Phenoxymethylpenicillin amidohydrolases from *Penicillium chrysogenum*

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Abstract A phenoxymethylpenicillin amidohydrolase which hydrolyses phenoxymethylpenicillin to 6-aminopenicillanic acid (6-APA) has been isolated from two species of *Penicillium chrysogenum*. The amidohydrolase had a molecular mass of approx. 42 kDa. Its activity with benzylpenicillin as substrate was only 1.5% of that with phenoxymethylpenicillin and it was inhibited by its products. No penicillin formation from 6-APA and phenoxyacetyl or phenylacetyl coenzyme A was observed. The enzyme is thus distinct from the phenylacetyl coenzyme A: 6-APA acyltransferase, which also has amidohydrolase activity and is involved in the final stages of the biosynthesis of penicillins.

Key words: Amidohydrolase; Phenoxymethylpenicillin; 6-Aminopenicillanic acid; *Penicillium*; Penicillin biosynthesis

1. Introduction

The first indication of the presence of an enzyme in *Penicillium chrysogenum* that hydrolysed benzylpenicillin to 6-aminopenicillanic acid and phenylacetic acid (Fig. 1) was obtained by Sakaguchi and Murao [1]. Subsequent studies by others [2–5] showed that phenoxymethylpenicillin and certain penicillins with aliphatic side-chains were hydrolysed more rapidly by *P. chrysogenum* than was benzylpenicillin, although it was believed [4] that the crude enzyme did not hydrolyse penicillin N or isopenicillin N which have D-α-aminoadipyl and L-α-aminoadipyl side-chains respectively (Fig. 1).

Formation of benzylpenicillin by the transfer of a phenylacetyl group from coenzyme A to 6-APA by a crude enzyme preparation from *P. chrysogenum* was described by Brunner et al. [6] and Gatenbeck and Brunsberg [7]. Later Fawcett et al. [8] showed that an extract of *P. chrysogenum* could also bring about the formation of benzylpenicillin from isopenicillin N, in the presence of phenylacetyl coenzyme A, while studies with partially purified acyl-CoA:6-APA acyltransferase led Spencer and Maung [9] to suggest that this enzyme could bring about an exchange of penicillin side-chains and also show penicillin amidohydrolase activity.

A highly purified preparation of the acyltransferase was then shown by Whiteman et al. [10] to convert isopenicillin N, as well as 6-APA, into benzylpenicillin. The acyltransferase protein could also function as an amidohydrolase, yielding 6-APA from isopenicillin N and phenoxymethylpenicillin.

During purification of the acyltransferase a different enzyme with phenoxymethylpenicillin amidohydrolase activity was detected. Meesschaert et al. [11], using a non-penicillin producing strain of *P. chrysogenum* (Wis 49-408) and Alvarez et al. [12] using a high penicillin producer (AS-P-78), have also noted the existence of a penicillin amidohydrolase in *P. chrysogenum* which was distinct from the acyltransferase. This enzyme was reported to have a molecular mass of 356 kDa [11]. The present paper describes our findings on the purification and properties of a phenoxymethylpenicillin amidohydrolase from a penicillin producing strain (SC6140, ATCC 2044) and the non-producing strain of *P. chrysogenum*.

2. Materials and methods

2.1. Growth of P. chrysogenum

P. chrysogenum SC 6140, ATCC 2044 was grown essentially as described by Fawcett et al. [8]. *P. chrysogenum* Wis 49-408, a non-penicillin producing strain, was obtained from Professor P. Claes, Katholieke Universiteit, Leuven, Belgium, and grown as described by Vanderhaeghe et al. [4].

2.2. Preparation of cell free extracts

The mycelium was normally ground with glass beads in a dynomill [13], but some extracts were prepared by shaking the mycelium in the presence of 0.2 M NaCl and 5 mM dithiothreitol (DTT) at 4°C [4].

2.3. Enzyme purification

After precipitation of nucleic acids from the crude dynomill extract with protamine sulphate and elution of the enzyme from DEAE-Sephacel with a NaCl gradient further purification was achieved on Phenyl Sepharose CL-4B (elution with a decreasing ammonium sulphate gradient), Affi-gel Blue chromatography (elution with a zero to 1.3 M NaCl gradient) followed by a TSK Gel Phenyl-5PW HPLC column in 50 mM phosphate buffer (pH 7.2) containing 0.5 M NaCl at a flow rate of 1 ml/min.

2.4. Enzyme assays

Phenoxymethylpenicillin amidohydrolase, isopenicillin N amidohydrolase and phenoxyacetyl/phenylacetyl CoA: 6-APA acyltransferase were determined by bioassay essentially as described previously [10]. Phenoxymethylpenicillin amidohydrolase was also assayed by determination of 6-APA and phenoxyacetic acid by HPLC on a Waters C18 column in 50 mM KH_2PO_4 (pH 4.65).

3. Results and discussion

Similar amounts of phenoxymethylpenicillin amidohydrolase activity were obtained from the penicillin producing and non-producing strains (Table 1). The extract prepared by shaking in NaCl contained only 15% of the activity present in the dynomill extract.

