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The use of a thermostable signature amidase in the resolution of the bicyclic synthon (*rac*)-γ-lactam

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Abstract—The resolution of the bicyclic synthon (*rac*)-γ-lactam (2-azabicyclo[2.2.1]hept-5-en-3-one) is an important step in the synthesis of a group of chemotherapuetic agents known as carbocyclic nucleosides. The archaeon *Sulfolobus solfataricus* MT4 produces a thermostable γ-lactamase that has a high sequence homology to the signature amidase family of enzymes. It shows similar inhibition patterns of amidases towards benzonitrile, phenylmethylsulfonyl fluoride and heavy metals such as Hg^{2+}, and is activated by thiol reagents. The enzyme selectively cleaves the (+)-enantiomer from a racemic mix of γ-lactam. It also exhibits general amidase activity by cleaving linear and branched aliphatic and aromatic amides. The enzyme catalyses the synthesis of benzoic hydrazide from benzamide preferentially to benzamide cleavage in the presence of excess hydrazine. This enzyme has potential for use in industrial biotransformations in the production of both carbocyclic nucleosides and hydrazides.

1. Introduction

The search for and development of new therapeutic agents drives the need for the synthesis of new enantiomerically pure compounds. Partially purified enzymes or whole cells have been used industrially in the production of fine chemicals and pharmaceuticals. Enzymes are especially useful in the production of fine chemicals and pharmaceuticals due to their chemo-, regio- and more particularly their enantioselectivity.[1]

Carbocyclic nucleosides, where the ribose oxygen of the nucleoside is replaced by methylene, are valuable as chemotherapeutic agents such as cardiac vasodilators and in the treatment of viral infection.[2] γ-Lactam (2-azabicyclo[2.2.1]hept-5-en-3-one) is a bicyclic lactam that is well established as an important synthon in the synthesis of carbocyclic nucleosides such as the anti-HIV agent (−)-carbovir.[2,3] The resolution of (*rac*)-γ-lactam by enantioselective cleavage using enzymes is an economical means of obtaining single enantiomers of the bicyclic lactam.

The use of whole cell preparations and impure enzymes in the resolution of (*rac*)-γ-lactam has been reported previously.[2–4] γ-Lactamases from *Rhodococcus* sp (ENZA1) and an *Aureobacterium* sp (ENZA25) cleave the [1(*R*),4(*S*)]-(−)-enantiomer, while enzymes from *Pseudomonas solanacearum* (ENZA20) *P. cepaecia* and *P. fluorescens* (ENZA22) cleave the [1(*S*),4(*R*)]-(+)-enantiomers of γ-lactam.[2,3] A (+)-γ-lactamase enzyme was isolated from *Comomonas acidovorans* and cloned and over-expressed in *Escherichia coli*.[3] The sequence of this protein showed 63% identity to a formamidase from *Methylophilus methylotropus*[5] and 56% identity to an acetamidase from *Mycobacterium smegmatis*[6] a family of enzymes for which there is currently no structural information available. This enzyme has been crystallised and preliminary X-ray data has been obtained.[7] A (−)-γ-lactamase was isolated from an *Aureobacterium* sp which has been cloned and over-expressed in *E. coli*.[8] This enzyme has a high sequence identity (68%) to two cofactor free haloperoxidase enzymes from *Streptomyces aureofaciens*.[9,10]

No thermophilic γ-lactamase has been described to date and it was considered that a more stable form of this enzyme would be useful for industrial processes.[11] This paper describes the cloning, purification and use of a γ-lactamase from the thermophilic archaeon *Sulfolobus solfataricus* MT4 and a description of its potential use in biotransformation reactions.

Keywords: β-Lactamase; Signature amidase; *Sulfolobus solfataricus*; Biotransformations.

Abbreviations: γ-Lactam, 2-azabicyclo[2.2.1]hept-5-en-3-one; DAN, diazoacetyl-norleucine methyl ester; PMSF, phenylmethylsulfonyl fluoride.

