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Selective Deprotection Of Phthalyl Protected Amines

Colleen A. Costello, Adam J. Kreuzman, and Milton J. Zmijewski*

Lilly Research Laboratories, A Division of Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285.

Abstract: Phthalyl amidase selectively deprotects phthalimido groups under very mild aqueous conditions in a one-pot reaction to produce phthalic acid and the free amine. The enzyme has been shown to deprotect several primary amines of distinctly different structure, and exhibits chiral selectivity when the substrate contains extensive β-branching. The enzyme has a definite requirement for *ortho* positioning of the functional groups on a fixed axis of rotation.
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The phthalimido group has always been an attractive alternative to amine protection in chemical synthesis. Until now, the drawback to using this protecting group has been the harsh conditions required for its removal.[1] Facile, inexpensive deprotection of the phthalimido group with phthalyl amidase under near neutral conditions has broad reaching implications in the oligosaccarride,[2] beta-lactam,[3] oligonucleotide,[4] and peptide industries. Native phthalyl amidase was first isolated and characterized from *Xanthobacter agilis*[5] and subsequently cloned and overexpressed in *Streptomyces lividans*.[6] Recently, it has been shown that phthalyl amidase is able to deprotect a variety of phthalimido substrates once the substrates are partially hydrolyzed to their monoacids[5,7] (Scheme 1). Assay conditions for optimal phthalyl amidase activity include incubation with substrate at 30°C in 200mM potassium phosphate buffer (pH 8.0).[5]

Base CH_3CN/H_2O or 0.2 M buffer (pH 8.0)

Phthalyl Amidase

1 2 3 4

Scheme 1

Here we will discuss work to further characterize the recombinant phthalyl amidase and its utility as the routine chemo-enzymatic reagent of choice in the deprotection of phthalimido groups.

Substrate structural requirements for free amine production (Table 1). Comparison of substrates with two and three carbon spacers between the free carboxylate and the benzoyl amine show the absolute requirement for a two carbon spacer for deprotection. From these studies, **5** and **7** were readily deprotected (k_{cat} of 4.1 s^{-1} and 3.2 s^{-1}, respectively). Deprotection was stopped with the introduction of an

additional carbon spacer in the substrate (**6** and **8**). Also, when the reactive carboxylate is blocked as a methyl ester (**9**), no enzymatic deprotection can occur.

Table 1

(1-X-2-Y-benzene)

X	Y	
COOH	-C(O)NH-(C_6H_3)(CO_2H)-NO_2	**5**
COOH	$-CH_2C(O)NH-$(C_6H_4)-Cl	**6**
COOH	-C(O)NH-N(imidazole)N-$CH(CH_2)_5CH_3$ (CO_2H)	**7**
CH_2COOH	-C(O)NH-N(imidazole)N-$CH(CH_2)_5CH_3$ (CO_2H)	**8**
$COOCH_3$	-C(O)NH-C(=O)(OH)-$CH(CH_2)_3CHCH_2$ / $NHCH_2COOCH_3$	**9**

Structural requirements for the aromatic ring. Comparisons of several substrates with varying degrees of conjugation show the enzymatic reaction is enhanced by presence of the phenyl group (Table 2). Under slightly basic aqueous conditions (pH 8.0), **10** was not stable, resulting in full non-enzymatic deprotection to the free amine at 30°C. Although free amine production occurs with **11**, replacement of the

Table 2

	k_{rel}		
10	**5**	**11**	**12**
not stable	100	0.33	0

R = HN-(C_6H_3)(COOH)-NO_2

phenyl group with a simple double bond reduces the relative rate of the reaction by 300 fold. Free rotation about the two carbon tether (**12**) results in complete loss of amidase activity.

Chiral Selectivity of phthalyl amidase. Phthalyl amidase appears to show some chiral selectivity towards substrates possessing extensive β-branching (Table 3). Although not a substrate for the enzyme, **16**, did bind to the enzyme ($K_I = 80\mu M$). PHT-L-Ala-L-Ala-OH and PHT-L-Ala-L-Ala-OH analogs were tested as substrates, but were found to be unstable to the assay work up conditions.[8]

Table 3

	Dipeptides	K_M (mM)	k_{cat} (s^{-1})
13	PHT-L-Phe-L-Ala-OH	0.23	2.3
14	PHT-D-Phe-L-Ala-OH	0.16	1.8
15	PHT-L-Phenylglycine-OMe	NS	NS
16	PHT-D-Phenylglycine-OMe	0.56	4.7

NS: Not a substrate, PHT: 2-carboxybenzoylamine.

