Esterase EstK from *Pseudomonas putida* mt-2: an Enantioselective Acetylesterase with Activity for Deacetylation of Xylan and Poly(vinylacetate)

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Abstract

An extracellular esterase gene *estK* was identified in *Pseudomonas putida* mt-2, and was overexpressed to high levels in *Escherichia coli*. The recombinant EstK enzyme was purified and characterised kinetically against *p*-nitrophenyl ester and other aryl-alkyl ester substrates, and was found to be selective for hydrolysis of acetyl ester substrates, with high activity for p-nitrophenyl acetate (k_{cat} 5.5 s^{-1}, K_M 285 μM). Recombinant EstK was found to catalyse deacetylation of acetylated beech xylan, indicating a possible *in vivo* function for this enzyme, and partial deacetylation of a synthetic polymer, poly(vinylacetate). EstK was found to catalyse enantioselective hydrolysis of racemic 1-phenylethyl acetate, generating 1*R*-phenylethanol with an enantiomeric excess of 80.4%.

Keywords: Esterase; *Pseudomonas putida* mt-2; xylan esterase; poly(vinylacetate)

Introduction

Bacterial lipase enzymes are widely used as biocatalysts for stereospecific hydrolysis of racemic ester substrates (1,2). While there are many examples of bacterial lipase enzymes whose optimal substrates are esters of long chain alkanoic acids (2), there are fewer examples of bacterial esterases, whose optimal substrates are esters of short chain alkanoic acids (3). Esterase enzymes are typically members of the α/β-hydrolase superfamily, containing an active site serine nucleophile within a Gly-x-Ser-x-Gly (GxSxG) amino acid sequence motif (4).

Soil bacteria that are able to break down the lignocellulose cell wall in plant biomass require a range of cellulose and xylanase enzymes to break down the cellulose and xylan polysaccharide backbones, but they also require esterases in order to hydrolyse ferulate-arabinose linkages in arabinoxylan (5), and to hydrolyse acetyl groups attached to the free hydroxyl groups of xylan (6). Feruloyl esterase enzymes have been identified in fungi such as *Aspergillus niger* (7,8) and *Sporotrichum thermophile* (9), but no homologues have been identified in bacteria to date. Xylan acetylesterases have been identified mainly in fungi such as *Schizophyllum commune* (10), but bacterial xylan esterases have been identified and characterised in *Thermoanaerobium sp* (11), *Bacillus pumilis* (12), *Clostridium cellulovorans* (13), and *Butyrivibrio proteoclasticus* (14).

We have previously shown that bacterial aromatic degraders *Pseudomonas putida* mt-2 and *Rhodococcus jostii* RHA1 have activity for breakdown of the lignin component of lignocellulose (15). Subsequently we have identified members of the dye-decolorizing peroxidase family with activity for lignin oxidation: DypB in *Rhodococcus jostii* RHA1 (16) and Dyp1B in *Pseudomonas fluorescens* Pf-5 (17) both show activity for oxidation of polymeric lignin in the presence of Mn(II). The release of ferulic acid from wheat straw lignocellulose by *Rhodococcus jostii* RHA1 (18) and *Pseudomonas putida mt-2* (see Supporting information Figure S1) suggests that an extracellular bacterial feruloyl esterase enzyme may be present in these bacteria, which could be an important accessory enzyme for lignocellulose breakdown.

Of the known bacterial esterase enzyme families, EstA from *Streptomyces scabies* (19) and EstK from *Pseudomonas mandelii* (20,21) have both been reported to be extracellular enzymes. Bioinformatic searches revealed an EstK homologue with 73% sequence identity to *P. mandelii* EstK (21) that is present in the genome of *Pseudomonas putida* mt-2 and *P. putida* KT2440 (accession number Q88GB2). In order to establish the function of this EstK enzyme, we have cloned and expressed the *P. putida mt-2 estK* gene, and have characterised the recombinant EstK enzyme. We report here its characterisation as an acetylesterase enzyme with activity as a xylan esterase, showing enantioselective deacetylation of a racemic synthetic substrate.

Materials and Methods

Materials

p-Nitrophenyl ester substrates were purchased from Sigma-Aldrich, with the exception of p-nitrophenyl ferulate, which was prepared using a literature method (22). Other chemicals and biochemicals were purchase from Sigma-Aldrich.

Cloning, expression & purification of recombinant *P. putida* EstK.

