviral agents [5]. (+/–)Carbovir (carbocyclic 2′,3′-didehydro-2′,3′-dideoxyguanosine) (Figure 1a) has been shown *in vitro* to be a potent and selective inhibitor of HIV-1 in human T-cell lines [12]. Glaxo developed a synthetic route from the chiral secondary metabolite aristeromycin to (–)carbovir, however this route is limited by the availability of aristeromycin [13] (Figure 1b). Vince and Hua [11] demonstrated a synthetic route to racemic carbovir with the key intermediate being γ-lactam (+/–)-2-azabicyclo[2.2.1]hept-5-en-3-one (Figure 1c). Chiroscience has developed a process using whole cell and immobilised enzyme biocatalysis

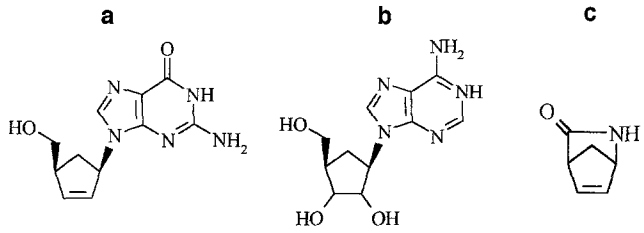


**Figure 1** Chemical structures of (a) Carbovir [12], (b) Aristeromycin [13], (c) γ-lactam [11].

to achieve enantiomeric separation of this carbovir precursor (+/–)-2-azabicyclo[2.2.1]hept-5-en-3-one (Figure 2) [9,10]. More recently, a process to produce the (+) isomer has been developed to operate on a large scale converting 5 kg day$^{-1}$ using a stable immobilised enzyme from an *Aureobacterium* species [6]. The ease of operation of this highly efficient process has prompted an investigation to isolate and utilise an equally stable (+)lactamase to produce the (–)lactam. In this study we examined the potential of novel hydrolytic enzymes for the chiral clarification of the

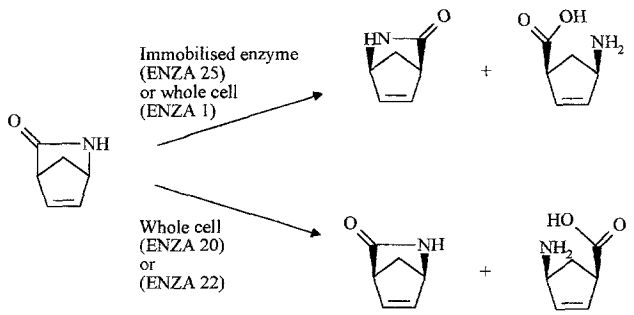


**Figure 2** Chiroscience's current process for the resolution of γ-lactam [9,10].

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racemic carbocyclic precursor (+/−)-2-azabicyclo-[2.2.1]hept-5-en-3-one to yield the (−) isomer.

## Materials and methods

### *Chemicals and reagents*

All chemicals were obtained from Sigma Chemical Co, Poole, Dorset, UK except for γ-lactam which was supplied by Chiroscience Ltd, Cambridge, UK. Protein purification columns and apparatus were supplied by Pharmacia LKB Ltd, St Albans, Herts, UK.

### *Maintenance and growth of cultures*

Cultures were grown in liquid medium containing (g $L^{-1}$): $K_2HPO_4$, 7; $NaHPO_4$, 2; $NH_4Cl$, 2; $MgSO_4$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.01; $FeCl_3 \cdot 6H_20$, 0.008; yeast extract (Lab M), 2; and glucose, 5; pH 7.0. Cells were cultivated at 30°C in shake flasks in an orbital shaker (200 rpm) for 24 h and then harvested by centrifugation at 10000 × g for 10 min. Strains were stored for long periods as 10% (v/v) glycerol suspensions at −80°C. These stocks were routinely raised on agar plates of the above medium.

