The identification of the acid–base catalyst of α-arabinofuranosidase from *Geobacillus stearothermophilus* T-6, a family 51 glycoside hydrolase

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Abstract **The α-L-arabinofuranosidase from *Geobacillus stearothermophilus* T-6 (AbfA T-6) belongs to the retaining family 51 glycoside hydrolases. The conserved Glu175 was proposed to be the acid–base catalytic residue. AbfA T-6 exhibits residual activity towards aryl β-D-xylopyranosides. This phenomenon was used to examine the catalytic properties of the putative acid–base mutant E175A. Data from kinetic experiments, pH profiles, azide rescue, and the identification of the xylopyranosyl azide product provide firm support to the assignment of Glu175 as the acid–base catalyst of AbfA T-6.**

Key words: α-L-Arabinofuranosidase; Glycoside hydrolase family 51; Acid–base catalyst; Aryl β-D-xylopyranoside; β-D-Xylopyranosyl azide

1. Introduction

α-L-Arabinofuranosidases (EC 3.2.1.55) catalyze the hydrolysis of α-1,2 and α-1,3 L-arabinofuranosidic bonds in hemicelluloses such as arabinoxylan, L-arabinan, and other L-arabinose-containing polysaccharides. These enzymes act synergistically with other hemicellulases for the complete degradation of xylan. Hemicellulases have attracted much attention in recent years because of their potential industrial use in biobleaching of paper pulp, bioconversion of lignocellulose material to fermentative products and for the improvement of animal feedstock digestibility [1–4]. α-L-Arabinofuranosidases are also capable of hydrolyzing the glycosides of monoterpenes, sesquiterpenes and other alcohols which constitute the aromatic potential of wine, and their exploitation in flavor improvement and wine aromatization has been studied in the last several years [5–7].

To date, α-L-arabinofuranosidases are assigned to four glycoside hydrolase families (GHs), 43, 51, 54 and 62, based on sequence similarities [8,9]. The GH51 and GH54 were shown to cleave the glycosidic bond with a retention of the anomeric configuration [10]. The GH43, which includes α-L-arabinofuranosidases as well as β-D-xylosidases, was shown to work via the inverting mechanism [10], whereas the stereochemistry of GH62 is not yet characterized. An extensive research was done on the wide and various substrate specificities α-L-arabinofuranosidases exhibit. Different α-L-arabinofuranosidases from bacterial, fungal and plant sources can hydrolyze arabinofuranose moieties at O-5, O-2 and/or O-3 as single substituent, as well as from O-2 and O-3 doubly substituted xylans and xylooligomers and arabinans [11–15]. However, very little is known on the exact biochemical mechanism and the specific catalytic residues of this group of enzymes. One of the major reasons for this is the difficulty in obtaining synthetic substrates bearing different leaving groups for α-L-arabinofuranosidases, an essential tool in the identification of catalytic residues of glycosidases [16,17]. To date, none of the catalytic residues of any of the four GH families is known.

Based on sequence similarities, Zverlov et al. [18] have made several predictions concerning the GH51 catalytic mechanism. It was suggested that this family is part of the GH-A clan, that the glutamic acids corresponding to E172 and E294 in *Clostridium stercorarium* are the catalytic residues of this family, and that these residues are located on the C-terminal ends of β-strands 4 and 7 in a $(\beta/\alpha)_8$-barrel fold. Recently, site-directed mutagenesis was applied on Abf51A from *Pseudomonas cellulosa* and the putative catalytic residues (Glu194 and Glu321 in *P. cellulosa*) were changed into alanines [15]. The reduction in the catalytic activity of the mutant enzymes was consistent with their suggested role as the catalytic residues, but no further evidences were presented to support this assumption.