Genomic DNA was extracted from *P. putida* KT2440 using the Wizard genomic DNA purification kit (Promega), using the manufacturer's instructions. The gene (accession number Q88GB2) was amplified from genomic DNA by polymerase chain reaction using the following oligonucleotide primers: forward 5'- ATG AAC ATT GTC CAC AAA GCC CTC-3'; reverse 5'- TCA CTG CAG ATG CAC TTT AAG TTC G -3'. The amplified 1020 bp gene was cloned into expression vector pET151 using the Champion pET151 Directional TOPO Expression Kit (Invitrogen) using manufacturer's instructions, and transformed into *E. coli* TOP10 competent cells (Invitrogen). The sequence of the cloned gene was confirmed by DNA sequencing, and the recombinant plasmid was transformed into *E. coli* BL21 (Invitrogen) for protein expression.

2L cultures of *E. coli* BL21/pET151-EstK were grown in Luria Bertani broth at 37 ^{o}C, and protein expression induced at A_{595} = 0.6 by addition of 0.5 mM IPTG, then cultures were grown for 16 hr at 15 ^{o}C and harvested by centrifugation at 5,000 g. Cell pellets were resuspended in 20 ml lysis buffer (50 mM sodium phosphate pH 8.0 containing 300 mM sodium chloride), and lysed using a Constant Systems cell disruptor, then cell debris removed by centrifugation at 15,000 g.

The cell extract was applied to a Sepharose Ni-NTA column, washed with 50 mM sodium phosphate buffer pH 8 containing 300 mM sodium chloride and 20 mM imidazole at 1 ml/min flow rate, then eluted in 50 mM sodium phosphate buffer pH 8.0 containing 300 mM sodium chloride and 250 mM imidazole. The purified enzyme was desalted by passage through a PD-10 gel filtration column, eluting with 50 mM sodium phosphate buffer pH 8.0 containing 300 mM sodium chloride and 20 mM imidazole.

The purified His_6-EstK protein was then incubated with His_6-TEV protease (1mg/ml) and incubated for 16 hr at 4 ^{o}C. The treated sample was applied again to the Ni-NTA column, and the cleaved EstK protein was eluted in 50 mM sodium phosphate buffer pH 8 containing 300 mM sodium chloride and 20 mM imidazole. The purified enzyme was desalted by passage through a PD-10 gel filtration column, eluting with 50 mM sodium phosphate buffer pH 7.0 containing 150 mM sodium chloride.

Assays of recombinant EstK

p-Nitrophenyl ester substrates were assayed in duplicate in 50 mM sodium phosphate buffer pH 8.0 containing 300 mM sodium chloride, and the appearance of p-nitrophenol monitored at 405 nm (ε 18,000 M^{-1} cm^{-1}). A range of commercially available esters were tested at 10 mM concentration in the above buffer, and ester hydrolysis monitored by silica thin layer chromatography, using ethyl acetate/petroleum ether (1:2 v/v) as eluent.

Hydrolysis of acetylated xylan and poly(vinylacetate)

Beechwood xylan (Sigma Aldrich) was acetylated using a published method (23). 100 mg xylan was added to dimethyl acetamide (2 mL) in a round-bottomed flask, and refluxed at 110 ^{o}C for 2 hr with stirring, then cooled. LiCl (0.175 g) was then added, and the mixture stirred for 16 hr at room temperature. The mixture was filtered, then pyridine (0.25 ml) and acetic anhydride (0.3 ml) were added, and the mixture heated at 50 ^{o}C for 6 hr. The mixture was then cooled and poured into 50 ml ethanol, giving a precipitate, which was collected by filtration, giving a yield of 30 mg acetylated xylan. ^{1}H NMR ($CDCl_3$) δ_H 5.00 (1H, d, J = 8 Hz, H-1), 4.70 (1H, m), 4.45 (1H, m), 3.90 (1H, m), 3.75 (1H, m), 3.30 (1H, m), 2.00 (6.3H, s) ppm.