Chromatography on Affi-gel Blue resolved the phenoxymethylpenicillin amidohydrolase from the acyl CoA: 6-APA acyltransferase although the latter also had some amidohydrolase activity (Fig. 2).

The phenoxymethylpenicillin amidohydrolase activity obtained from the Phenyl-5PW HPLC column coincided precisely with the intensity of the 42 kDa protein band, revealed by Coomassie staining, on SDS polyacrylamide gel electrophoresis (Fig. 3A,B). The pooled fractions from this column gave a single band on SDS gel (Fig. 3B, lane 6). To confirm the identity of the protein in this band with that of the purified enzyme, the latter was run on a gel under non-denaturing

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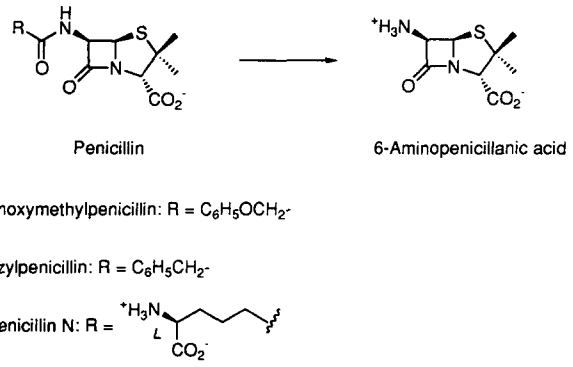

Fig. 1. Conversion of penicillin to 6-aminopenicillanic acid.

conditions. A single protein band was revealed by Coomassie staining. Protein in this position was eluted and shown to possess amidohydrolase activity. On rerunning on SDS gel, it gave a single 42 kDa band.

Gel filtration through Sephacryl S200 HR in 50 mM Tris-HCl (pH 7.5) containing 10% ethanediol also indicated that the amidohydrolase from both strains had a molecular mass of 41–44 kDa. Gel permeation HPLC on a Waters protein pak I 125 column in 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4 (pH 6.8) containing 20% ethanediol gave a molecular mass of 39–42 kDa. In contrast, an active phenoxymethylpenicillin amidohydrolase obtained from *P. chrysogenum* Wis 49-408 by Meesschaert et al. [11] was reported to have a much higher molecular mass of 356 kDa and was believed to be a tetramer. However, no evidence for the presence of an active tetramer was obtained in the present study.

In an attempt to discover whether the penicillin amidohydrolase had any similarity to the acyl coenzyme A: 6-APA acyltransferase or other amidohydrolases four small peptides, obtained after enzymic hydrolysis, were sequenced [10]. But a computer search revealed no obvious similarities to sequences in other known proteins.

The optimum pH for the phenoxymethylpenicillin amidohydrolase in phosphate buffer (50 mM) was 7.6. The optimum temperature was 34°C. An apparent K_m value of 0.54 mM for phenoxymethylpenicillin was observed under these conditions.

In dilute buffers the amidohydrolase was relatively unstable but it was partially protected by the presence of 1 mM DTT, 50 μg/ml bovine serum albumin and 0.5 M NaCl. In the presence of 20% ethanediol it showed no loss in activity after storage at −20°C for 4 days.

6-Aminopenicillanic acid (6-APA, 10 mM) and 7-aminocephalosporanic acid (7-ACA, 5 mM) inhibited the amidohydrolase activity against phenoxymethylpenicillin (1 mM) by about 50%. A similar inhibition was obtained with 1 mM phenoxyacetic acid. Phenylacetic acid was a less effective inhibitor than phenoxyacetic acid.

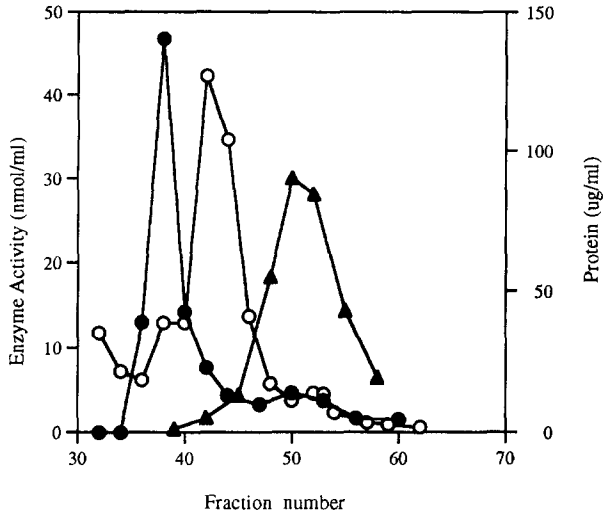

Fig. 2. Chromatography on Affi-gel Blue of a crude eluate from DEAE-Sephacel to show the separation of the phenoxymethyl-penicillin amidohydrolase (●) from the acylcoenzyme A: 6-APA acyltransferase (▲). Enzyme activities were determined by bioassay. Protein concentration (○) was determined by the method of Bradford (BioRad).