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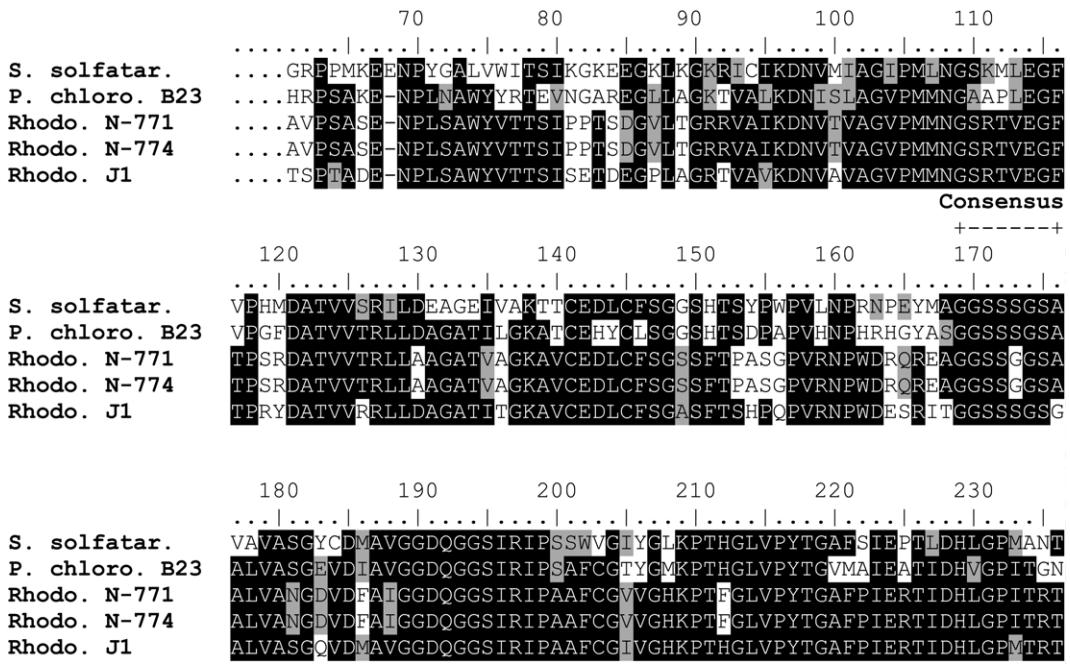


Figure 1. Partial alignment of the more conserved regions of the γ-lactamase/amidase from *Sulfolobus solfataricus* MT4 (*S. solfat.* MT4) with 4 other amidases. Residues boxed in black are entirely conserved. The alignment was created using ClustalW.[27] Consensus=the conserved GGSS(S/G)SA sequence of signature amidases. *P. chloro.* B23=*Pseudomonas chlororaphis* B23;[13] *Rhodo.* N-771=*Rhodococcus* sp. N-771 (Accession number BAA36596); *Rhodo.* N-774=*R. erythropolis* N-774;[28] *Rhodo.* J1=*R. rhodochrous* J1.[15]

2. Results

2.1. γ-Lactamase sequence

The thermostable amidase from *S. solfataricus* has been described previously.[12] Figure 1 shows an alignment of the amino acid sequences of the γ-lactamase from *S. solfataricus* MT4 with four other amidases from *Pseudomonas chlororaphis* B23,[13] *Rhodococcus* sp. N-771 (Accession number BAA36596), *R. erythropolis* N-774[28] and *R. rhodochrous* J1.[15] The γ-lactamase shows a 41–44% sequence identity to these amidases.