Assays of xylan esterase activity (total volume 3.0 ml) contained acetylated xylan (7.5 mg dissolved in 200 µl acetone) in 50 mM sodium phosphate buffer pH 8.0 containing 300 mM sodium chloride, to which were added either 100 µl 1M NaOH, or 100 µl EstK (7.5 mg/ml protein). Samples were incubated for 30 min at 20 ^{o}C, then boiled at 100 ^{o}C for 5 min to remove protein, then centrifuged (microcentrifuge 10 min) before HPLC analysis. Samples (50 µl) were injected onto a BioRad Aminex HPX87H Organic Acids HPLC column, eluted isocratically with 5 mM H_2SO_4 at a flow rate of 0.2 ml/min. Acetic acid eluted at 17.9 min. Assay of poly(vinylacetate) was carried out as above, except using a sample of 1.0 mg poly(vinylacetate) dissolved in 200 µl acetone.

EstK-catalysed hydrolysis of racemic 1-phenylethylacetate

Racemic 1-phenylethylacetate was prepared by acetylation of 1-phenylethanol with acetic anhydride in pyridine, and was characterised by ^{1}H NMR spectroscopy and mass spectrometry (see Supporting Information Figure S7). Samples of 7.5 mg 1-phenylethyl acetate were treated with 1.5 mg EstK in 50 mM sodium phosphate buffer pH 8.0, and left for 16 hr at 20 ^{o}C. Products were extracted with ethyl acetate (2 ml) and analysed by chiral GC (Chrompac cyclodextrin-β-236M-19 column, 50 m length, carrier gas He, T = 115 ^{o}C). Retention times: 1-phenylethylacetate 5.58, 6.24 min; 1-phenylethanol 8.38, 9.06 min for *R* and *S* enantiomers, respectively, as previously determined (24). Product yields and enantiomeric excess were calculated from peak areas.

Results

Sequence analysis, expression and purification of recombinant *P. putida* EstK

Analysis of the amino acid sequence of *P. putida* KT2440 EstK (Q88GB2) using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov) showed related EstK sequences in *P. fluorescens* (A0A0P8X2W8, 73% sequence identity) and *Burkholderia xenovorans* (Q13Q49, 64% sequence identity). Analysis using the SSDB motif search on the EMBL European Bioinfomatics Institute server (http://www.ebi.ac.uk) identified that *P. putida* KT2440 EstK is a member of α/β-hydrolase fold family 3, which includes *Sulfolobus tokadaii* carboxylesterase (accession Q976W8, 30% sequence identity) (25), *Lactobacillus plantarum* carboxylesterase (accession F9US10, 12% sequence identity) (26), and a thermophilic carboxylesterase from *Thermogutta terrifontis* (accession A0A0M3KKY6, <10% sequence identity) (27), whose crystal structures have each been determined. These amino acid sequences were aligned using Clustal Omega software (see Figure 1). The sequences contain a GxSxG sequence motif at Ser-182 of *P. putida* EstK (see Figure 1), characteristic of an α/β-hydrolase (4). The alignment indicates that conserved His-309 and Asp-283 are likely to be the other amino acid residues in the Ser-His-Asp catalytic triad, as verified for *Lactobacillus plantarum* carboxylesterase (26)

The *estK* gene (accession number Q88GB2) was amplified by polymerase chain reaction from *P. putida* mt-2 genomic DNA, and the gene was cloned into a pET151 expression vector. Expression in *Escherichia coli* BL21 as an N-terminal His_6 fusion protein with induction by 0.5 mM IPTG gave a new protein band at 36 kDa (see Supporting Information Figure S2) corresponding to the predicted molecular weight for EstK (36.3 kDa). Purification by Ni-NTA affinity chromatography, followed by proteolytic cleavage with TEV protease and elution from Ni-NTA gave purified recombinant EstK enzyme in a yield of 25 mg per litre of bacterial culture.

Figure 1. Amino acid sequence alignment of EstK

Kinetic characterisation of *P. putida* EstK

Recombinant EstK was first assayed against a series of p-nitrophenyl ester substrates. No activity was observed using synthetic p-nitrophenyl ferulate, implying that EstK is not a ferulate esterase. EstK was very active towards p-nitrophenyl acetate, but showed no activity towards p-nitrophenyl valerate (C_5 acid), p-nitrophenyl caproate (C_6 acid), p-nitrophenyl palmitate (C_{16} acid), or p-nitrophenyl benzoate, and no activity towards p-nitrophenyl phosphate. Hence EstK appears to act as a selective acetylesterase enzyme.