In the present work, we describe kinetic studies of AbfA from *Geobacillus stearothermophilus* T-6 and its E175A mutant (the equivalent of Glu172 from *C. stercorarium*). Due to the structural similarity between β-D-xylopyranoside and α-L-arabinofuranoside, the wild-type AbfA T-6 and its E175A mutant showed low but sufficient activity on aryl β-D-xylopyranosides, and enabled the analysis of their activity on substrates bearing different leaving groups. This analysis, together with a comparison of the pH dependence, and a chemical

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Abbreviations: pNPAF, *p*-nitrophenyl α-L-arabinofuranoside; oNPX, *o*-nitrophenyl β-D-xylopyranoside; 2,5-DNPX, 2′,5′-dinitrophenyl β-D-xylopyranoside; 3,4-DNPX, 3′,4′-dinitrophenyl β-D-xylopyranoside; GH, glycoside hydrolase family

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azide rescue of the activity of E175A, confirmed for the first time the assignment of the acid–base catalyst of GH51.

2. Materials and methods

2.1. Cloning of the abfA T-6 gene

Based on the DNA sequence of the *abfA* T-6 gene (AF159625), two PCR primers were designed in a way that enabled the in-frame cloning of the gene into the T7 polymerase expression vector pET9d (Novagen). The N-terminal primer (5′-GGAATACCATGGCTACGAAAAAAGC-3′) included an ATG translational start codon inside the *Nco*I restriction site (CCATGG). The C-terminal primer (5′-GCGTAGGGATCCTAAGCTTAGGGTTCATGC-3′) included a *Bam*HI restriction site (GGATCC) after the end of the gene. PCR amplification was performed on *G. stearothermophilus* T-6 chromosomal DNA and the PCR product was digested with *Nco*I and *Bam*HI. Since an *Nco*I site is present inside the *abfA* gene, two segments were obtained, which were separated using agarose gel. The cloning of the complete gene included two steps. In the first step, the *Nco*I–*Bam*HI segment was cloned into *Nco*I/*Bam*HI-digested pET9d vector. The resulting plasmid was linearized again with *Nco*I, treated with alkaline-phosphatase, and then ligated to the second *Nco*I–*Nco*I segment, resulting in pET9d-*abfA*. The direction of the cloning was verified using restriction enzymes digestion and by DNA sequencing.

2.2. Purification of Abfa T-6

Expression of the *abfA* gene was carried out by growing overnight cultures of *Escherichia coli* BL21(DE3) carrying pET9d-*abfA* in Terrific Broth medium [19] (500 ml in 2 l shake flasks, shaken at 220 rpm, 37°C), supplemented with kanamycin (25 μg/ml), without induction. Following growth, cells from 1 l of overnight culture (D_{600} of 12–14) were harvested (14 000×*g* for 10 min), resuspended in 50 ml of 100 mM Tris–Cl, pH 7.0, and disrupted by two passages through a French® press (Spectronic Instruments, Inc., Rochester, NY, USA) at room temperature. The cell extract was centrifuged (14 000×*g* for 15 min) and the soluble fraction was heat-treated (60°C, 30 min) and centrifuged again at room temperature. The soluble fraction (about 50 ml) contained the recombinant AbfA as the main product at a concentration of about 15 mg/ml. Final purification of the enzyme was performed by gel filtration using a Superdex 200 26/60 column, AKTA explorer (Pharmacia), running at 2.5 ml/min with 50 mM Tris–Cl buffer pH 7.0, 100 mM NaCl and 0.02% sodium azide. The enzyme appeared as a distinct protein peak, which was then collected and used for biochemical characterization. Protein concentrations were determined by the Bradford method, with bovine serum albumin as a standard [20].

2.3. Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutagenic primers for E175A were as follows (the mutated nucleotides are shown in bold): 5′-CGTGGTGCTTGGGCAA**C**G**C**GATGGACGGTCCG-3′ and 5′-CGGACCGTCCATC**GC**GTTGCCCAAGCACCACG-3′. The mutated gene was sequenced to confirm that only the desired mutations were inserted. The mutated protein was overexpressed and purified as the wild-type AbfA.

