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Discovery, purification, and properties of *o*-phthalyl amidase from *Xanthobacter agilis*

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Abstract

A selective screen for organisms that would metabolize *o*-phthalyl protected beta-lactams resulted in the discovery of a *Xanthobacter agilis* strain that contains an *o*-phthalyl amidase. The low level of enzyme expression in this organism could be enhanced by growing it on medium containing *o*-phthalate. The enzyme was purified to near homogeneity by a 6-step procedure. The phthalyl amidase was characterized for its molecular mass, amino acid composition, internal sequences, catalytic and kinetic properties. No metal ion was required by or stimulatory to the enzyme. The amidase catalyzed conversion could be complete with a reaction stoichiometry of 1:1. The pH and temperature stability of the enzyme is improved significantly by increasing ionic strength of the buffer. The enzyme exhibits a broad substrate specificity for *o*-phthalylated amides; however, it demonstrates an absolute requirement for the *o*-phthalyl protecting group. The broad substrate acceptance, high catalytic activity, and stability at high salt or substrate concentration of the enzyme indicates that it can serve as a gentle method for deprotecting phthalimido and *o*-phthalyl protected amides in new chemo-enzymatic synthetic routes.

Keywords: Enzyme discovery; Purification; Characterization; Specificity

1. Introduction

Phthalimido protection of amines has been used by chemists to develop new syntheses of a number of compounds including oligosaccharides [1] and beta-lactams [2]. The advantage of phthalimido as a stable protecting group in beta-lactam chemistry has been largely negated by the inability of conventional chemical methods of removing this group effectively. Low yields or the need to use hazardous chemicals have plagued efforts to remove this otherwise attractive protecting group. In the course of examining the stability of a phthalimido azetidinone (Fig. 1) to long term incubation at neutral pH, it was found that this compound and all similar compounds examined opened to the *o*-phthalyl derivative. This compound was stable upon further incubation. Removal of this protecting

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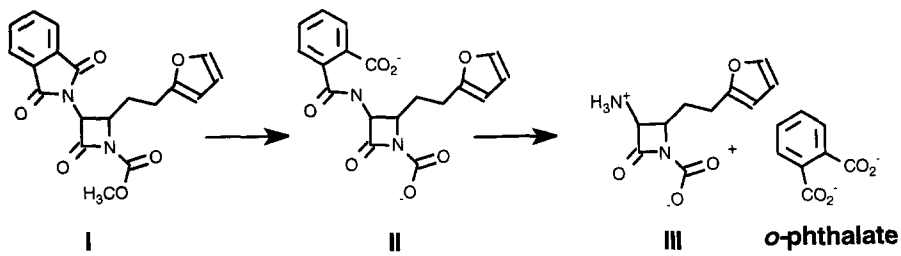


Fig. 1. Removal of phthalimido protecting group.

group using a biocatalytic approach now seemed possible. However, no existing enzyme capable of removing *o*-phthalyl group from any amide had been previously described. To overcome the problems with removing phthalimido, it was envisioned that an amidase, specific for *o*-phthalyl groups, might cleave the amide linkage to liberate the beta-lactam (compound III) without affecting the beta-lactam bond. This mild approach could have dramatic implications for beta-lactams, and, depending on the enzyme's specificity, could have broader implications for general chemical methodology.

We now report for the first time, the discovery of a natural enzyme that will dephthalylate *o*-phthalylated amides. The formation of the *o*-phthalyl amides from the corresponding phthalimido compounds as well as the enzymatic dephthalylation are carried out under mild aqueous conditions. For selection of a novel industrial relevant *o*-phthalyl amidase, a contemporary paradigm was undertaken for microbial screening, enzymology, gene cloning, and enzyme process scale-up. A new enzyme, which catalyzes the reaction as shown in Fig. 1, was discovered from *Xanthobacter agilis*. The amidase was purified to near homogeneity and, via 'reverse genetics', the gene encoding the amidase was cloned and over-expressed extracellularly by *Streptomyces lividans* [3]. Using the natural amidase, an enzyme process was developed with phthalyl carbacephem as the substrate [4]. Here we describe the discovery, purification, and broad substrate specificity of *o*-phthalyl amidase from *Xanthobacter agilis* as well as other essential properties of the enzyme.

2. Materials and methods

2.1. Reagents

Some of the compounds that were tested as substrates for the phthalyl amidase were synthesized at Lilly Research Laboratories. All other chemicals were of the highest purity commercially available.

2.2. Screening for o-phthalyl amidase

2.2.1. Screening of known bacteria

Trypticase soy broth (Difco) was inoculated with lyophils of cultures and incubated at 30°C on a rotary shaker overnight. This culture was used to inoculate trypticase soy broth or brain–heart infusion broth and was incubated as stated above for 24 h before the addition of *N*-*o*-phthalylglycine sodium salt at a final concentration of 1 mg/ml. It was hoped that this substrate would induce a phthalyl amidase if one was present. After an additional incubation of 24 h, broths were centrifuged and cells were washed once with 50 mM potassium phosphate buffer pH 7.0. The cells were

suspended in the phosphate buffer containing glucose and compound II (Fig. 1), both at final concentrations of 1 mg/ml. Gram negative organisms also were suspended in phosphate buffer containing compound II (1 mg/ml), Ethomeen 18/25 (4 mg/ml), and xylene (0.01 mg/ml) to permeabilize the cell membrane. Reaction mixtures were assayed by HPLC at 24 and 96 h of incubation for the hydrolysis of II.