### *Purification of γ-lactamase*

Enzyme purification was performed at 4°C, by the methods detailed below. Buffer A consisted of 10 mM $NaH_2PO_4$, 5 mM EDTA, 2 mM dithioerythritol (DTE), 1 mM phenylmethylsulfonylfluoride (PMSF), 2 mM benzamidine (BAM), 10% (v/v) glycerol, 1.5 M $NH_4SO_4$, 0.5 μg $ml^{-1}$ leupeptin and 0.7 μg $ml^{-1}$ pepstatin.

*i) Preparation of cell extract:* A freeze/thawed wet cell pellet (20 g) was suspended at a concentration of 0.1 g $ml^{-1}$ in Buffer A. Cells were generally lysed by this freeze/thawing but were further lysed by sonication (3 × 30 s at 16 μm peak to peak). Cell debris was removed from the resultant lysate by centrifugation at 16000 × *g* at 4°C for 15 min. DNA was precipitated by the addition of protamine sulphate (0.1% (w/v) final concentration) and removed by centrifugation as above.

*ii) Ammonium sulphate fractionation:* The clarified lysate was brought to 60% saturation by step-wise addition of ammonium sulphate (enzyme grade) and allowing for complete solubilisation of the salt before further addition with stirring. After stirring the suspension for 30 min, precipitated protein was removed by centrifugation at 16000 × *g* (30 min). The ammonium sulphate concentration was then raised to 80% saturation and the precipitated protein was removed by centrifugation. This pellet was resuspended and dialysed overnight in Buffer B [Buffer A containing 1.5 M $(NH_4)_2SO_4$].

*iii) Phenyl-sepharose hydrophobic interaction chromatography:* All column chromatography was performed on a Fast Protein Liquid Chromatography or High Load system (Pharmacia) at 4°C. The dialysed protein solution was applied to the column which had previously been equilibrated with two column volumes of Buffer B at a flow rate of 2.0 ml $min^{-1}$. The column was then washed with another two column volumes of Buffer B. Bound protein was eluted with a linear $(NH_4)_2SO_4$ gradient (1.5 M to 0 M) with three column volumes. Fractions were collected, assayed as described previously, and fractions with enzymic activity were pooled. Pooled protein was dialysed overnight against Buffer A. Semi-purified enzyme referred to later was purified to this stage.

*iv) Fast Flow Q anion exchange chromatography:* Dialysed protein was applied to a Fast Flow Q column pre-equilibrated with two column volumes of Buffer A at a flow rate 4.0 ml $min^{-1}$. Bound protein was then washed with a further column volume of Buffer A, before eluting the protein with two column volumes of Buffer A and a linear NaCl gradient (0–1 M). Fractions with enzyme activity were pooled, concentrated to 5 ml using a Centricon 30 molecular weight filter (Amicon, Stonehouse, Gloucestershire, UK), and dialysed overnight against Buffer A.

*v) Superose 12 gel filtration:* Concentrated protein (5 ml) was applied to a pre-equilibrated preparative Superose 12 column in Buffer A. Protein was eluted at a flow rate 0.5 ml $min^{-1}$. Fractions containing lactamase were pooled and concentrated using a Centricon 30 filter to 2 ml final volume.

### *Determination of lactamase activity*

γ-Lactamase activity was assayed by the addition of 2 mg of racemic lactam to 50 μl of suitable dilute buffered cell-free lysate. The reaction was then incubated at 25°C for 30 min before being analysed by HPLC or GC.

### *HPLC analysis*

One microlitre of the assay mixture was diluted with 400 μl of HPLC mobile phase and used for HPLC analysis. Twenty microlitres of this solution were applied to a Kromasil C8 column and eluted with a mobile phase MeOH : 10 mM potassium phosphate pH 7.0 (1 : 1 v/v). The lactam and amino acid were monitored for at 225 nm. At a flow rate of 0.5 ml $min^{-1}$ the amino acid and lactam have retention times of 3.6 and 4.8 min, respectively.