2.2. General properties

The γ-lactamase was purified to homogeneity as detected by SDS-PAGE (results not shown). The molecular mass of the monomer was estimated to be 55 kDa by SDS-PAGE which is consistent with the calculated molecular mass of 55.7 kDa. Figure 2 shows the temperature profile of the γ-lactamase. The highest activity was achieved at pH 7.0 at 85 °C with the substrate γ-lactam. This high optimal temperature is not surprising as *Sulfolobus* is a thermophilic archaeon. This enzyme is known to have a half life of 25 h at 80 °C.[12]

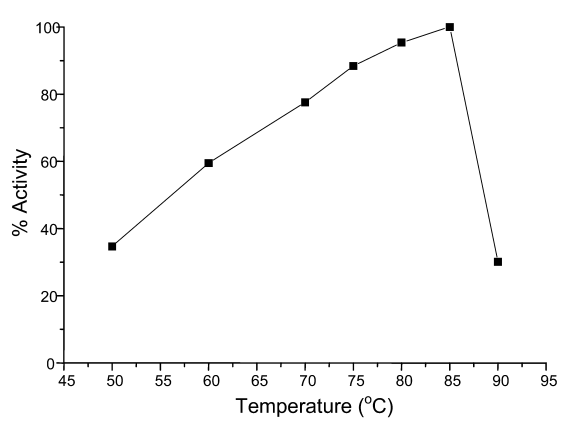


Figure 2. Temperature profile of γ-lactamase with 20 mM (*rac*)-lactamide in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0.

2.3. Inhibitors

Table 1 shows the effect of inhibitors on γ-lactamase. The enzyme was significantly inhibited by benzonitrile which has been suggested to be an amidase dead-end inhibitor.[14] The serine protease inhibitors PMSF and benzamidine

Table 1. Effect of inhibitors on the activity of γ-lactamase

Inhibitor	Class of inhibition	Activity remaining (%)
10 mM Benzonitrile	Amidase dead-end inhibitor	48.1
1 mM PMSF	Serine protease	38.4
1 mM Benzamidine	Serine protease	44.5
1 mM Dithiothreitol	Cysteine protease	145.5
1 mM Iodoacetic acid	Cysteine protease	120.9
10 μM Hg^{2+}	Binds to thiols	53.6
10 μM Pb^{2+}	Binds to thiols	79.2
10 μM Cu^{2+}	Binds to thiols	28.1
10 μM Fe^{3+}	Binds to thiols	111.3
10 mM EDTA	Metallo-enzyme inhibitor	105.8
1 mM *o*-Phenanthroline	Metallo-enzyme inhibitor	84.0
100 μM Pepstatin	Aspartic protease	99.5
10 mM DAN[a]	Aspartic protease	52.2

Activity was determined by the benzamide HPLC method as described.
[a] DAN=diazoacetylnorleucine methyl ester.

partially inhibited the enzyme, while the sulfhydryl reagents dithiothreitol (DTT) and iodoacetic acid (IAA) significantly activated the enzyme. Heavy metals such as Hg^{2+}, Pb^{2+} and Cu^{2+} were inhibitory.

The metal chelators *o*-phenanthroline and EDTA had slight to no inhibitory effect, respectively. There was no inhibition by the aspartic protease inhibitor pepstatin, but significant inhibition by the aspartate-modifying reagent diazoacetyl-norleucine methyl ester (DAN).

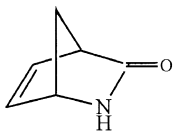
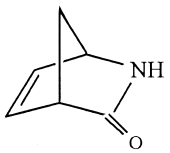
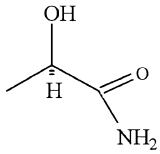
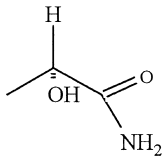
2.4. Substrate specificity

Table 2 shows the specific activity of the γ-lactamase towards a variety of amide substrates. The highest activity and enantioselectivity were detected with the (+)-enantiomer of γ-lactam. It had a slightly lower activity towards the substrate (*R*)-(+)-lactamide and a more relaxed enantioselectivity. The enzyme also exhibits activity towards a range of aliphatic and aromatic amides cleaved by other signature amidases[15,16] though with a much lower specificity. The enzyme had a low specificity towards acrylamide, urea and *N*-substituted amides.