2.2.2. Screening of molds and Actinomycetes

Lyophils of molds and *Actinomycetes* were inoculated into medium #172 and incubated on a rotary shaker at 25°C for molds and 30°C for actinomycetes. After 24 h, this vegetative culture was used to inoculate another flask containing medium 172 or CSM medium (see below). This production culture was incubated for an additional 96 h. At this time, 1 mg/ml of *N*-phthalylglycine was added and the culture was incubated for 24 additional hours. Cells were washed one time with 50 mM potassium phosphate buffer pH 7.0 and then suspended in buffer containing glucose (1 mg/ml) and II (1 mg/ml). The suspension was examined at 48 and 144 h of further incubation for hydrolysis of compound II by HPLC.

2.2.3. Screening of enzymes

A reaction mixture of compound II (1 mg/ml), enzyme preparation (1 mg/ml), and 50 mM potassium phosphate buffer pH 7.0 was incubated at 28°C for 16 h and then examined by HPLC for conversion of compound II to compound III and *o*-phthalate.

2.2.4. Isolation of microorganisms from soils

Samples of soils from around the world (8–15 mg) were inoculated into modules with 10 ml of BL medium containing II (10 mg/ml) as the primary carbon source and incubated at 30°C on a rotary shaker at 250 rpm for 2 weeks. Broths were examined by TLC at 7 day intervals for presence of III (Fig. 1) and *o*-phthalate. Those soil broths that showed the presence of III were transferred several times using this enrichment procedure to enhance for II hydrolyzing organisms. The final suspensions were plated to isolate colonies, and the pure organisms were grown in BL medium and Bac MI medium containing II (10 mg/ml). Pure cultures that converted compound II to III were examined further. No breakdown of compound II was seen in the absence of the soil suspension.

2.2.5. Taxonomy of organisms isolated from soil enrichment

Pure organisms were grown on agar prepared for that purpose, incubated under controlled conditions of time and temperature. Cells were harvested from agar plates and saponified with the appropriate reagents. The cell extracts were methylated to form fatty acid methyl esters (FAMEs). The FAMEs were extracted into an organic phase from an aqueous suspension and subjected to GC analysis. The identity of the cell extract FAMEs was determined by comparison to standard FAMEs. The pattern obtained from the cell extracts were then compared to known organisms via an HP 5898A microbial identification system. This procedure generates a similarity index which is used to classify the unknown organism [5].

2.2.6. Comparison of dephthalylating activity of isolates

Organisms that were shown to have dephthalylating activity were grown for 24 h at 30°C in Bac MI medium. Centrifuged cells were divided equally and then added to BL medium or Bac MI medium with phthalic acid at a final concentration of 1 mg/ml. After 48 h of incubation, broths were centrifuged, and the supernatant was discarded. Cells were washed once with 0.05 M phosphate buffer

pH 7.0 and then suspended in the same buffer containing Compound II at final concentration of 1 mg/ml. Samples were taken at different times and examined on HPLC for presence of III and *o*-phthalate and the disappearance of II.

Broth samples of whole cell bioconversions were submitted to LC/MS for analysis and confirmation of the formation of III and *o*-phthalate from II. One hundred microliters of each incubation solution was injected through a Rheodyne injector onto an 8 × 10 Waters RCM reverse phase HPLC cartridge column equipped with a pre-column of the same composition. The chromatographic separation of II and III was achieved by pumping 1 ml/min of mobile phase using the following gradient conditions: 100% mobile phase A (90:10 0.05 M ammonium acetate: methanol, pH 7.0) for 5 min, then linearly change to 100% mobile phase B (50:50 0.05 M ammonium acetate: methanol, pH 7.0) during the next 8 min, remain at 100% mobile phase B for 5 min and then linearly return to 100% mobile phase A during the final 3 min. The gradient is pumped through the column using a Waters 600-MS HPLC pump with Silk software. The 1 ml/min flow was supplemented with a 0.7 ml/min flow of 0.1 M ammonium acetate delivered post-column through a low dead volume 'T' fitting. The HPLC eluant was delivered to a Nermag thermospray ion source. The ion source block temperature was maintained at 180°C with a probe tip temperature of 221°C and a repeller voltage of 200 eV. The mass spectrometer was equipped with a photoelectron multiplier set at 600 eV gain in positive ion detection. For single scan operation Q1 and Q2 were kept in rf only operation while Q3 scanned the mass range of 200–450 amu every second during the entire analysis except for the analysis of *o*-phthalate which was scanned from 100–450 amu every second. The data was collected DEC PDP 11/73 microcomputer interfaced to the mass spectrometer equipped with SIDAR software.

2.2.7. *Crude enzyme preparation*

Cells exhibiting dephthalylating activity were suspended in 50 mM Tris pH 8.0 buffer at ratio of 1 g wet weight cells to 8 ml buffer. A solution of lysozyme, 2 mg in 1.0 ml 50 mM disodium EDTA pH 8.2, was added at a ratio of 1 ml per 8 ml of cell suspension. After mixing well and holding at room temperature for one hour, the suspension was refrigerated overnight at 4°C. The viscous solution was sonicated only long enough to liquefy the viscous mass. The material was centrifuged at 10000 rpm for 15 min. The pellet was discarded and the supernatant tested for phthalyl amidase activity.