### *Determination of stereospecificity*

The stereospecificity of each enzyme was determined by chiral gas chromatography. Lactamase activity was assayed as previously described, and the reaction products were extracted into ethyl acetate (0.5 ml). The ethyl acetate was dried by the addition of anhydrous magnesium sulphate, which was subsequently removed by filtration. A sample (1 μl) was applied to a chiral Lipodex D column (oven temp 165°C, injector temp 190°C). The (+) and (−)lactams have retention times of 5.3 and 6.0 min, respectively.

### *Temperature and pH optima*

The pH optima of the semipurified (phenyl-sepharose eluate) lactamase was determined by replacing buffer A with universal buffer over the pH range 4–12 in the lactamase assay method described previously [3].

The temperature optimum for semipurified lactamase was determined by incubating lactamase assays as previously described for 60 min over the temperature range 4–60°C.

### *Determination of enzyme stability*

The stability of the semipurified enzyme was determined by pre-incubation of enzyme samples at 4°C or 37°C. Samples were removed intermittently and residual enzyme activity determined at 25°C as per the standard lactamase assay previously described.

### *Effect of metal ions and co-factors*

The effect of various co-factors and metal ions upon the semi-purified γ-lactamase was carried out by the addition of each chemical to an enzyme solution so as to produce a final concentration of 5 mM in the lactamase assay. The lactamase assay was then carried out as previously described.

### *Electrophoresis*

Column eluates were examined by SDS-PAGE electrophoresis in 12% (w/v) polyacrylamide gels. After electrophoresis, proteins were fixed and stained with Coomassie blue R-250 in 30% (v/v) methanol and 10% (v/v) acetic acid for 2 h. Gels were destained with 10% (v/v) methanol and 5% (v/v) acetic acid until bands were clearly visible.

### *Enzyme immobilisation*

Immobilisation of semi-purified enzyme was carried out according to the protocol provided by Acti Disk (FMC Corporation, Pine Brook, NJ, USA).

## Results

### *γ-Lactamase activity and specificity*

A range of microorganisms isolated for their ability to utilise *N*-acetylphenylalanine as sole carbon source was screened for γ-lactamase activity and the stereospecificity of this activity. Most of the 28 isolates either showed no activity against the substrate or showed no stereospecificity. Five strains not identified had stereoselective γ-lactamase activity to some degree (results not shown). An isolate identified as CMC 3060 produced much higher enzyme levels than the other strains and was therefore selected for further study. This isolate, CMC 3060, a highly mucoidal Gram-negative rod was subsequently identified as a strain of *Pseudomonas fluorescens*.

### *General properties of CMC 3060 lactamase*

Small scale whole cell biotransformations were used to examine the activity, stereospecificity and potential of CMC 3060 for use in a scaleable resting whole cell process. In these biotransformations it was found that both enantiomers were degraded sequentially over 100 h. The (+) isomer was rapidly degraded with total conversion to the amino acid in 90 min, with no apparent degradation of the (−)lactam (Figure 3). However over the next 100 h the (−)lactam was also totally degraded. When fresh cells were harvested from culture, lysed, and enzyme activity measured, only (+)lactamase activity was detected. Also if cells were grown and then used in a biotransformation with the addition of azide or streptomycin, no (−)lactamase activity was detected (Figure 3). The (+)lactamase enzyme was found to be relatively unstable in crude extracts despite the inclusion of various protease inhibitors (PMSF, BAM, pepstatin, leupeptin) and DTE. Activity was however stabilised to a small degree by the inclusion of 1..5 M ammonium sulphate and 10% (v/v) glycerol in the buffer.