Effects of organic solvents on phthalyl amidase activity. The utility of phthalyl amidase as a reagent in organic synthetic reactions was explored by determining the effect of various organic solvents on phthalyl amidase activity. Solvents were classified into two categories: 1) water miscible (CH_3CN, THF, and DMF) and 2) not water miscible (toluene, heptane, and CH_2Cl_2).[9] We found that greater than 90% residual activity remained when assays were carried out in the presence of 1% toluene, 20% heptane, or 5% THF. No effect on activity was seen in the presence of 1% CH_2Cl_2, 10% DMF or 20% CH_3CN (activity $\geq$ 95%).

Table 4

	Proteases				
Inhibitor	**Serine**	**Cysteine**	**Aspartate**	**Metallo**	**Phthalyl Amidase**
1 mM PMSF	+				--
20μg/mL SBTI	+				--
1 mM *p*-HMB		+			+
10 mM Iodoacetate		+			±
1 mM NEM		+			--
1 mM DTNB		+			--
1 mM DTT		Activate			--
10μg/mL Pepstatin A			+		--
10 mM DANME			+		--
1 mM EDTA				+	--
1 mM Phenanthroline				+	--
2 mM PGO					--

(+): inhibition; (--): no inhibition; DTT: dithiothreitiol; *p*-HMB: *p*-hydroxy mercuricbenzoate; DTNB: 5,5'-dithiol-bis-2-nitrobenzoate; NEM: N-ethylmaleimide; DANME: diazoacetylnorleucine methylester; PGO: phenylglyoxal monohydrate; PMSF: phenylmethylsulfonyl fluoride; EDTA: ethylenediaminetetraacetic acid; SBTI: soybean trypsin inhibitor.

Effects of protease inhibitors on phthalyl amidase activity. The reaction mechanism was probed by subjecting the enzyme to several known protease inhibitors and testing for phthalyl amidase inactivation (Table 4). Inactivation was seen with *p*-hydroxy mercuricbenzoate with only 65% residual activity remaining after treatment. Partial inactivation of phthalyl amidase was observed when the enzyme was treated

with iodoacetate (91% and 46% residual activity remaining in the presence of 1mM and 10mM iodoacetate, respectively). All other inhibitors exhibited no effect on phthalyl amidase activity suggesting that the enzyme follows a unique mechanism for deamidation.

Discussion. Phthalyl amidase offers a highly selective one-pot method for mild deprotection of amines. Substrate specificity studies show the enzyme can accept a broad range of protected amines which lends support to its use as a general synthetic tool.[5] Studies using chiral substrates suggest the enzyme is stereoselective only when extensive β-branching of the substrate exists. Analysis of the reaction shows that phthalyl amidase utilizes a mechanism unique to any known amidase to achieve phthalimido cleavage. Characterization of the reaction using substrate and inhibition kinetics (Tables 1, 2, & 4) suggests the enzyme exploits the chemical pre-disposition of its substrate for hydrolysis via neighboring group effects.[10] Phthalimido deprotection is potentially facilitated through acid/base chemistry of the enzyme active site (Scheme 2). Studies continue to further decipher the mechanism of action of phthalyl amidase.

Enz–B–H Enz–B–H Enz–B⁻ H_2O

2 17 18 4 3

Scheme 2

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8. Assays were initiated with the addition of enzyme, reaction aliquots (750μl) were removed at specific time intervals and quenched with methanol (750μl). The resultant mixture was loaded directly onto a Waters™ 4μ C_{18} radial pak HPLC column (8mm x 10mm) equilibrated in 99:1 10mM phosphate buffer (pH 7.0): CH_3CN.
9. Abbreviations: CH_3CN: acetonitrile; THF: tetrahydrofuran; DMF: dimethylformamide; CH_2Cl_2: methylene chloride. Assays were monitored at spectrophotometrically at 380nm using 200μM **5** at 30°C.
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