Several other aryl-alkyl esters were tested as substrates for EstK, using thin layer chromatography to monitor hydrolysis. Turnover was observed using 4-acetoxybenzoic acid, but not with methyl benzoate, methyl 4-hydroxybenzoate, or methyl cyclohexane-1-carboxylate (structures shown in Figure 2). Using optimal substrate p-nitrophenyl acetate, steady-state kinetic parameters were measured, giving k_{cat} 5.5 ± 0.5 s^{-1} and K_M 285 ± 30 µM (see Figure 3A), comparable to data reported for this substrate with *P. mandelii* EstK (Lee et al, 2013). A pH-rate profile was also evaluated with this substrate (see Figure 3B), showing optimal activity at pH 7.5-8.5, and an inflexion in activity below pH 7.5. *P. mandelii* EstK was reported to show optimal activity at pH 8.5, with reduction in activity at pH <8.5 (21).

Figure 2. Substrate specificity shown by *P. putida* EstK

Figure 3. Kinetic characterisation of EstK with p-nitrophenyl acetate

Assay of xylan esterase activity

In order to test for xylan esterase activity, acetylated xylan was prepared. Commercially available beech xylan, found by ^{1}H NMR spectroscopy to contain very low levels of acetyl groups, was dissolved in dimethylacetamide/LiCl, and acetylated using acetic anhydride/pyridine (23). The acetylated xylan was characterised by ^{1}H NMR spectroscopy, and the two O-acetyl groups observed at δ 2.00 ppm (integration 6.3H relative to ring C-H signals, see Supporting Information Figure S3).

Release of acetate from the acetylated xylan was monitored using a BioRad HPX87H Organic Acids HPLC column. Calibration with acetic acid gave a peak at retention time 17.9 min, observed at 208 nm or 230 nm, with a sensitivity of 50 nmol acetic acid (see Supporting Information Figure S4). Samples of 7.5 mg acetylated xylan were treated with either 1M NaOH or 0.75 mg EstK in 50 mM sodium phosphate buffer pH 8.0. Treatment with 1M NaOH gave rise to a visible change from cloudy to clear assay solution, and released 5.1 µmol acetate by Organic Acids HPLC (see Figure 4). Treatment of acetylated xylan with EstK also generated a smaller amount of acetate by Organic Acids HPLC (see Figure 4), and led to partial clearing of the acetylated xylan solution after 30 min (see Supporting Information Figure S5). From calibration of the HPLC data, treatment with EstK released 0.21 µmol acetate from a 30 min assay, corresponding to a specific activity of 9.2 nmol acetate min^{-1} mg $protein^{-1}$. Incubation of the acetylated xylan with the reaction buffer and no EstK gave no observable acetate release.

Figure 4. Release of acetate from acetylated xylan, monitored by Organic Acids HPLC

Deacetylation of synthetic poly(vinylacetate)

Having observed deacetylation of acetylated xylan, we also examined whether EstK could catalyse deacetylation of a synthetic polymer, poly(vinylacetate). Samples of 1.0 mg poly(vinylacetate) (gift of Dr. M. Gibson, Department of Chemistry, University of Warwick) were treated with either 1M NaOH or 0.75 mg EstK in 50 mM sodium phosphate buffer pH 8.0. Treatment with 1M NaOH released 240 nmol acetate by Organic Acids HPLC, and treatment with EstK for 30 min also generated a smaller peak corresponding to 84 nmol acetate (see Supporting Information Figure S6), giving a specific activity of 3.7 nmol acetate min^{-1} mg $protein^{-1}$. No release of acetate was observed in a control incubation with the reaction buffer and no EstK.

Figure 5. Polymeric substrates for *P. putida* EstK

EstK-catalysed hydrolysis of racemic 1-phenylethylacetate

In order to investigate the enantioselectivity of EstK-catalysed hydrolysis using a model substrate, racemic 1-phenylethyl acetate was prepared (see Supporting Information Figure S10 for characterisation data). Chiral gas chromatography was used to separate the two enantiomers of 1-phenylethylacetate (retention times 5.58, 6.24 min) and 1-phenylethanol (retention times 8.38, 9.06 min for *R* and *S* enantiomers, respectively). Samples of 7.5 mg 1-phenylethyl acetate were treated with 1.5 mg EstK in 50 mM sodium phosphate buffer pH 8.0, and left for 16 hr at 20 ^{o}C. Products were extracted with ethyl acetate and analysed by chiral GC. Peaks for 1*R*-phenylethanol (33.1% peak area) and 1*S*-phenylethanol (3.6% peak area) were observed, with a 10-fold preference for 1*R*-phenylethanol (see Figure 6, and Supporting Information Figure S8). The calculated enantiomeric excess is 80.4%, with an overall yield of 33% of 1*R*-phenylethanol.