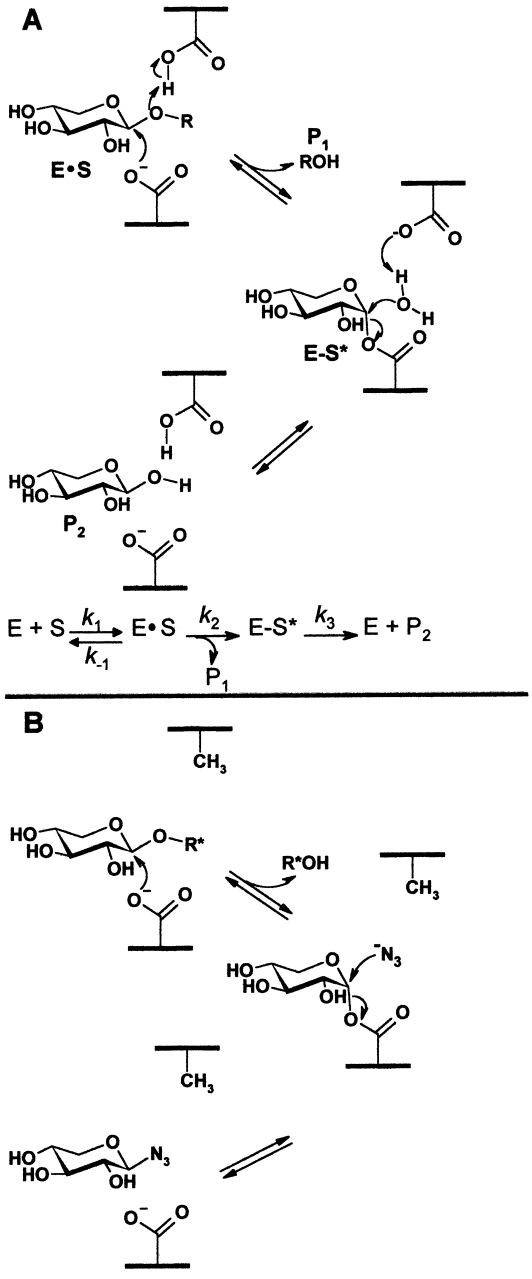
2.4. Kinetic studies

The substrates 2′,5′-dinitrophenyl β-D-xylopyranoside (2,5-DNPX) and 3′,4′-dinitrophenyl β-D-xylopyranoside (3,4-DNPX) were synthesized as described by Ziser et al. [21]. *o*-Nitrophenyl β-D-xylopyranoside (oNPX) and *p*-nitrophenyl α-L-arabinofuranoside (pNPAF) were obtained from Sigma. All kinetic studies were carried out either continuously or by stopped assay conditions. Continuous reactions were performed at 40°C in a 1 ml cuvette. Assay solutions contained 600 μl of appropriately diluted enzyme with different substrate concentrations, in 100 mM citric acid–Na_2HPO_4 buffer (pH 6.0). The increase in absorbance was followed using a Biochrom 4060 spectrophotometer (Pharmacia). The extinction coefficients used and wavelength monitored for each of the substrates were as follows: *o*-nitrophenyl, 420 nm, $\Delta\varepsilon = 0.55$ mM^{-1} cm^{-1}; 2′,5′-dinitrophenyl, 440 nm, $\Delta\varepsilon = 3.61$ mM^{-1} cm^{-1}; 3′,4′-dinitrophenyl, 400 nm, $\Delta\varepsilon = 11.98$ mM^{-1} cm^{-1}. Values of K_m and k_{cat} were determined by non-linear regression analysis using the program GraFit 5.0 [22].

Stopped assay reactions were used for pH dependence studies. Reactions were carried out in 1.7 ml microtubes placed in a water bath at 55°C with pNPAF as a substrate. The mixtures contained 600 μl of 3 mM substrate and appropriately diluted enzyme in 100 mM of the appropriate buffer. The buffers used were: citric acid–Na_2HPO_4 (pH 3.5–6.5), phosphate buffer (pH 5.5–7.0), Tris–HCl buffer (pH 7.0–8.5) and $NaHCO_3$–NaOH (pH 9.0). The reactions were stopped by the addition of Na_2CO_3 (final concentration of 0.3 M), and the released *p*-nitrophenyl was determined spectrophotometrically using a 1 ml cuvette and an extinction coefficient of $\Delta\varepsilon = 18$ mM^{-1} cm^{-1}.