2.2.8. *Growth and screening medium*

BL medium consisted of 6.00 g/l Na_2HPO_4, 3.00 g/l KH_2PO_4, 0.50 g/l NaCl, 2.00 g/l NH_4Cl, 0.111 g/l $CaCl_2$, 0.247 g/l $MgSO_4 \cdot 7H_2O$, 0.0692 g/l $ZnSO_4 \cdot 7H_2O$, 0.270 g/l $FeCl_3 \cdot 6H_2O$, 0.080 g/l $MnSO_4$, 0.0074 g/l $CuCl_2$, 0.0281 g/l $CoSO_4 \cdot 7H_2O$, 0.003 g/l H_3BO_3, and 1.00 g/l yeast extract. Final pH was 7.0. Bac MI medium consisted of peptone 10.00 g/l, beef extract 5.00 g/l, yeast extract 2.00 g/l, and NaCl 5.00 g/l. Final pH was 7.0. Medium 172 consisted of 5.00 g/l glucose, 10.00 g/l soluble starch, 2.50 g/l yeast extract, 2.50 g/l *N-Z* amine type A, 0.50 g/l $CaCO_3$. Final pH was 7.0–7.5. CSI medium consisted of 15.00 g/l soybean flour, 1.00 g/l *N-Z* amine type A, 25.00 g/l glucose, 3.00 g/l black strap molasses, 2.50 g/l and $CaCO_3$, 2.00 ml of Czapek's mineral stock. Final pH was 7.2–7.5. Czapek's mineral stock solution consisted of two parts. Part A was made up with 100.00 g of KCl, 100.00 g of $MgSO_4 \cdot 7H_2O$, and 900.00 ml distilled water. Part B consisted on 2.00 g of $FeSO_4 \cdot 7H_2O$, 2.00 ml of 1 N HCl, and 100.00 ml of distilled water. After dissolution, Part A and B are mixed and refrigerated. CSM consisted of 30.00 g/l T-soy broth (Difco), 3.00 g/l yeast extract, 2.00 g/l $MgSO_4 \cdot 7H_2O$, 5.00 g/l glucose, and 4.00 g/l maltose. The pH of the medium was not adjusted.

2.2.9. Thin layer chromatography

Compound II was chromatographed on Silica gel 60 F254, 5% NaCl/CH_3CN (1:1), and detected by short wave UV and iodine at a R_f value of 0.09. Compound III moved to an R_f of 0.13 and *o*-phthalate moved to 0.62.

2.2.10. High pressure liquid chromatography

Waters Radial Pak containing Nova Pak C18 cartridge with μBondapak C18 guard column were used to separate compound II, III, and *o*-phthalate. Solvent A = 90% 50 mM potassium phosphate buffer pH 7/10% CH_3CN. Solvent B = 50% Buffer/50% CH_3CN.

Gradient:	0% B	⟶	100% B	⟶	0% B
	3 min	10 min	5 min	3 min	9 min

Retention times: Compound II, 11.7 min: Compound III, 5.7 min: *o*-phthalate, 2.7 min.

2.3. Enzyme assays

2.3.1. For purification

The enzyme activity was determined using 4-(2′-carboxy-*N*-benzoyl)amino-2-carboxy-nitrobenzene (IV, Fig. 2) as a chromogenic substrate. A typical reaction mixture in a total volume of 1 ml contained 0.2 mg of the chromogenic substrate and an aliquot of phthalyl amidase in 50 mM potassium phosphate, pH 8. The enzymatic reaction was conducted at 30°C for 10–15 min. The amine product was monitored with a DU70 spectrophotometer at 380 nm (or 430 nm) and quantitated from a standard curve of the product. One unit of the enzyme is defined as the amount of the amidase required to cause formation of 1 μmol of the product/min from substrate under the reaction conditions. The specific activity is defined as units per milligram of protein. The protein content was determined as described by [6] using bovine serum albumin as the standard.

2.3.2. For characterization

Unless specified, a typical reaction mixture consisted of 1 ml total volume and contained 0.1 mM *o*-phthalyl carbacephem (V, Fig. 2), 0.1 μM *o*-phthalyl amidase in 200 mM potassium phosphate buffer at a pH 8 and 32°C for 20 min. The enzymatic reaction was stopped by the addition of 1 ml methanol. After removal of precipitate by centrifugation, an aliquot of the supernatant fraction (typically 30 μl) was monitored for the beta-lactam nucleus and/or phthalic acid by HPLC using a Zorbax C8 column (0.46 × 15 cm). The two reaction products were eluted by a mobile phase

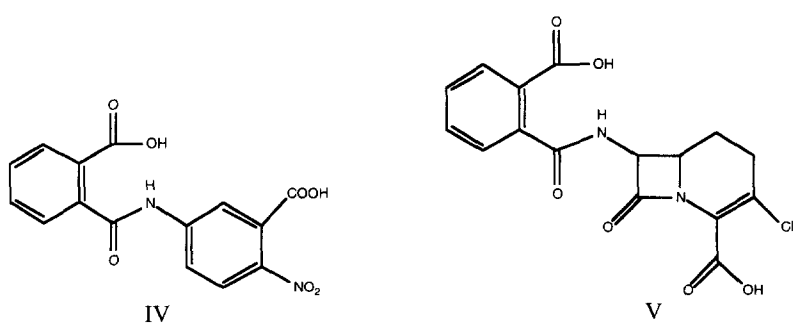


Fig. 2. Chromogenic substrate (IV) and *o*-phthalyl carbacephem (V).

constructed as continuous mixed gradients from (a) 1% ACN (acetonitrile)/0.2% TFA (trifluoroacetic acid) and (b) 80% ACN/0.2% TFA as shown below: 0% (b), 3 min; 0–50% (b), 0.5 min; 50–100% (b), 3 min; 100% (b), 2.5 min; 100–0% (b), 0.1 min and 0% (b), 5 min. At a flow rate of 1.5 ml/min and detection at 254 nm, the retention times of the beta-lactam nucleus and phthalic acid were 2.3 and 7.2 min, respectively. The enzyme unit and specific activity are defined by formation of either product similarly as described under *For purification.*

2.3.3. Enzyme purification

2.3.3.1. Crude extract. Growth of *Xanthobacter agilis*, purification for substrate specificity studies and process development using the phthalyl amidase will be described elsewhere [4]. For characterization of the enzyme, cells of *X. agilis* (200 g, wet weight), which contained a high activity of *o*-phthalyl amidase, were resuspended to 1800 ml of 50 mM Tris–HCl, pH 8, plus 5 mM EDTA. The cells were broken by sonication maximal power for 22 min below 8°C. DNase (1 μg/ml) and $MgSO_4$ (10 mM) were added during the sonication to minimize viscosity and thus improved cell breakage. After a high-speed centrifugation, the resulting supernatant was used as the crude extract for the enzyme purification at 4°C.