Figure 6. EstK-catalysed hydrolysis of racemic 1-phenylethylacetate

Discussion

Our investigation of *P. putida* EstK was initially based on the hypothesis that this enzyme might be an extracellular feruloyl esterase enzyme. After expression and purification of the recombinant enzyme, assay against p-nitrophenyl ferulate revealed that EstK is not a feruloyl esterase, however, the enzyme appeared to be a selective acetylesterase enzyme that could have applications for biotechnology. EstK is highly active against p-nitrophenyl acetate, but showed no activity against p-nitrophenyl valerate or caproate, indicating a high selectivity for hydrolysis of acetyl esters. *P. putida* EstK shows some enantioselectivity for hydrolysis of 1-phenylethyl acetate,

and therefore could be a useful biocatalyst for resolution of racemic alcohols via their acetate esters (3).

The catalytic properties of *P. putida* EstK are similar to those of a cold-adapted EstK enzyme from *Pseudomonas mandelii* (20), which also has specificity towards p-nitrophenyl acetate (21). A further esterase from *Pseudomonas putida* ECU1011 was reported during the completion of this work, which is sequence-related to *P. putida* mt-2 EstK, which catalyses the stereoselective hydrolysis of aromatic α-acetoxycarboxylates (28). *P. putida* EstK is a member of the α/β-hydrolase family 3, which includes a carboxylesterase from *Lactobacillus plantarum* (26), a carboxylesterase from the thermoacidophilic archaeon *Sulfolobus tokodaii* (25), and a thermophilic carboxylesterase from *Thermogutta terrifontis* (27), whose crystal structures have been determined. Other esterase enzymes present in pseudomonads are intracellular enzymes that are from different α/β-hydrolase families: a 332 amino acid EstF esterase has been characterised from *P. fluorescens* DSM 50106 that has high activity towards lactone substrates (29); and a 218 amino acid a broad specificity carboxylesterase from *P. fluorescens* with optimum activity towards methyl esters of short to medium chain (C_2 to C_{10}) fatty acids, whose structure has been determined (30).

In order to explore the biological function of EstK, we have prepared acetylated xylan, and have shown that EstK can catalyse the partial deacetylation of acetylated xylan, removing 4% of the acetyl groups hydrolysed by NaOH over 30 min, but sufficient to cause solubilisation of the xylan sample in aqueous buffer. Deacetylation of xylan could therefore be a possible *in vivo* function for EstK, and is a further member of the group of bacterial xylan esterases (11,12,13,14). EstK also catalyses the partial deacetylation of poly(vinylacetate) to polyvinyl alcohol, a polymer with a number of biomedical applications such as cartilage replacement and orthopaedics (31). Enzymatic methods for poly(vinylacetate) deacetylation could therefore be of interest for biotechnology. The only reported enzymatic deacetylation method for poly(vinylacetate) involves cutinase, reported to show activities of 0.7-10 μmol nmol enz^{-1} hr^{-1} (32). The activity shown by EstK corresponds to 6.2 nmol nmol enz^{-1} hr^{-1}, so is less efficient than the cutinase-catalysed deacetylation, but could potentially be optimised via protein engineering.

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Figure Legends

Figure 1. Amino acid sequence alignment of *P. putida* KT2440 EstK (accession Q88GB2) with *P. mandelii* EstK (accession H6VXL7, 96% sequence identity), *Burkholderia xenovorans* EstK (accession Q13Q49, 64% sequence identity), *Sulfolobus tokadaii* carboxylesterase (accession Q976W8, 30% sequence identity), and *Lactobacillus plantarum* carboxylesterase (accession F9US10, 12% sequence identity), using Clustal Omega software. GxSxG sequence motif at Ser-182 is highlighted in red, putative catalytic His-309 and Asp-283 residues highlighted in cyan.

Figure 2. Substrate specificity shown by *P. putida* EstK

Figure 3. Kinetic characterisation of *P. putida* EstK with p-nitrophenyl acetate. A, Michaelis-Menten kinetic plot for p-nitrophenyl acetate; B, pH-rate profile with p-nitrophenyl acetate.

Figure 4. Release of acetate (17.9 min peak) from acetylated xylan by treatment with 1M NaOH (green line) or EstK in 50 mM sodium phosphate buffer pH 8.0 after 30 min (red line), monitored by Organic acids HPLC analysis (absorbance at 208 nm).