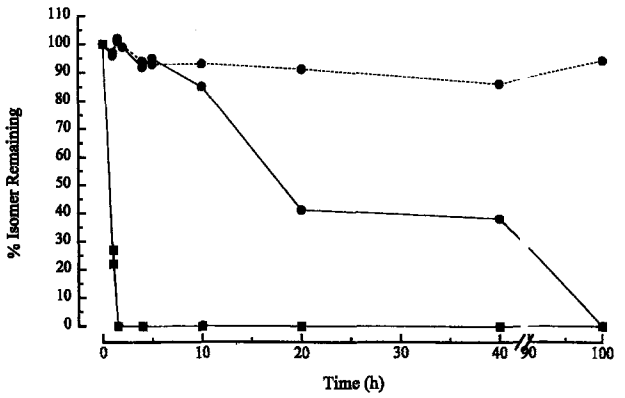


**Figure 3** Biotransformations using whole cells with (----) and without (—) the addition of 0.05% (w/v) sodium azide. (•) (−)γ-lactam, (■) (+)γ-lactam. Mixtures contained (20 ml) 0.2 g wet cell paste or clarified extract and racemic 0.1 g γ-lactam.

### *Purification*

(+)γ-Lactamase activity was purified from a strain of *Pseudomonas fluorescens* using an ammonium sulphate precipitation and three fast protein liquid chromatography steps. Wet cell paste (20 g) was lysed and the γ-lactamase purified as described below and in Table 1. Figure 4(a,b,c) shows the three successive purification steps.

### *Step 1*

Hydrophobic interaction chromatography was carried out using a phenyl-sepharose column and an ammonium sulphate gradient 1.5–0 M by the method detailed. Approximately 50% of the total protein failed to bind to the column, however the majority of the lactamase activity did. The lactamase was eluted over a concentration range approximately 1.1–0.7 M $(NH_4)_2SO_4$ (Figure 4a). Enzyme containing fractions (18–23) were pooled and dialysed against Buffer A to remove all of the $(NH_4)_2SO_4$. After this stage approximately 31% of enzyme activity was left with a six-fold purification.

### *Step 2*

All of the pooled activity from the phenyl-sepharose column was applied to a Fast Flow Q column in Buffer A and eluted over a gradient 0–1 M NaCl. The enzyme activity was contained in fractions 11 and 16 with approximate salt concentrations of 0.3–0.45 M (Figure 4b).

### *Step 3*

Final purification was performed on a preparative Superose 12 gel filtration column. All of the activity was contained in fractions 13–15 with the majority (>90%) in fraction 14, corresponding to a single protein peak as shown in Figure 4c. This fraction was concentrated using a Centricon 30 molecular weight filter. The final yield of enzyme activity was 2.82% with a specific activity of 28.6 μmol $min^{-1}mg^{-1}$ protein, a 22.5-fold purification over the crude extract. Examination of the Centricon concentrate by SDS-

**Table 1** Purification of (+)γ-lactamase from *Pseudomonas fluorescens* CMC 2803

| Step | Volume (ml) | Protein (mg) | Total activity ($\mu$mol min$^{-1}$) | Specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$ protein) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Crude lysate | 200 | 1040 | 1320 | 1.27 | 1 | 100 |
| 60–80% $(NH_4)_2SO_4$ | 20 | 252 | 603 | 2.39 | 1.88 | 45.7 |
| Phenylsepharose | 60 | 52.1 | 409 | 7.85 | 6.18 | 31.0 |
| Fast Flow Q | 60 | 12.3 | 186 | 15.1 | 11.9 | 14.1 |
| Superose 12 | 20 | 1.9 | 43.9 | 23.1 | 18.2 | 3.33 |
| Centricon 30 | 2 | 1.3 | 37.2 | 28.6 | 22.5 | 2.82 |