Purification of the *o*-phthalyl amidase was performed between 2 and 6°C, as described below.

2.3.3.2. Q-Sepharose chromatography. The crude extract was loaded onto a Q-Sepharose column (4.4 × 23 cm; Pharmacia), previously equilibrated with 50 mM potassium phosphate, pH 8 (buffer A). After washing with buffer A, the *o*-phthalyl amidase was eluted as a single activity peak with a linear gradient of 0–1.5 M KCl in buffer A. The enzyme elution occurred between 1 and 1.1 M KCl. Selected fractions containing most of the enzyme activity were pooled as Q-Sepharose eluate (step 1, Table 1).

2.3.3.3. Ammonium sulfate fractionation. The Q-Sepharose eluate was subjected to $(NH_4)_2SO_4$ fractionation. Most of the enzyme activity was recovered from 67–77, 77–87 and 87–97% $(NH_4)_2SO_4$ pellets. Those pellets were combined and solubilized in buffer A with 0.2 M $(NH_4)_2SO_4$ (step 2, Table 1).

2.3.3.4. Phenyl-Sepharose chromatography. Ammonium sulfate was added to the 67–97% $(NH_4)_2SO_4$ enzyme pool to a final concentration of approximately 2 M. The enzyme pool was loaded onto a

Table 1
Purification of *o*-phthalyl amidase from *Xanthobacter agilis*

Step	Protein (mg)	Activity [a] (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	5475	345	0.063	1	100
Q-Sepharose eluate	230	279	1.214	19	81
Ammonium sulfate: 67–97% fraction	145	224	1.547	25	65
Phenyl-Sepharose eluate	63	158	2.505	40	46
Hydroxylapatite eluate	28	154	5.52	88	45
Mono P eluate	16.5	119	7.2	119	34
Superdex 200 eluate	16.3	80	6.3	100	23

[a] μmol/min/mg protein using compound IV as substrate.

Phenyl-Sepharose column (2.6 × 16 cm; Pharmacia), which was previously equilibrated with buffer A plus 2.6 M $(NH_4)_2SO_4$. The phthalyl amidase bound to Phenyl-Sepharose was eluted with a linear gradient of 2.6–0 M $(NH_4)_2SO_4$ in buffer A. The enzyme was eluted as a single activity peak between 0 and 0.4 M $(NH_4)_2SO_4$. Selected fractions containing the most of the enzyme activity were pooled as Phenyl-Sepharose eluate (step 3, Table 1).

2.3.3.5. Hydroxylapatite chromatography. The Phenyl-Sepharose eluate was dialyzed against buffer A and then loaded onto a hydroxylapatite column (1.5 × 90 cm; Clarkson Chemical Company), which was previously equilibrated with buffer A. After washing the column with buffer A, the enzyme was eluted as a single activity peak with a linear gradient of 50–500 mM potassium phosphate, pH 8. The enzyme elution occurred between 150 and 190 mM potassium phosphate. Selected fractions containing most of the enzyme activity were pooled as hydroxylapatite eluate (step 4, Table 1).

2.3.3.6. Mono P chromatography. After a dilution of the buffer strength from 120 to 50 mM potassium phosphate, the hydroxylapatite eluate was loaded onto a Mono P column (0.5 × 20 cm; Pharmacia), which was previously equilibrated with buffer A. After washing with 3 column volumes of buffer A, the enzyme was eluted as a single activity peak with a linear gradient of 0–1.5 M KCl in buffer A. The enzyme elution occurred between 0.72 and 0.8 M KCl. Those fractions containing the majority of the enzyme activity were pooled as Mono P eluate (step 5, Table 1). The most active enzyme preparation was derived from Mono P FPLC (fast protein liquid chromatography).

2.3.3.7. Superdex 200 gel-filtration. The Mono P eluate was concentrated by ultrafiltration to a small volume (about 2 ml) and loaded onto a Hi Load Superdex 200 (1.6 × 60 cm; Pharmacia), previously equilibrated with buffer A. The enzyme was eluted as a major activity peak (step 6, Table 1). A slight activity loss appeared to occur in this step.

2.4. Other methods

Previously described procedures were used for SDS-PAGE [7], gel-filtration [8] and electrospray ionization mass spectrometry [9] in molecular weight determination of the enzyme, isoelectric focusing in isoelectric point estimation [10], anion-exchange chromatography in amino acid composition analysis [11], and automated Edman degradation for amino acid sequence determination (Beckman Instruments). The gel-filtration was conducted with a Hi Load Sephadex 200 column in Buffer A with phosphorylase b (92 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (31 kDa) as molecular mass standards.