Figure 5. Polymeric substrates for *P. putida* EstK

Figure 6. Enantioselective hydrolysis of racemic 1-phenylethyl acetate by *P. putida* EstK in 50 mM sodium phosphate buffer pH 8.0 for 16 hr at 20 ^{o}C, showing the peaks for 1*R*-phenylethanol (33.1% peak area) and 1*S*-phenylethanol (3.6% peak area) obtained by chiral GC analysis. The calculated enantiomeric excess is 80.4%, enantioselectivity E = 15 (extent of conversion 36.7%, overall yield of 1*R*-phenylethanol 33%).

Figure 1.

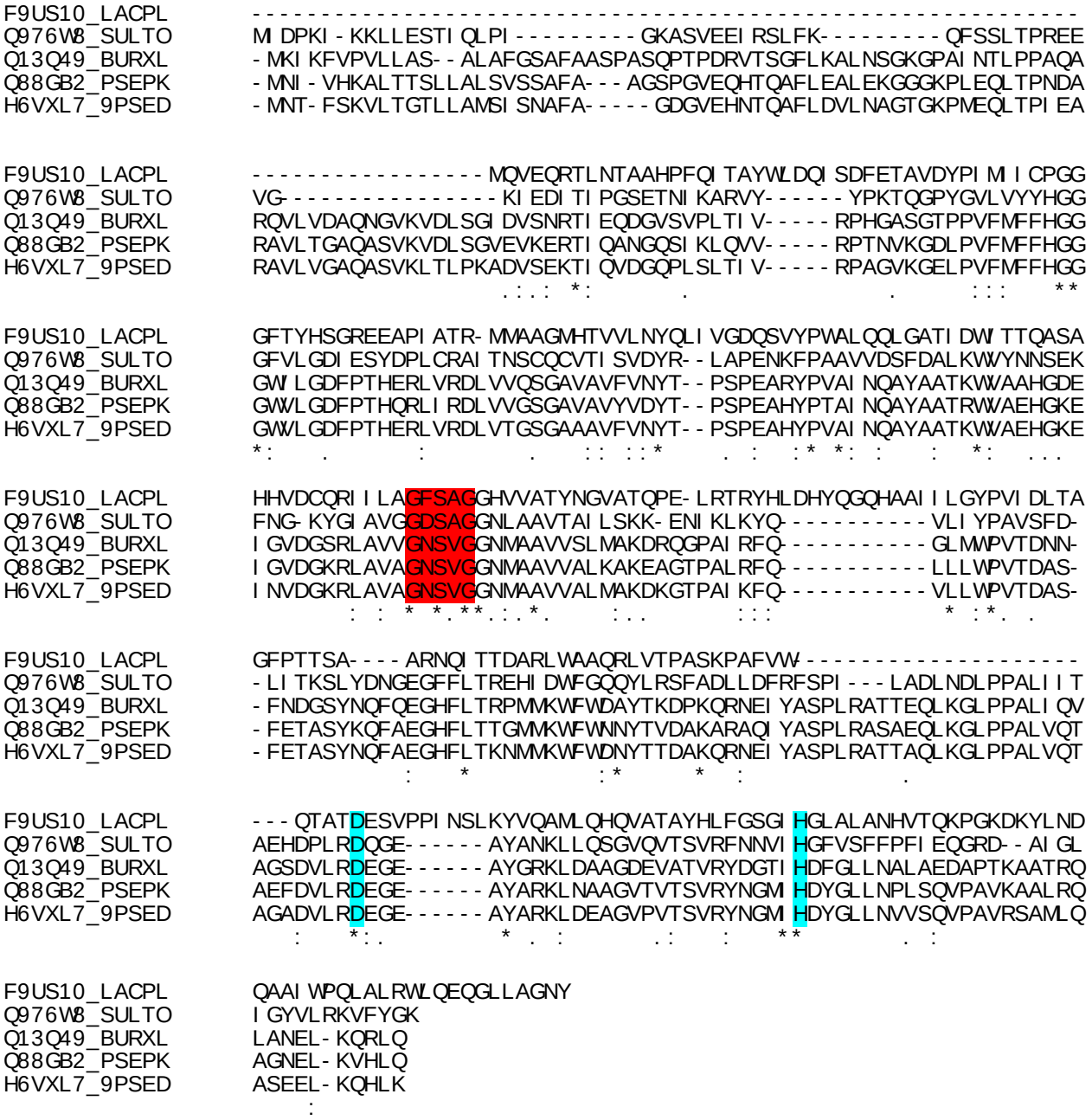
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Figure 2.