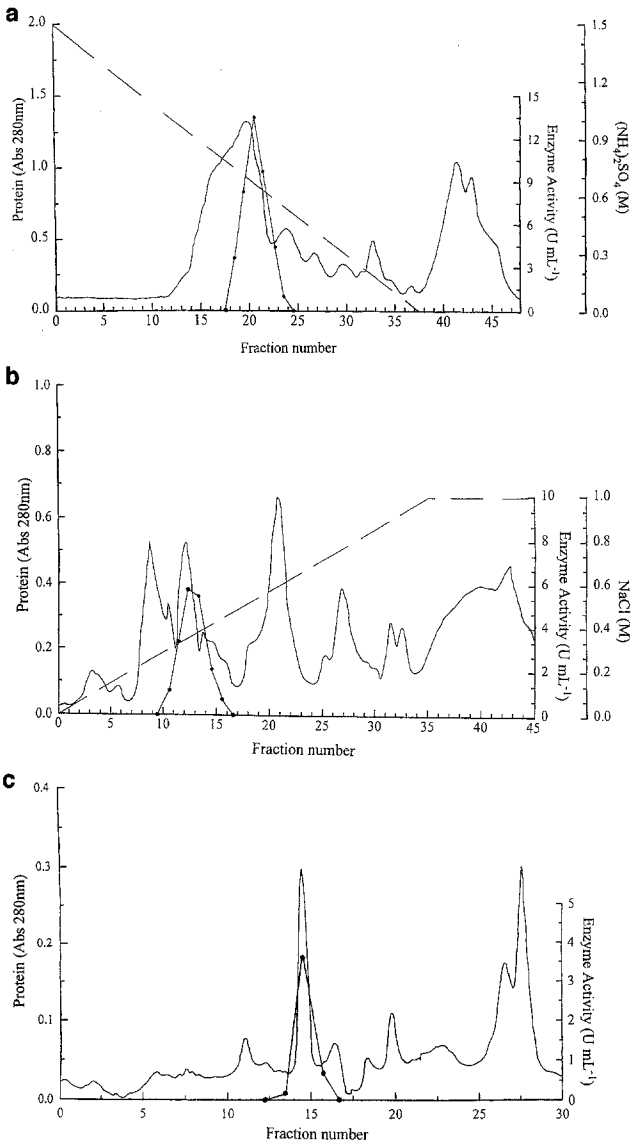


**Figure 4** Chromatographic steps used in the purification of γ-lactamase from *P. fluorescens* CMC 3060. For each step: (—) absorbance 280 nm (protein); (—●—) enzymic activity (U ml$^{-1}$); and (----) salt concentration (M); (a) Elution profile from Phenyl-Sepharose hydrophobic interaction chromatographic over a linear 1.5–0 M $(NH_4)_2SO_4$ gradient; (b) Fast Flow Q ion exchange chromatography over a linear 0–1 M NaCl gradient; (c) Gel filtration chromatographic using a Superose 12 gel filtration column.

PAGE electrophoresis yielded one band of approximate molecular weight 61 kDa.

### *Substrate specificity*

The semi-purified (+)γ-lactamase obtained from step 1 was highly selective and had no activity against a wide range of similar compounds over a 72-h period (all substrates were recovered at >95% initial mass) (Figure 5). Significantly the enzyme was stereospecific and incapable of hydrolysing the (–)lactam isomer. These results are similar to those reported by Bevan *et al* [1] who tested the purified γ-lactamases of *Pseudomonas fluorescens* and an *Aureobacterium* strain against a similar range of compounds and found no hydrolytic activity.

### *Temperature and pH optima*

The pH optimum of the (+)γ-lactamase, determined at 25°C in universal buffer, was in the range 7.0–7.5 (Figure 6). Outside of this optimal range, activity was surprisingly pH-resistant with up to 80% of maximum activity over the pH range 6.0–9.5. The temperature optimum of the enzyme was determined as 37°C with a rapid decrease in activity above 40°C and which fell to below 20% at 50°C (Figure 7). The temperature optimum is however a compromise between increased reaction rates at higher temperatures and thermal denaturation of the enzyme. A more important parameter is that of thermal stability.

### *Enzyme stability*

The enzyme was relatively unstable with activity rapidly lost at 37°C (Figure 8). At a lower storage temperature (4°C), although the enzyme denatured less quickly, it still lost activity quite rapidly. This loss of activity was more

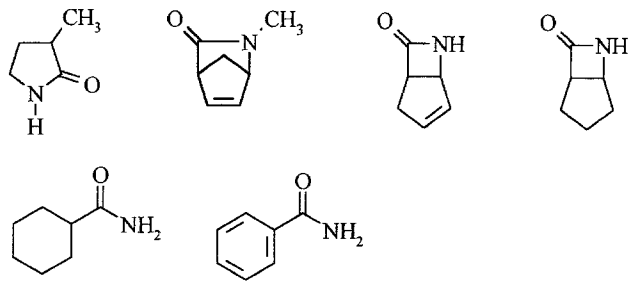

**Figure 5** Substrate specificity of semi-purified (+)lactamase. Phenyl-Sepharose eluate was assayed for activity against the substrates below by the lactamase method previously described.