2.5. Purification and structure determination of the enzymatic reaction product with sodium azide

The enzymatic reaction included 9.5 mg of AbfA T-6 E175A, 14 mg of 2,5-DNPX and 1 M sodium azide in a final volume of 4.5 ml of 100



Scheme 1. A: Proposed mechanistic pathway for retaining glycosidase. The kinetic constants are as follows: $k_{cat} = k_2k_3/(k_2+k_3)$; $K_m = (k_{-1}+k_2)k_3/(k_2+k_3)k_1$; $k_{cat}/K_m = k_1k_2/(k_{-1}+k_2)$. B: The azide rescue of activity for an acid–base catalyst mutant, using a substrate with good leaving group ability.

mM citric acid–Na_2HPO_4 buffer (pH 6.0). The reaction mixture was incubated at 40°C for 1 h (complete hydrolysis), and lyophilized. The crude material was purified by flash chromatography (MeOH/$CHCl_3$ 1:9) on a silica gel (Merck, 63–200 mesh) column to yield a pure product (4 mg). The structure of this product was assigned to be 1-azido-1-deoxy-β-D-xylopyranoside: $R_f = 0.5$ (MeOH/$CHCl_3$ 1:4); ^{1}H-nuclear magnetic resonance (NMR) (200 MHz, CD_3OD) δ 3.00 (t, 1H, $J = 8.6$ Hz, H-2), 3.20 (m, 2H, H-5 and H-3), 3.37 (m, 1H, H-4), 3.81 (dd, 1H, $J = 5.0$, 11.0 Hz, H-5′), 4.31 (d, 1H, $J = 8.5$ Hz, H-1); ^{13}C-NMR (50.3 MHz, CD_3OD) δ 69.0, 70.8, 74.8, 78.1, 92.7 (C-1); negative CIMS *m/z* 174.0 ($M-H^-$, $C_5H_9O_4N_3$ requires 175.1); Fourier transform infrared spectroscopy (FTIR) (MeOH) ν 2118 cm^{-1} (N_3). ^{1}H-NMR spectrum was recorded at an ambient temperature on a Bruker AM-200 spectrometer. Mass spectrum was obtained on a TSQ-70B mass spectrometer (Finnigan Mat) by negative chemical ionization in isobutane. FTIR spectrum was recorded on a Bruker vector 22 spectrometer.

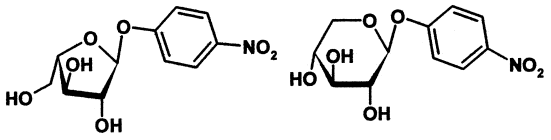
3. Results and discussion

3.1. Cloning and sequence analysis of AbfA

The *abfA* gene from *G. stearothermophilus* T-6 was cloned, efficiently overexpressed in *E. coli* and purified to homogeneity. The amino acid sequence found for *abfA* proved to be identical to the N-terminal sequence of the previously characterized α-L-arabinofuranosidase from *G. stearothermophilus* T-6, confirming that it is the same protein [23]. The sequence of the protein was scanned with the BLAST2 [24] program and showed 78%, 72%, 54%, 54%, 54%, 54% and 53% to GH51 α-L-arabinofuranosidases from *Bacillus halodurans*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Mesorhizobium loti*, *Streptomyces coelicolor*, *Streptomyces lividans* and *Sinorhizobium meliloti* respectively.

3.2. Identification of the acid–base catalyst

GH51 α-L-arabinofuranosidases hydrolyze the glycosidic bond using a two-step double-displacement mechanism (the retaining mechanism) as illustrated in Scheme 1A [10]. In the first step of the reaction (glycosylation), the acid–base residue acts as a general acid, by protonation of the glycosidic oxygen and stabilizing the leaving group. In turn, the nucleophilic residue attacks the anomeric center of the scissile bond, forming a covalent glycosyl-enzyme intermediate. In the second step (deglycosylation), the acid–base residue, this time acting as a general base, activates a water molecule that attacks the anomeric center of the glycosyl-enzyme intermediate, liberating the free sugar with an overall retention of the anomeric configuration. Both steps involve the formation of an oxocarbenium ion-like transition state [25]. The identification of catalytic acid–base residues in glycosidases is based mainly on the replacement of the putative carboxylic acid with a non-ionizable amino acid, and characterizing the mutant enzyme. This includes kinetic analysis with substrates bearing different leaving groups, restoration of activity in the presence of external nucleophiles such as azide ion, and comparing the pH dependence of the wild-type and mutant enzyme [17,26].