The γ-lactamase achieved 50% conversion of industrial grade (*rac*)-γ-lactam at 10 g l^{-1} at 85 °C, which corresponds to about 100% conversion of (+)-γ-lactam. No reaction was detectable with (−)-γ-lactam.

The γ-lactamase was able to synthesize benzoic hydrazide

Table 2. Substrate specificity of γ-lactamase

Substrate	Structure	Specific activity (μmol/min/mg)
(+)-γ-Lactam		21.5
(−)-γ-Lactam		0.0
(*R*)-(+)-Lactamide		18.9
(*S*)-(−)-Lactamide		2.4
Formamide	$HCONH_2$	0.0
Acetamide	CH_3-CONH_2	0.2
Propionamide	$CH_3-CH_2-CONH_2$	0.8
Isobutyramide	$(CH_3)_2CH-CONH_2$	4.6
Trimethylacetamide	$(CH_3)_3C-CONH_2$	1.7
Butyramide	$CH_3-CH_2-CH_2-CONH_2$	1.4
Valeramide	$CH_3-CH_2-CH_2-CH_2-CONH_2$	3.1
Benzamide	$(C_6H_5)-CONH_2$	7.4
N,*N*-Dimethyl formamide	$HCON(CH_3)_2$	0.0
N,*N*-Dimethyl acetamide	$CH_3-CON(CH_3)_2$	0.4
Acrylamide	$CH_2=CH-CONH_2$	0.3
Urea	$(NH_2)_2CO$	0.1
Thiourea	$(NH_2)_2CS$	0.0

Activity towards γ-lactam and benzamide was determined by the HPLC methods described. Activity with all other substrates was determined by the ammonia detection method as described.

from benzamide and excess hydrazine (specific activity=8.6 μmol/min/mg) with negligible benzamide cleavage. In comparison, the enzyme cleaved benzamide to benzoic acid in the absence of hydrazine with a slightly lower specific activity of 7.4 μmol/min/mg. γ-Lactamase did not possess nitrile hydratase or nitrilase activity towards benzonitrile (results not shown). This is not surprising as benzonitrile is an inhibitor of the enzyme. It also had no detectable aminoacylase, protease or esterase activity (results not shown).

3. Conclusion

We have described the cloning, sequencing and characterization of a thermostable γ-lactamase/amidase from *S. solfataricus* MT4. It belongs to the signature amidase family which all contain the sequence GGSS(S/G)GS located in residue positions 173–179 (Fig. 1 numbering). The amino acid sequence of γ-lactamase contains the highly conserved putative catalytic residues Asp191 and Ser195, but not the highly conserved Cys207 residue (Asp194, Ser199 and Cys207 in Figure 1). Kobayashi et al., showed that the residues Asp191 and Ser195, and to a lesser extent Cys203, in signature amidases are important for catalytic activity.[15,17] The inhibition pattern of *S. solfataricus* amidase shows partial inhibition by PMSF and benzamidine, similar to the amidase from *R. rhodochrous* J1. There was also a significant inhibition by the aspartate-modifying reagent DAN. However, this is not a key indicator of the presence of a catalytic aspartate residue as the amidase from *R. rhodochrous* J1 was not inhibited by DAN.[15]

This class of amidases has a mixed response to the presence of thiol reagents. *S. solfataricus* and *R. rhodochrous* M8[18] amidases are activated by DTT while the amidase from *R. rhodochrous* J1 which contains the highly conserved Cys203 (valine in γ-lactamase) was inhibited by thiol reagents.[15] The lack of significant inhibition to metal chelators suggests that metal ions are not required for catalysis.

The highest activity and enantioselectivity were detected with γ-lactam as it specifically cleaves only the (+)-enantiomer. This makes it an industrially useful enzyme as it can be used at one stage in the synthesis of carbocyclic nucleosides. This is an example of the usefulness of enzymes as biocatalysts as their specificity can increase the yield of enantiomerically pure biochemicals and pharmaceuticals.