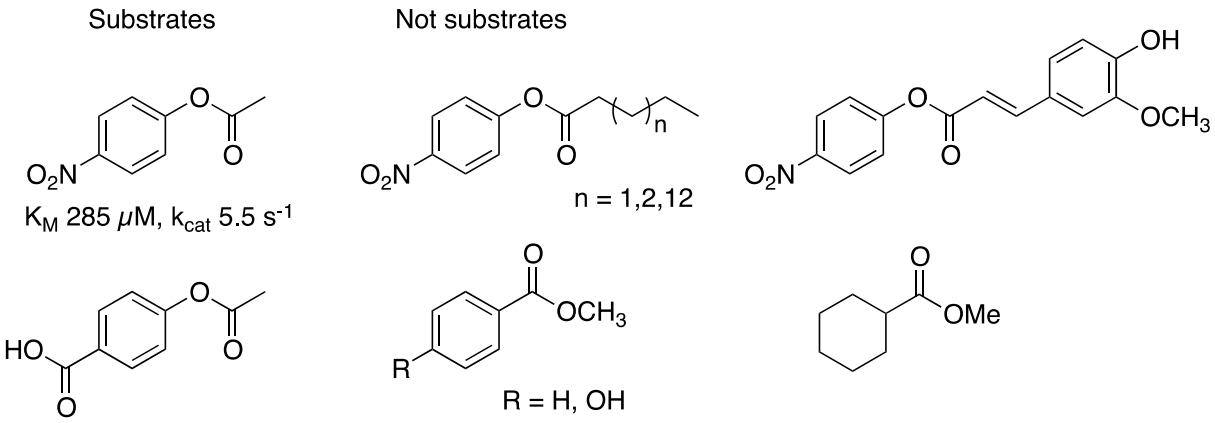

Figure 3.

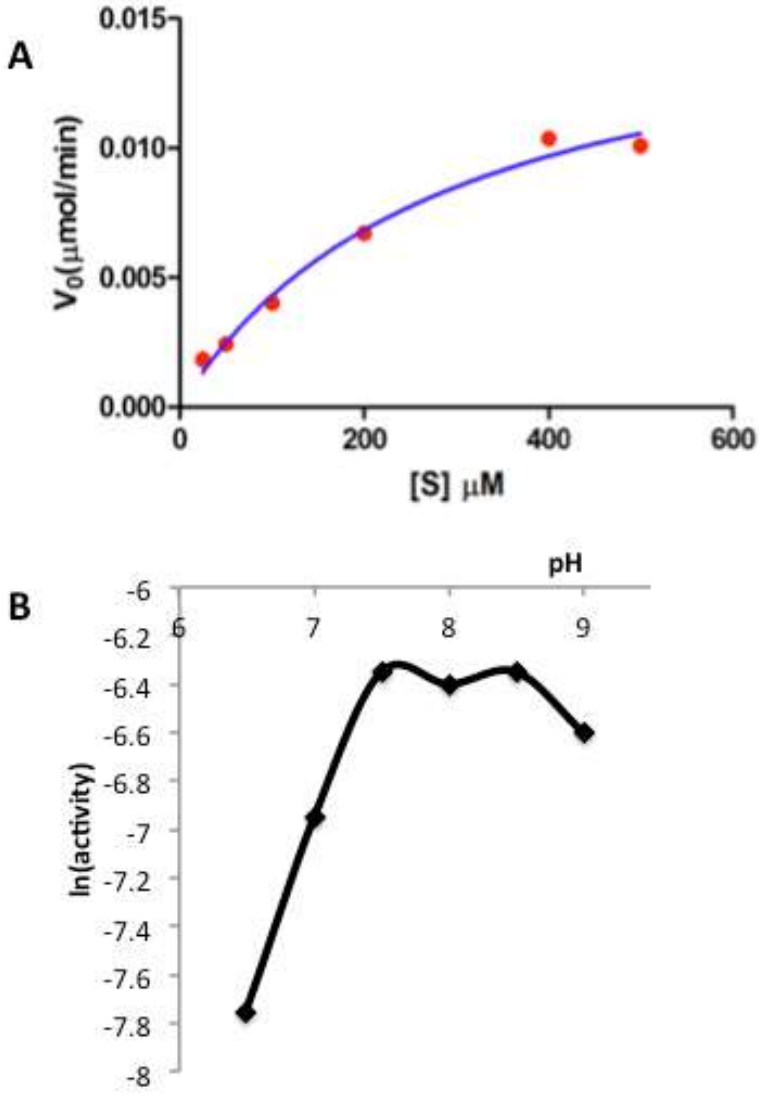

Figure 4.