2.5. Examples of chemical synthesis of representative compounds

2.5.1. Ethyl (±) 4-nitroimidazole-2-octanoate

To a suspension of sodium hydride (14.8 g, 0.37 mol, 60% dispersion in mineral oil) in DMF (300 ml) under N_2 was added 4-nitroimidazole (41.8 g, 0.37 mol) in small portions. After gas evolution ceased, ethyl 2-bromooctanoate (92.0 g, 0.366 mol) was added dropwise. The solution was refluxed for 2 h, cooled, added to ice–water (1 l), and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and concentrated to yield 104 g of a yellow oil. MS 284. Calcd. for $C_{13}H_{21}N_3O_4$: C, 55.11; H, 7.47; N, 14.83. Found: C, 54.89; H, 7.62; N, 14.95.

2.5.2. Ethyl (±) 4-aminoimidazole-2-octanoate

Ethyl (±) 4-nitroimidazole-2-octanoate (4.0 g, 0.014 mol) in 100 ml of ethanol was reduced over 5% palladium on carbon (1.0 g) at room temperature, 40 psi for 2 h. The solution was filtered through Celite and condensed to yield 3.5 g of amine which was used without further purification.

2.5.3. Ethyl (±) [4-[(2-carboxybenzoyl)amino]-1H-imidazol-1-yl]octanoate

Ethyl (±) 4-aminoimidazole-2-octanoate (0.014 mol) was added to a solution of phthalic anhydride (2.2 g, 0.015 mol) in 10 ml of DMF and 200 ml ethanol and stirred at room temperature for 1.5 h. The solution was concentrated and extracted with ethyl acetate, dried over sodium sulfate and concentrated. The product was triturated with ether and filtered to yield 2.6 g (46%). m.p. 142–143°C. FD MS 382. Calcd. for $C_{21}H_{27}N_3O_5$: C, 62.83; H, 6.78; N, 10.47. Found: C, 63.11; H, 6.85; N, 10.45.

2.5.4. (±) [4-[(2-Carboxybenzoyl)amino]-1H-imidazol-1-yl]octanoic acid (compound XIV)

Ethyl (±) [4-[(4-carboxybenzoyl)amino]-1H-imidazol-1-yl] octanoate (0.5 g, 1.2 mmol) was dissolved in 25 ml methanol and treated with 4.0 ml of 1 N sodium hydroxide. The reaction was stirred for 45 min at room temperature and concentrated. Water (5 ml) was added and the pH slowly adjusted to 4.4 with 6 N HCl. The product was extracted with ethyl acetate, dried over sodium sulfate and concentrated to yield 0.16 g product. MS 374 and 356. Calcd. for $C_{19}H_{23}N_3O_5$: C, 61.12; H, 6.21; N, 11.25. Found: C, 61.12, H, 5.93; N, 11.01.

2.6. Substrate specificity studies

2.6.1. Representative enzyme reaction using purified enzyme

Enzyme used in these studies was purified as described elsewhere [4]. Each reaction contained 2.5 μmol of compound, 0.3 units (based on the chromogenic substrate) of the preparative scale purified enzyme, and 1 ml of 50 mM phosphate buffer at pH 8.0 and 30°C. Samples of the reaction mixture were taken at appropriate times, and methanol (1 ml) was added to stop the reaction. The samples were examined by HPLC to determine the extent of substrate hydrolysis. The amount of compound hydrolyzed was calculated from a standard curve of the test compound. All substrates were stable in buffer at 30°C and pH 8.0 in the absence of enzyme for 24 h.

3. Results and discussion

3.1. Screening of known enzymes and organisms

The possibility that a known enzyme or organism would have dephthalylating activity was determined using compound II. Of the 70 select organisms and 45 known enzymes, none converted compound II to compound III and *o*-phthalate. An alternative approach was apparently required for this biotransformation.

3.2. Soil enrichment for o-phthalyl amidase

A soil enrichment using Compound II as a substrate was used to find an organism that would grow on this compound as a carbon source. One organism identified as *Xanthobacter agilis* completely

converted compound II to compound III and *o*-phthalate within a 24 hour period as determined by LC–MS. The molecular weight of the enzyme in the crude enzyme lysate was found to be approximately 50000 Da and its activity in this organism was found to be 39 nmol/min/mg crude cell protein, as determined using compound II as substrate. Growth studies indicated that the enzyme could be induced by adding *o*-phthalate to the growth medium (Briggs unpublished data).

3.3. Purification of phthalyl amidase

The 6-step enzyme purification procedure is summarized in Table 1. Based on SDS-PAGE and laser densitometric scanning, the *o*-phthalyl amidase from the Superdex 200 chromatography was $> 95\%$ pure.

3.4. Characterization of phthalyl amidase

3.4.1. Physical, chemical and structural properties

The molecular weight of the *o*-phthalyl amidase was determined to be 49,900 Da based on amino acid determination (Table 2) and SDS page gel. The enzyme was monomeric. The isoelectric point was estimated to be 5.5. For amino acid composition analysis, 16 residues of the protein were determined by reverse-phase HPLC. The cysteine and tryptophan were derived from the nucleotide sequence of the gene. Repeated attempts to determine the amino-terminal residue of the purified enzyme failed, suggesting that the enzyme was blocked. Five internal sequences were obtained and are shown in Table 3. The internal sequences and the precise molecular weight of the enzyme effectively guided molecular cloning and extracellular over-expression of the amidase gene from *X. agilis* to *S. lividans* [3]. The molecular weight of the enzyme based on the DNA sequence was 49,724 Da.