With benzylpenicillin as substrate the amidohydrolase activity was only 1.5% of that observed with phenoxymethylpenicillin at the same concentration and the rate of hydrolysis of phenoxymethylpenicillin was reduced by up to 80% in the presence of an equivalent concentration of benzylpenicillin. No amidohydrolase activity was detected in the presence of phenoxyacetyl-7-aminocephalosporanic acid (1 mM or 5 mM) and this cephalosporin completely inhibited the hydrolysis of phenoxymethylpenicillin (1 mM). The purified enzyme showed no activity against phenoxyacetyl-L-alanine, although the latter was rapidly hydrolysed by a crude extract of the *Penicillium*.

These findings suggested that the kinetics of the hydrolysis of phenoxymethylpenicillin by the amidohydrolase could be partly governed by product inhibition. However, in the presence of the very low concentration of the relatively unstable enzyme available the concentrations attained by the products were too low for a significant inhibition to be detected directly.

The inhibition of the enzyme by 7-ACA is presumably due to its competition for the site on the enzyme which is occupied by the 6-APA moiety of phenoxymethylpenicillin. But the failure of phenoxyacetyl-7-ACA to undergo hydrolysis may reflect an inability of this cephalosporin to reach a required position at the enzyme's active site.

The phenoxymethylpenicillin amidohydrolase did not hydrolyse isopenicillin N (1 mM) nor did it exhibit any phenoxyacetyl or phenylacetyl coenzyme A: 6-APA acyltransferase

Table 1
Yields of phenoxymethylpenicillin amidohydrolase activity obtained on grinding in a dynomill or shaking in sodium chloride

Strain	SC 6140		Wis 49-408	
	Activity (pmol/s)	Spec. act. (pmol/s/mg)	Activity (pmol/s)	Spec. act. (pmol/s/mg)
Dynomill extract	25153	16.6	22675	10.6
NaCl extract	650	1.1	3042	8.5

The values represent activity obtained from 100 g damp dry mycelium and were determined by bioassay in the presence of 1 mM phenoxymethylpenicillin.

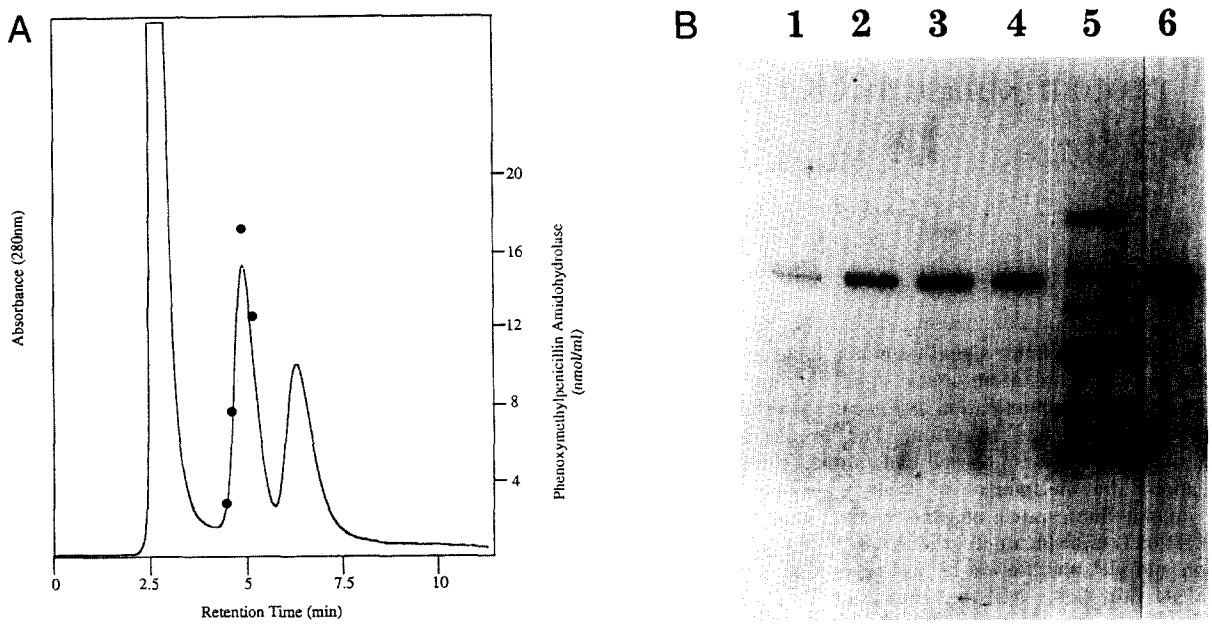

Fig. 3. (A) Elution of phenoxymethylpenicillin amidohydrolase activity (●) from a Phenyl-5PW HPLC column and (B) SDS-PAGE (3–27% acrylamide) analysis of fractions from the column. Lanes: (1) 4.5 min, (2) 4.75 min, (3) 5 min, (4) 5.25 min, (5) SDS-7 molecular weight markers (Sigma) containing 7 proteins of molecular weights 66, 45, 36, 29, 24, 20 and 14 kDa, (6) pooled Phenyl-5PW fractions.

activity. The purified acyltransferase shows amidohydrolase activity in the presence of both isopenicillin N and phenoxymethylpenicillin [10,12] but is a distinct enzyme.

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