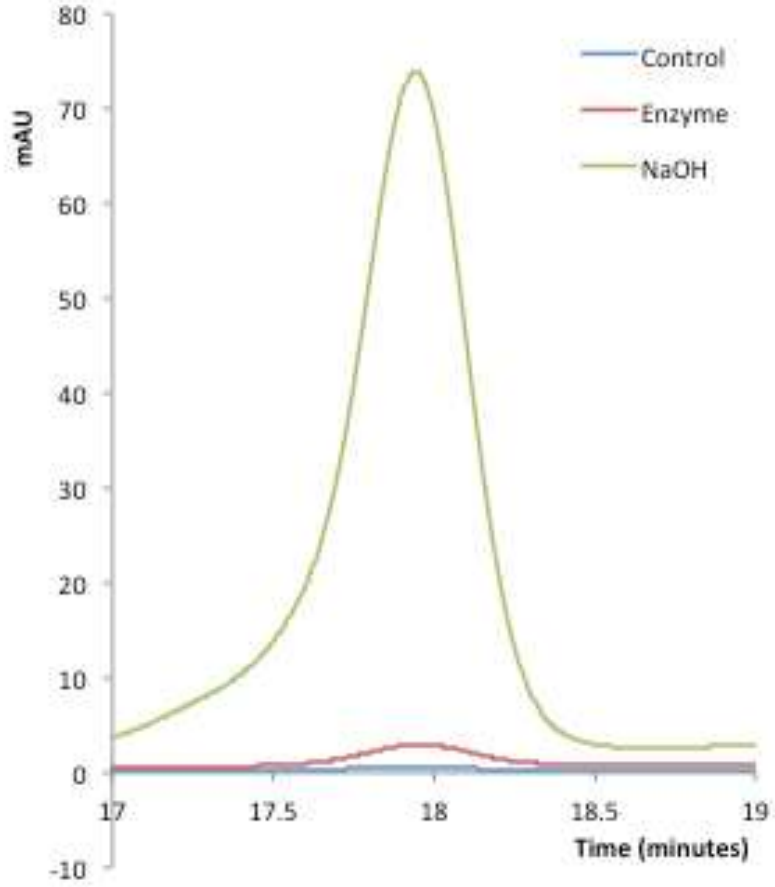

Figure 5.

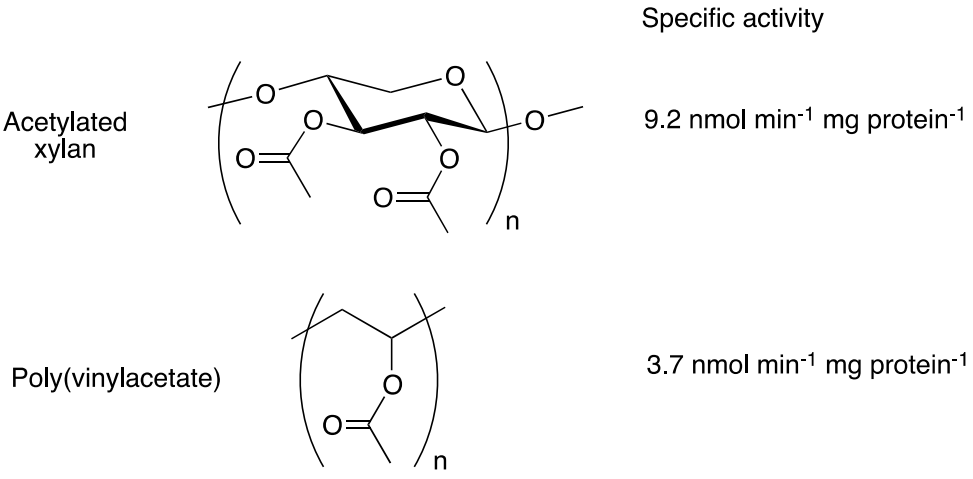

Figure 6