The more relaxed enantioselectivity towards lactamide could be due to the more flexible structure of lactamide compared to the bicyclic lactam allowing the unfavourable enantiomer to bind significantly to the enzyme. Activity towards aliphatic amides, such as acetamide and valeramide, showed that as the length of the hydrocarbon chain increased, the specific activity increased. The enzyme was over 3-fold more active towards isobutyramide than butyramide suggesting that branched-chained substrates are more favourable. The specific activity increased further with the aromatic substrate benzamide.

Compounds such as isoniazid (isonicotinic acid hydrazide) are widely used as anti-tuberculosis drugs.[17] The amidase from *R. rhodochrous* J1 was found to be capable of synthesizing hydrazides. Similarly, γ-lactamase was able to synthesize benzoic hydrazide from benzamide and excess hydrazine with negligible benzamide cleavage. The competing amidase reaction, cleavage of benzamide, occurred significantly only in absence of hydrazine. Thus, there is potential to develop processes using amidases in the synthesis of hydrazides in industry.

Protein crystals grew in droplets using 16–20% polyethylene glycol 4000 as the precipitant in 100 mM sodium acetate buffer pH 4.5–5.0. The crystals were small needles with dimensions 0.05×0.05×0.5 mm^3. These are not yet of sufficient quality for X-ray structural studies, however, they grow readily. These crystals are ideal for use after being chemically cross-linked with glutaraldehyde to further stabilize the enzyme and enable its easy recovery and potential reuse for commercial biotransformation applications.

The thermophilic archaeal γ-lactamase described in this paper belongs to the signature amidase class of enzyme which is different to the γ-lactamase enzymes previously described.[2,3,7,8] Due to its thermostability and substrate specificity the enzyme has use for commercial biotransformation applications.

4. Experimental

4.1. Cloning of the γ-lactamase

A search of the complete genomic sequence of the hyperthermophile *Sulfolobus solfataricus* strain P2[19] revealed the presence of a putative amidase (Swiss-Prot accession number P95896). Total genomic DNA was prepared from *Sulfolobus solfataricus* strains MT4 and P1 (obtained from CAMR, Salisbury) by the method described by Taylor et al.[3] The open reading frames encoding an amidase from each strain were amplified by the polymerase chain reaction (PCR). Restriction endonuclease cleavage sites for *Sph*I and *Bam*HI were incorporated within the 5′ and 3′ oligonucleotide primers, respectively. Seven open reading frames were detected and sequenced by Cambridge Bioscience (Cambridge, UK) and found to be identical. The PCR fragments were digested with *Sph*I and *Bam*HI (New England Biolabs, Essex), ligated into pUCCER11 expression vector[3] and transformed into *Escherichia coli* strain DH5 (Dibco BRL, Scotland).

4.2. Production and purification of recombinant γ-lactamase

The *E. coli* strain DH5 harboring the γ-lactamase/amidase was grown in a 2 l ST Applikon bioreactor, as described by Toogood et al.[20] and a cell-free enzyme extract from 10 g of cell paste was prepared by sonication. The enzyme was precipitated with 40% (saturated) ammonium sulfate and recovered by centrifugation at 11,000*g* for 30 min at 4 °C. The precipitate was resuspended and dialysed against 10 mM NaH_2PO_4/Na_2HPO_4 pH 7.0, then loaded onto a

60 ml phenyl Sepharose Fast Flow column (Amersham Pharmacia, Buckinghamshire) pre-equilibrated in 20 mM Tris–HCl pH 8.0 containing 1 M ammonium sulfate. Protein was eluted with a gradient of equilibration buffer to 20 mM Tris–HCl pH 8.0, followed by a gradient of the latter buffer to 20 mM Tris–HCl pH 8.0 containing 50% ethylene glycol. The active fractions were concentrated and equilibrated in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 by ultrafiltration using a Centriprep-10 (Amicon, Gloucestershire) concentrator. The enzyme was loaded onto a HiLoad Superdex 200 gel filtration column (Amersham Pharmacia, Buckinghamshire) and eluted with 50 mM Tris–HCl pH 8.0 containing 100 mM NaCl.