Table 2
Amino acid composition of the phthalyl amidase

Amino acid	No. of residues per 49.9-kDa protein
Asp + Asn	62
Thr	20
Ser	32
Glu + Gln	54
Pro	26
Gly	33
Ala	52
Cys	2
Val	23
Met	12
Ile	18
Leu	38
Tyr	20
Phe	14
His	11
Lys	4
Arg	20
Trp	13

Table 3
Internal sequences of the phthalyl amidase

No.	Sequence
1	G–R–A–R–M–A–L–A–F–A–L–G–Q–W–S–P–W–L–A–D–N–Q–P–Q–P–D–L
2	F–M–A–L–D–G–W–E–I–P–E–Y–N
3	A–S–Q–Y–A–L–D–Y–W–N–T–P–G–R–N–V–I–G–D
4	N–A–A–R–G–Q–Q–L–S–W–N–D–D–I–D
5	N–S–F–L–D–G–G–F–S–L–A–S–L–A–G–E–Y–Y–V–E–A–G–A–G–P–L

3.4.2. Catalytic and kinetic properties

o-Phthalyl amidase was optimally active between pH 7.8–8.4 and 28–34°C in 100–200 mM potassium phosphate. Under the optimal conditions, the enzymatic reaction was linear with time up to

Table 4
Influence of effectors on phthalyl amidase activity

Effector compound [a]	% of control activity
Inhibitors	
p-HMB	65
DTNB	98
NEM	100
Iodoacetate, 1 mM	91
Iodoacetate, 10 mM	46
Phenanthroline	104
EDTA	103
Cofactors	
β-Mercaptoethanol	105
DTT	100
NAD	101
NADH	96
NADP	99
NADPH	99
ATP	96
PLP	106
THF	100
CoASH	102
THF + DTT	100
FAD	101
FAD + DTT	100
Metal cations (as chloride salts)	
Na^+	104
K^+	100
Ca^{2+}	89
Co^{2+}	101
Cu^{2+}	36
Fe^{2+}/Fe^{3+}	102
Mg^{2+}	102
Mn^{2+}	84
Ni^{2+}	94
Zn^{2+}	100

[a] Concentration: 1 mM unless specified; DTT: dithiolthreitol; *p*-HMB: *para*-hydroxy mercuric benzoate; DTNB: 5,5′-dithiol-bis-2-nitrobenzoate; NEM: *N*-ethylmaleimide; NAD: nicotinamide adenine nucleotide; NADP: nicotinamide adenine dinucleotide phosphate; NADPH: reduced form of NADP; ATP: adenosine 5′-triphosphate; THF: tetrahydrofolate; FAD: flavin adenine dinucleotide; PLP: pyridoxyl phosphate.

Table 5
Effect of organic solvents on phthalyl amidase activity

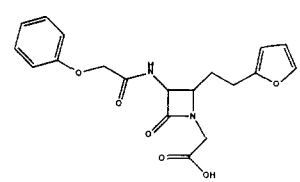
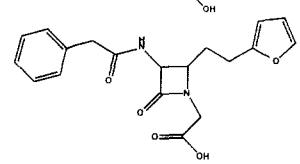
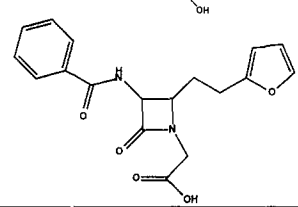
Solvent	% Residual enzyme activity		
	1.0%	5.0%	10.0%
Ethanol	99	85	45
DMSO	101	80	71
Glycerol	100	94	85
Methanol	100	90	69

DMSO: dimethyl sulfoxide.

25 min and with enzyme concentration up to 0.15 μM. The enzyme activity decreased in a lower ionic strength buffer, e.g., to 40% of the maximal activity at 10 mM potassium phosphate. The enzyme was 80% and 30% as active in Tris–HCl and ammonium acetate at 100 mM buffer, respectively, as in potassium phosphate. For each buffer, the enzyme activity improved with increasing ionic strength; the effect is of a general nature and does not appear to depend on specific anions or cations.

The effect of various compounds on the enzyme activity of *o*-phthalyl amidase was determined under optimal conditions. All compounds were tested at 1 mM final concentration unless otherwise indicated. As shown in Table 4, iodoacetate, *p*-HMB, and copper ions significantly decreased *o*-phthalyl amidase activity. None of the tested compounds was stimulatory to the enzyme. At a concentration of 10%, four organic solvents decreased the enzyme activity to various degrees (Table

Table 6
Substrate specificity of phthalyl amidase against beta-lactams

Compound number	Structure	Relative activity
IV		100
II		47.9
VI		0
VII		0
VIII		0

Each reaction mixture (1 ml) contained 2.5 μmol of compound, 0.3 units of enzyme (activity based on compound IV) in 50 mM phosphate buffer at pH 8.0 and 30°C. Samples of the reaction mixture were withdrawn at various times and equal amounts of methanol added to stop the reaction. Samples were analyzed by HPLC and the extent of the reaction compared to the hydrolysis of compound IV.

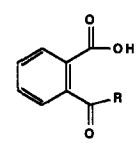
5). Because the amidase remained mostly active in the presence of 5% solvent, it might be useful in enzymatic de-blocking of an insoluble substrate.

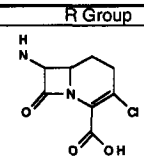
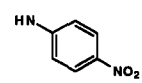
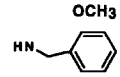
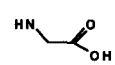
Kinetic constants of the *o*-phthalyl amidase were determined under 50 mM potassium phosphate, pH 8 and 30°C. The K_m of enzyme for compound V was 0.9 mM and V_{max} of the enzymatic reaction was 7.6 μmoles of product formed per minute per milligram of the enzyme. The amidase was saturated at 5 mM of compound V. The enzyme activity was lower at a higher substrate concentration. The cause of this is not clear. The turnover number of the enzyme (i.e., K_{cat}), based on the 49.9-kDa protein, was 6.32 mol product/s/mol enzyme. Thus, the catalytic efficiency of the enzyme (i.e., K_{cat}/K_m) was 7.02 mol product/s/mol enzyme/mM compound V.