Scheme 2. Schematic structures of aryl α-L-arabinofuranosides and aryl β-D-xylopyranosides.

3.2.1. Catalysis with substrates bearing different leaving groups. As the acid–base residue has a double role in the catalysis, its replacement should affect the rates of both steps, though the effect on each step will be different. The effect on the glycosylation step will depend strongly on the leaving group ability of the aglycone. The hydrolysis rates of substrates with a poor leaving group, which require the protonic assistance of the catalytic acid, will be affected much more than the hydrolysis rates of substrates with good leaving groups. In contrast, the deglycosylation step will be affected equally for all substrates with different leaving groups, as the same glycosyl-enzyme intermediate is hydrolyzed during this step [26].

Currently, substrates bearing different leaving groups for α-L-arabinofuranosidase (e.g. aryl α-L-arabinofuranosides) are not available. Yet the low enzymatic activity AbfA T-6 has on aryl β-D-xylopyranosides allowed kinetic analysis of the wild-type and the E175A mutant (Table 1). This low activity results probably from the structural similarities between α-L-arabinofuranoside and β-D-xylopyranoside (Scheme 2), as it has been suggested for the α-L-arabinofuranosidases/β-D-xylosidases from GH43 [27]. As expected, the k_{cat} values measured for the E175A mutant were considerably reduced with all substrates used, and were 5×10^2–3×10^3-fold lower than the wild-type′s. For both, the wild-type and the E175A mutant, it seems that on these three substrates, the second step is rate-limiting, as the k_{cat} values do not vary significantly with substrate variation. Although the E175A replacement did not cause a change in the rate-limiting step for these substrates, it did have an effect on the glycosylation step. This effect can be seen from the k_{cat}/K_m values (Table 1), which reflect the first step of hydrolysis. For the mutant, the k_{cat}/K_m values decline as the leaving group ability of the substrate decreases, indicating that for the substrate which requires strong acid assistance (oNPX), the first step was much slower than for those substrates who need less acid assistance. In addition, as the leaving group ability of the substrates increases, the K_m values of the E175A mutant decrease, suggesting that more glycosyl-enzyme intermediate accumulates, as would be expected if the rate of the first step is higher [26]. This kinetic behavior could be explained only if the residue

Table 1
Kinetic parameters for hydrolysis of aryl β-D-xylopyranosides by AbfA T-6 and its E175A mutant

Phenol substituent	pK_a	Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
2,5-Dinitro	5.15	WT	74.4	8.0	9.30
		E175A	0.026	0.1	0.26
3,4-Dinitro	5.36	WT	10.3	4.4	2.34
		E175A	0.022	0.9	0.024
2-Nitro	7.22	WT	20.1	15.3	1.3
		E175A	0.022	5.3	4.2×10^{-3}

that has been removed is indeed the acid–base catalyst.

3.2.2. pH profiles of the wild-type and the E175A mutant. The assignment of Glu175 as the acid–base residue can also be examined by a comparison of the pH dependence profiles for the wild-type and the E175A mutant using pNPAF as a substrate (Fig. 1). As expected, the pH dependence of the wild-type enzyme is a typical bell-shaped curve, reflecting the ionization state of the two catalytic carboxylic acids. However, with the E175A mutant, the reduction in activity at high pH values is much more moderate, suggesting that the protonated group (the acid catalyst) has been removed. In addition, the optimal pH of the E175A mutant is higher than that of the wild-type enzyme by about one pH unit. In retaining glycosidases the average distance between the two catalytic residues is about 5 Å, leading to charge repulsion between these two residues. Thus, the removal of the acid–base catalyst in the E175A mutant seems to have affected the ionization state of the nucleophile, elevating its pK_a, reducing its acidity, and shifting the optimal pH to higher values.