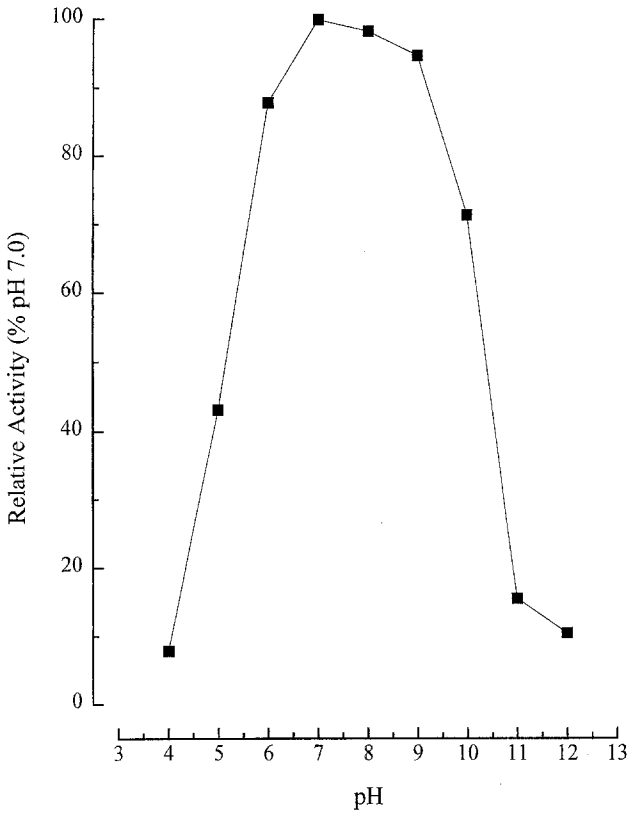


**Figure 6** Effect of pH on the activity of semipurified CMC 3060 (+)γ-lactamase. Lactamase activity was determined over a pH range 4–12 in universal buffer at 25°C as described in the methods section. Assays were carried out for 1 h. The activity at pH 7.0 was 7.43 μmol $min^{-1}$ $mg^{-1}$ protein.

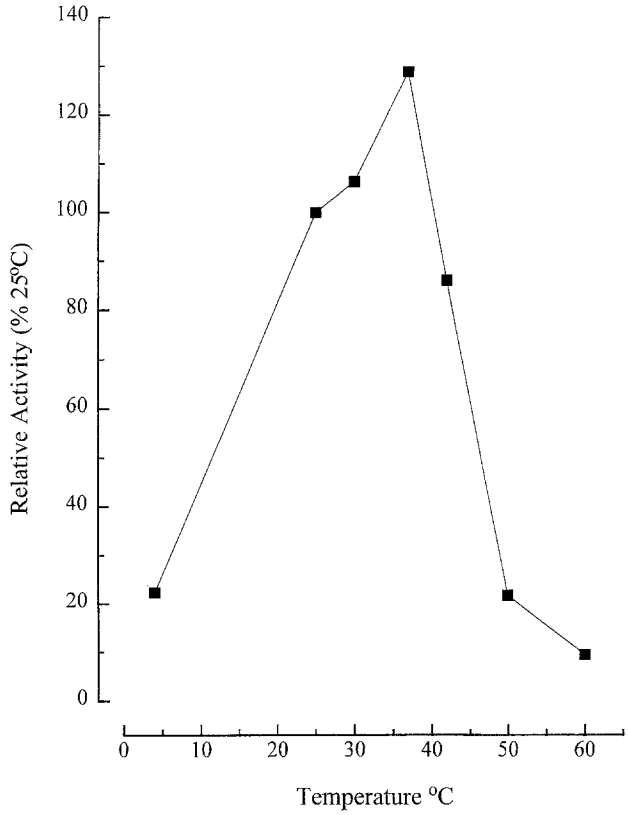


**Figure 7** The effect of temperature on the activity of semipurified CMC 3060 (+)γ-lactamase. Lactamase activity was determined over a temperature range 4–60°C. Assays were carried out for 1 h. The activity at 25°C was 6.3 μmol $min^{-1}$ $mg^{-1}$ protein.