3.4.3. Reaction stoichiometry

During the *o*-phthalyl amidase catalyzed reaction, both disappearance of compound V and formation of the two products (carbacephem nucleus and *o*-phthalate) were monitored simultaneously by reverse-phase HPLC. Based on formation of the beta-lactam product, the enzymatic reaction was essentially complete at 2 h and a molar ratio for product formation/substrate disappearance was maintained at 1.15. The formation of *o*-phthalate was slightly lower with a molar ratio of 0.92. After an apparently complete enzymatic reaction at 17 h, the two molar ratios were 1.16 and 0.96, respectively. Thus, no competing side-reaction existed in the amidase-catalyzed reaction. The complete conversion of the substrate to either product also indicated that, at either product concentration, the enzymatic reaction was not subjected to any product inhibition.

Table 7
Comparison of the reactivity of phthalyl amides with phthalyl amidase



Compound number	R Group	Relative Activity
V		100.0
IX		544.6
X	OCH_3	207.6
XI		40.2
XII		31.8
XIII		9.7
XIV	L-Asp-L-Phe-OMe	118.5
IV		295.7
XV	DL-methionine	220.1
XVI	DL-leucine	90.2

Reaction conditions were the same as Table 6. The relative reactivity of each compound was compared to compound V.

3.4.4. Stability of phthalyl amidase

The pH stability of the amidase was determined in the pH range of 6–9 at 30°C and at 20 and 200 mM potassium phosphate buffer. In 20 mM phosphate buffer, all enzyme activity was lost within 2 h at any pH of the incubation medium. At 200 mM buffer, the enzyme retained at least 80% of its activity for 100 h irrespective of the pH of the incubating medium. Similar results were obtained with 20 mM buffer that was supplemented with 200 mM KCl or NaCl. This indicates that the enzyme stabilization was primarily dependent on the high ionic strength of the buffer.

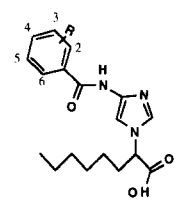
The temperature stability of the enzyme was determined at pH 8.2 and in the temperature range of 4–50°C for 48 h in 50 and 200 mM potassium phosphate buffer. Below 25°C and at 50 mM buffer, the enzyme retained 90% of its activity for 48 h. Above 40°C, all enzyme activity was lost within 48 h. At 200 mM buffer, 80% of the enzyme activity was still retained up to 35°C and 30% of the enzyme activity was retained at 40°C after 48 h.

3.5. Substrate specificity of phthalyl amidase

3.5.1. Chemical structure requirements for enzyme activity

The activity of phthalyl amidase against 25 compounds was determined. The compounds were divided into beta-lactams (Table 6), phthalyl amides (Table 7), aromatic ring substituted amides (Table 8) and chiral studies (Table 9).

Table 8
Comparison of the reactivity of compound XVII derivatives with phthalyl amidase

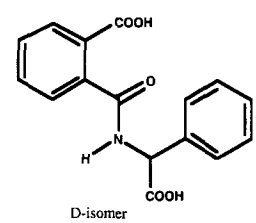
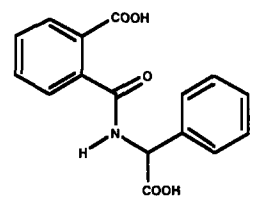
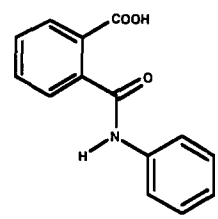
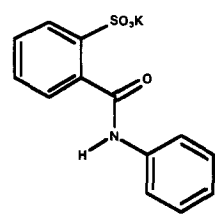


Compound number	R Group	Relative Activity
XVII	2-COOH	100.0
XVIII	6-F, 2-COOH	159.00
XIX	6-NH_2, 2-COOH	10.2
XX	N replace C-6, 2-COOH	85.9
XXI	3-OH, 2-COOH	1.3
XXII	 at C2	0.2
XXIII	4-COOH	0
XXIV	3-COOH	0
XXV	2-OH	0
XXVI	3-OH	0
XXVII	3,5- OH	0
XXVIII	2-H	0

Reactions were carried out in a total volume of 1 ml. Reaction mixture contained 0.009 mg (0.6 units) of enzyme, 2.5 μmol of substrate and 50 mM potassium phosphate buffer at pH 8.0. All reactions were run at 30°C for 2 min except for slower reacting substrates and they were run for either 30 min or 24 h.

As shown in Table 6, the enzyme recognized mono- and bicyclic beta-lactam compounds containing an *o*-phthalyl group attached to the exocyclic nitrogen. However, the side chain apparently required an *o*-carboxylate group; e.g., *o*-phthalyl, since no hydrolysis was observed in the absence of this functional group. A wide variety of *o*-phthalyl amides were substrates for the enzyme (Table 7). Substrates included phthalylated amino acids, dipeptides, monocyclic and bicyclic beta-lactams, phenyl, benzyl, and aliphatic amines. The enzyme also exhibited esterase activity as demonstrated by its ability to hydrolyze *o*-phthalate mono methyl ester (compound X). In this series, compound IX was the most active compound found.

Table 9
Chiral and additional substrate selectivity of phthalyl amidase

Compound Number	Structure	Relative Activity
IV		100
XXIX	 D-isomer	136
XXX	 L-isomer	1.3
XXXI		200
XXXII		0.0

Reactions were carried out in a total volume of 1 ml. Reaction mixture contained 0.009 mg (0.6 units) of enzyme, 2.5 μmol of substrate and 50 mM potassium phosphate buffer at pH 8.0. All reactions were run at 30°C for 2 min except for compounds XXX and XXXII which were run for 30 min at the same temperature. The hydrolysis of the substrate and the formation of *o*-phthalate was monitored by HPLC.