4.3. Protein determination

The protein concentration was determined by a modification of the Bradford assay method[21] kit supplied by BioRad (Hertfordshire, UK). BSA was used as a standard in the range of 0–100 μM.

4.4. Determination of molecular mass

Enzyme extract samples were run on SDS-PAGE[22] to determine their degree of purity and molecular mass. Protein bands were stained using a coomassie staining method.[23] Molecular weight standards in the range of 14.4–97 kDa were obtained from Amersham Pharmacia (Buckinghamshire). Native molecular mass of the purified enzyme was determined by gel filtration using a Superdex 200 Hiload 16/60 column (Pharmacia). The column was equilibrated with 10 mM Tris–HCl pH 8.0 buffer at 0.3 ml/min and calibrated with the standards ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa).

4.5. Enzyme assays

All enzyme assays were carried out with 0.05–0.5 μM enzyme. Activity towards γ-lactam was determined by incubating the enzyme in 1 ml of 10 g l^{-1} (91 mM) (*rac*)-γ-lactam in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C. The reaction was terminated by adding 100 μl of the reaction to 900 μl of 50:50 methanol:10 mM NaH_2PO_4/Na_2HPO_4 pH 7.0. Analysis was performed by HPLC as described by Gonsalvez et al.[7]

General amidase activity was determined by incubating the enzyme in 1 ml of 20 mM amide substrates in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 50 °C. The amount of ammonia produced in the reaction was determined by the phenol/hypochlorite method of Silman et al.[24] Activity towards benzamide was determined with 10–20 mM substrate in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C. The reaction was terminated by adding 100 μl of the reaction to 900 μl of 50:50 methanol:10 mM NaH_2PO_4/Na_2HPO_4 pH 7.0. Analysis was performed by HPLC as described by Kobayashi et al.[15] Nitrilase and nitrile hydratase activity with benzonitrile was determined according to the method of Kobayashi et al.[25] Synthesis of benzoic acid hydrazide from benzamide and hydrazine was carried out according to the method of Kobayashi et al.[17]

Proteolytic activity was determined by incubating the enzyme in 1 ml of 0.2% azocasein in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C. Detection of the cleavage products was performed by the trichloroacetic acid (TCA) precipitation method of Peek et al.[26] Esterase activity with *p*-nitrophenyl acetate was determined by incubating the enzyme in 1 ml of 0.4 mM substrate in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 containing 0.8% (w/v) Triton X-100 and 10% (v/v) acetonitrile with continuous monitoring of the absorbance at 400 nm for 1–3 min at 30–50 °C. Aminoacylase activity was determined in 1 ml of 10 mM *N*-acetyl-DL-tryptophan in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C according to the method of Toogood et al.[20]

4.6. Optimization of reaction conditions

The optimal temperature was determined by reacting the γ-lactamase in 1 ml of 20 mM (*rac*)-lactamide in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h in the temperature range of 50–90 °C using the ammonia detection method of Silman et al.[24] To determine the optimal pH, the γ-lactamase was reacted as above for 10 min at 85 °C in acetate (pH 3.0–4.5), phosphate (pH 4.5–8.5) and borate (pH 8.5–10.0) buffers.

4.7. Inhibitor studies

To determine the effect of inhibitors on activity the enzyme was incubated with the inhibitors for 2 h at 25 °C, then assayed for remaining activity using the above benzamide substrate. The concentration of the inhibitors in the assay was the same as in the preincubation with the enzyme.

4.8. Crystallisation

Crystallisation experiments were carried out using either the sitting or hanging drop vapour diffusion method. The purified protein was concentrated to 10 mg/ml using a Vivaspin 2 (Sartorius AG, Germany) and diluted with an equal volume of well precipitant (16–20% polyethylene glycol 4000 in sodium acetate buffer pH 4.5–5.0). The crystallization dish was incubated at 17 °C.

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