3.2.3. Azide rescue. The chemical rescue methodology is probably the most important method for the identification of the acid–base catalyst in retaining glycosidases, and has been successfully applied to several GHs [17,28–31]. In retaining glycosidases, the replacement of the acid–base catalyst with a smaller residue (such as alanine) forms a small cavity in which exogenous nucleophiles (like azide) can penetrate. The azide ion, which does not require the general base assistance of the catalytic acid–base, can then attack the glycosyl-enzyme intermediate forming a glycosyl-azide product (Scheme 1B). In the cases where the second step of hydrolysis is the rate-limiting step, the addition of azide will accelerate the reaction. Indeed, in the presence of 1 M azide, the k_{cat} values increased about 35-fold for the hydrolysis of 2,5-DNPX by E175A (Fig. 2). As a consequence of the acceleration of the second step, the K_m values also increased, since less glycosyl-enzyme intermediate accumulated. The net result is that the k_{cat}/K_m, which reflects the first step of hydrolysis, remained almost constant, suggesting that the presence of azide had no effect on the glycosylation step. The addition of azide had also accelerated the hydrolysis of 3,4-DNPX and oNPX by the E175A mutant (data not shown). This gives an additional support to the assumption that the second step is the rate-limiting step for the hydrolysis of all three substrates by the E175A mutant. Azide had no acceleration effect on the wild-type enzyme.

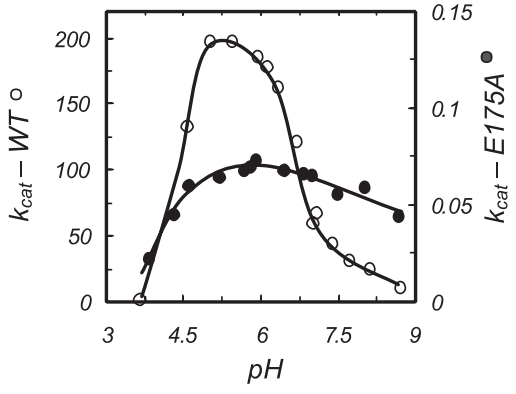


Fig. 1. pH dependence for the hydrolysis of pNPAF by wild-type AbfA T-6 and its E175A mutant. (k_{cat} values are in s^{-1}).

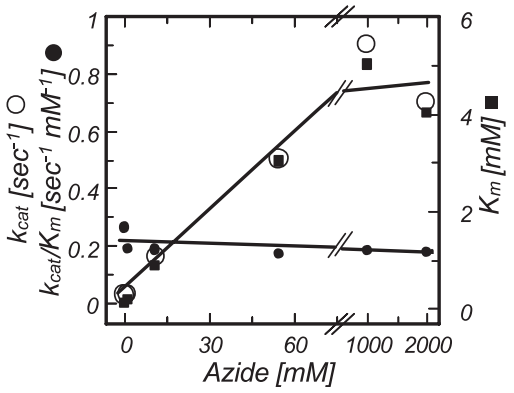


Fig. 2. Kinetic parameters for hydrolysis of 2,5-DNPX by AbfA T-6 E175A in the presence of various azide concentrations.

In the cases that the introduced mutation replaces the acid–base catalyst, the azide ion will attack the anomeric carbon at the deglycosylation step, and the glycosyl-azide product will be in the same configuration of the substrate (i.e. β-D-xylopyranosyl azide from aryl β-D-xylopyranosides) (Scheme 1B). In the alternative case, where the introduced mutation replaces the nucleophilic residue, the addition of azide may also accelerate the reaction, but this time the configuration of the glycosyl-azide product will be inverted, as the azide ion will attack at the glycosylation step. To determine the anomeric configuration of the xylopyranosyl azide product of the accelerated reaction, the final product of the hydrolysis of 2,5-DNPX by E175A in the presence of 1 M azide was characterized by ^{1}H-NMR, ^{13}C-NMR spectroscopy, mass spectrometry and FTIR analyses. The NCI mass spectrum showed a molecular peak fitting exactly to the expected molecular mass of a xylopyranosyl azide (175.1). The ^{13}C-NMR confirmed the presence of five carbons, and the FTIR results indicated the presence of an azide moiety [32]. The ^{1}H-NMR spectrum was consistent with the product being a monosaccharide with a single anomeric proton, which corresponds to H-1 of the β-anomer (see Section 2 for complete assignments). All these observations taken together suggest that the resulting product of the azide rescue reaction is a β-D-xylopyranosyl azide. The fact that only the β product was formed during this reaction provides definite evidence to the identification of Glu175 as the acid–base catalyst of AbfA T-6.

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