rapid when the enzyme was stored as a clarified sonic lysate than as a whole cell suspension.

### *Effects of metal ions*

During the course of the purification, enzyme activity was rapidly lost. This loss of activity was overcome to some degree by the inclusion of a wide range of protease inhibitors and protein stabilisers as detailed above. A range of metal ions and co-factors was therefore added to a semi-purified sample which had lost over 50% of its initial activity to determine whether this loss was attributable to co-factor/metal ion loss. Metal ions added included $Ca^{++}$, $Mg^{++}$, $Co^{++}$, $Fe^{++}$, $Zn^{++}$, and $Mn^{++}$. Co-factors added included ADP, ATP, $NAD^+$, NADH, $NADP^+$, FMN, FAD, and NADPH. No significant increase or return of activity was detected upon addition of any of the co-factors or metal ions (results not shown).

### *Enzyme immobilisation*

Enzyme immobilisation can lead to increased stability [4]. An attempt was made to immobilise the semi-purified (+)lactamase onto a glutaraldehyde-activated disk (Acti Disk GTA, FMS Corporation, Pine Brook, NJ, USA). The phenyl-sepharose eluate (18.7 mg) immobilised on the disk had a specific activity of 115 μg $h^{-1}$ $mg^{-1}$ protein. After 48 h, 103 mg of γ-lactam, equivalent to 69% of the (+)lactam, was degraded. However after 100 h no activity was detected. Experiments carried out at the same time with

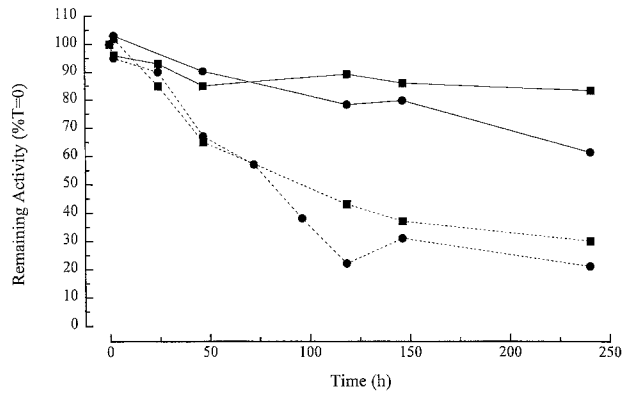


**Figure 8** Stability of the semipurified CMC 3060 (+)γ-lactamase at 4°C and 37°C: (----) 37°C, (—) 4°C, (•) sonic lysate, (■) whole cells.

whole CMC 3060 cells showed a significant loss of activity over a similar period (results not shown). It was postulated that this loss of activity was due to impurities in the substrate, however use of a new higher purity substrate did not increase the stability of the enzyme.

## Discussion

A number of microorganisms isolated from distinct ecosystems for their ability to utilise *N*-acetylphenylalanine as sole carbon source were screened for activity against γ-lactam. Some of these isolates produced stereospecific

hydrolytic enzyme activity against the target compound. *Pseudomonas fluorescens* strain CMC 3060 produced a lactamase capable of hydrolysing (+)lactam at much higher levels than the other strains tested. (+)Lactamase activity was produced constantly by strain CMC 3060; activity was detected in cells which had been grown in the absence of either lactam isomer in glucose–yeast-extract–salts medium. However in small scale biotransformations (−)lactamase activity was detected after 20 h. The addition of metabolic and protein synthesis inhibitors to the whole cell biotransformation inhibited the production of this second enzyme. It therefore appears that the (+)lactamase is a constitutive enzyme whereas the (−)lactamase is inducible, being produced only in the presence of the substrate.