Using compound XVII as a standard in Table 8, a variety of aromatic ring substituted compounds were examined for reactivity with the enzyme. Aromatic ring substitutions at the 6 position of the phthalylated ring such as F and NH_2 were accepted by the enzyme. A nitrogen within the aromatic ring (compound XX) was also acceptable but a hydroxyl group at the 3 position (compound XXI) of the ring was not. Low levels of hydrolysis occurred if a tetrazole was substituted for the *o*-carboxylate group (compound XXII). Moving the carboxylate group to the 3 (compound XXIV) or 4 (compound XXIII) position of the aromatic ring completely eliminated hydrolytic activity. Compounds lacking the *o*-carboxylate (XXV–XXVIII) were not substrates for the enzyme. These results are consistent with the enzyme being a novel catalyst that removes phthalyl protecting groups from a variety of amines under mild conditions.

While new substrates as shown in Table 9 were being prepared, the enzyme was stored in the freezer ($-80°C$). After thawing the enzyme and determining the specific activity of the enzyme, it was found that the activity had increased. Apparently an unstable enzyme inhibitor in the enzyme preparation, the nature of which was not clear, had decomposed during storage. Since the results with the new substrates cannot be compared to the previous results, these substrates were run separately and compared to the activity of the more active enzyme preparation against compound IV. The results (Table 9) show that the enzyme had a marked preference for the D isomer of *N*-phthalyl-phenylglycine (XXIX). The L isomer was an extremely poor substrate for the enzyme. Compound XXXI was twice as good as a substrate for the enzyme than compound IV. However, by substituting a sulfonate group for the carboxyl group of the phthalate (compound XXXII), enzyme reactivity was completely lost. Again, these results show the exquisite selectivity of this enzyme for *N*-phthalylated amines and suggest that the enzyme has a chiral preference on the amine side of the substrate. The chiral selectivity of the enzyme may be substrate dependent since in some cases L-isomers are operated on by the enzyme.

The substrate specificity studies combined with the inhibition studies (Table 4) suggest that the enzyme might be working by protonating the *o*-carboxylate of the *o*-phthalyl side chain. The subsequent formation of the *o*-phthalic anhydride would expel the charged amine from the active site. The anhydride formed would rapidly hydrolyze in the aqueous environment to *o*-phthalate. Additional work to determine the true mechanism of amide bond hydrolysis is underway.

Table 10
Kinetic parameters for selected substrates of phthalyl amidase

Parameter	Substrate				
	IV	V [a]	XII	XVI	XVIII [b]
K_m, mM	0.05	0.9	0.14	0.09	0.17
V_{max} [c]	5.95	7.6	0.27	1.41	1.94
K_{cat} [d]	4.95	6.33	0.22	1.18	1.61
K_{cat}/K_m [e]	99.0	7.0	1.6	13.1	9.5

[a] The carbacephem nucleus was quantitatively monitored and the specific activity was calculated from μmol of this compound formed.
[b] For the other compounds, phthalic acid was the product monitored during the reaction. Compounds IV, V and XII were tested using 5.14 μg/ml of enzyme. Compounds XVI and XVIII were tested using 0.9 μg/ml of enzyme. Substrate concentrations were between 0 and 25 mM and reaction time was between 2 and 20 min, depending on the substrate used. All reactions were run at 32°C and at pH 8.2.
[c] μmol/min/mg protein.
[d] μmol/s/μmol enzyme.
[e] μmol/s/μmol enzyme/mM substrate.

3.5.2. Kinetic parameters for five substrates

The kinetic parameters of the enzyme were determined for five variously reactive substrates. The K_m, V_{max}, K_{cat}, and K_{cat}/K_m values for these substrates are shown in Table 10. It is of interest to note that *o*-phthalyl carbacephem (compound V) exhibited a high K_{cat} value (i.e., turnover number) that appears suitable for a large-scale enzyme process [4].

4. Conclusion

Chemo-enzymatic synthesis is becoming increasingly more relevant in the generation of industrially important organic compounds. At Lilly, three chemo-enzymatic routes have been developed for use in the synthesis of carbacephems. As related directly to this report and described previously [12,13], penicillin G amidase catalyzes a selective acylation of a carbacephem nucleus intermediate. As also related and described more recently [14], serine hydroxy methyltransferase mediates condensation of glycine and an amide substrate leading to the formation of a diastereospecific intermediate, which can be converted to this carbacephem by further chemical synthesis. In an alternative synthesis of the same carbacephem, a phthalimido group was used for blocking of an analogous diastereospecific intermediate and a third enzyme (*o*-phthalyl amidase) was explored for de-blocking of the *o*-phthalyl group in a latter step of the overall pathway.

An *o*-phthalyl amidase has been purified from *X. agilis* to near homogeneity by a conventional 6-step procedure. The amidase is a novel 49.9-kDa biocatalyst with no cofactor requirement. It is most active and stable in a high ionic strength buffer such as potassium phosphate. The amidase exhibits a broad substrate specificity against phthalyl beta-lactams and amides with an essential requirement for *o*-carboxylate group and marked preference for one D-isomer. This study in enzymology has effectively guided molecular cloning and over-expression of the amidase gene to *S. lividans* as well as an enzyme process scale-up with *o*-phthalyl carbacephem. The broad substrate specificity and ready availability of the extracellular recombinant amidase can make this simple biocatalyst an economic choice in de-blocking of industrial substrates.

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