Enzyme activity was detected over a wide pH range suggesting that the enzyme is stable over a wide pH range or that the nature of the reaction is not pH-sensitive in the range studied. Activity was however markedly affected by temperature, with rates increasing up to 37°C but rapidly falling at higher temperatures. This result suggested that above 37°C the enzyme is so unstable that denaturation is the significant reaction factor. Activity was stable for long periods when stored as frozen cells; the loss of stability at all temperatures was rapid in cell-free lysates. This activity could not be stabilised. The failure to stabilise activity by the inclusion of a large number of protease inhibitors indicated that loss of activity was due to an inherent instability within the protein. This conclusion was also supported by the increased stability of the enzyme with the addition of glycerol and ammonium sulphate. Experiments to evaluate enzyme stability at 37°C showed that activity was rapidly lost in both whole cells and clarified sonic lysates. Trials to test the shelf life of the enzyme at 4°C showed that enzyme activity was more stable than at 37°C but that this method of long term storage was not applicable. Freezing whole cells would therefore appear to be the best method for enzyme storage. This loss of activity is similar to that reported for ENZA 22 (+)lactamase whereas the ENZA 25 (−)γ-lactamase activity is very stable, retaining activity up to 80°C [1].

The (+)lactamase lost activity during purification and storage. The addition of metal ions and co-factors failed to stimulate activity in the semipurified enzyme or to regenerate activity in enzyme samples that had lost activity during purification. This result indicated that the hydrolytic activity of CMC 3060 lactamase does not require cofactors and that this enzyme does not have any metal ion requirement. We have noted that ENZA 22 and ENZA 25 γ-lactamase do not require metal ions so this result is not surprising (results not shown).

The natural substrate for this enzyme has so far not been determined, although substrate specificity tests did show that it is highly specific for the (+)lactam. As this substrate is not a normal part of metabolism and has not been found in nature, the natural substrate and role of this enzyme in metabolism is therefore not known. Pseudomonads are well known for their ability to utilise a wide variety of compounds as carbon and nitrogen sources however the production of two independently produced enantiomer-specific enzymes for the same substrate is novel. Two other (+)lactamases have been found in *Pseudomonas* (ENZA 20 and 22) species, however neither of these organisms had dual enantiomer activities [10]. Lactamase has not been detected in any other organisms at a significant level. The γ-lactamase enzyme may therefore play an important role in pseudomonad metabolism not found in other organisms.

Bevan *et al* [1] found that the N-terminal sequences of two γ-lactamases have no homology to each other or to any of the purified β-lactamases. The two purified γ-lactamases from ENZA 22 and 25 were also found to differ from one another, with molecular weights 33–35 kDa and 43 kDa, respectively. The γ-lactamase in this study appears to be very different with a molecular weight of 61 kDa by SDS-PAGE electrophoresis.

The organism appears to have potential for the production of chirally pure γ-lactam by hydrolysing the (+)lactam specifically with no action against the (−)isomer. This high specificity and activity gave yields in excess of 48% with an enantiomeric excess >99% in small scale biotransformations. Problems associated with the production of the inducible (−)lactamase could be overcome by stopping the reaction as soon as all of the (+)lactam has been degraded, or by the addition of DNA transcription and protein synthesis inhibitors such as streptomycin to inhibit (−)lactamase synthesis. The enzyme does not appear to be stable enough for use in an immobilised system on the scale of the ENZA 25 enzyme, which is currently used in a flow-through reactor batch process producing 1.42 kg $day^{-1}$ of (+)lactam (94.5% hydrolysis of the (−)lactam) with little loss of activity (78% initial activity after 400 days; results not shown). However given its high specific activity *P. fluorescens* 3060 may have potential as a whole cell biocatalyst under these conditions, as the enzyme appears to be stable when frozen and could be stored and used when needed [10].

## Acknowledgements

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