Bacterial class A acid phosphatases as versatile tools in organic synthesis
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Chapter 6

Directed evolution of the acid phosphatase from *Salmonella enterica* to improve the stereoselectivity in dephosphorylation of O-phospho-DL-serine

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Abstract
Directed evolution was performed on the bacterial non-specific acid phosphatase from *Salmonella enterica* ser. *typhimurium* in order to increase the enantioselectivity in the hydrolysis of *O*-phospho-DL-serine. Two variants with increased selectivity have been found; N151D and V78L show *E*-values of 18.1 and 4.1, respectively compared to 3.4 for the wild type (WT) enzyme. Furthermore, both variants show a slightly higher hydrolysis rate compared to the WT enzyme. The variants were also tested in the hydrolysis of *O*-phospho-DL-threonine in which the WT enzyme is very selective (*E* > 200). Both variants show a slightly higher hydrolysis rate, but comparable stereoselectivity compared to the WT enzyme. Sulfoxidation of methyl phenyl sulfide by the N151D and V78L variants resulted in slightly higher ee values of 47% and 42%, respectively, in comparison with the wild-type enzyme (37% ee).

Introduction
The stereoselective synthesis of chiral organic compounds is of considerable interest. Enzymes are attractive tools in asymmetric catalysis and resolution, and efficiently complement (traditional) chemical methods. Many enzymes are commercially available and are able to catalyze the enantioselective transformation of a number of unnatural compounds. However, the substrate specificity and enantioselectivity of enzymes are not always sufficient. This means that a vast number of substrates are not converted with acceptable degrees of stereoselectivity. Many different strategies are used to optimize chirality during biocatalysis, including substrate engineering, solvent engineering (e.g. polarity, hydrophobicity) or changes of reaction conditions (e.g. temperature, pH, ionic strength, pressure). These approaches have in common that the biocatalyst itself is not modified, but result in altered enzyme-substrate interactions, which could sometimes also alter the active-site geometry of the enzyme. By contrast, genetic engineering methods, i.e. site-directed mutagenesis or directed evolution, directly alter at least the primary structure of an enzyme, and often alter the secondary and tertiary structure as well. This may result in a desired change in catalytic activity.

Recently we described the use of the acid phosphatase from *Salmonella enterica* ser. *typhimurium* LT2 (PhoN-Se) in a kinetic resolution (Chapter 5). PhoN-Se is a non-specific bacterial acid phosphatase showing a broad substrate specificity. It is able to distinguish between the D- and L-*O*-phosphothreonine, preferring the L-enantiomer, showing an *E*-value of >200. The stereoselectivity for *O*-phospho-DL-serine however was inversed, preferring the D-enantiomer with a much lower *E*-value of 3.4 ± 0.1. To increase the enantioselectivity for this reaction, directed evolution was applied to the WT enzyme and the variants were screened for higher stereoselectivity. Some interesting variant enzymes were purified and tested for their stereoselectivity in the hydrolysis of *O*-phospho-DL-serine and *O*-phospho-DL-threonine.

Results and discussion
Error prone PCR mutagenesis was successfully used to create a PhoN-Se mutant library and DNA sequencing revealed 1.8 base changes on average per mutant. Screening of 8000 mutants was performed using enzymatic hydrolysis of both D- and L-*O*-phosphoserine in separate incubations using identical enzyme lysates and subsequent assay for inorganic phosphate (P<sub>i</sub>) to identify variant enzymes with changed stereoselective hydrolysis. Lysates from *E. coli* carrying an empty vector showed no significant hydrolysis of *O*-phospho-DL-serine under the assay conditions. After the first screening, 61 variants were selected and screened with a more sensitive P<sub>i</sub> assay. Because the D- and L-substrates are tested pairwise
separately, the enzyme does not compete for the two substrates and so some uncertainty in its stereoselectivity is introduced. Therefore, the three most promising variants were produced in larger amounts, purified and assayed in time dependent reactions, determining their stereoselectivity by chiral HPLC measurements. Sequencing revealed that two of the variants carried the same mutation (N151D) indicating that this site is a ‘hot spot’. The other variant carried a V78L mutation.

It has been reported that a lipase with low enantioselectivity was evolved into an enzyme with inversed selectivity.\cite{11} Since the WT PhoN-Se is close to showing no selectivity at all, the screening results were also evaluated for variants showing inversed selectivity. No such variants were found during this study.

The best mutant generated, N151D (1), has a 5.3 times increased \(E\)-value compared to the WT enzyme in the hydrolysis of \(O\)-phospho-DL-serine under our screening conditions as shown in Table 1 and Figure 1. This variant appears to be quite active, reaching a conversion of 61% compared to 31% for the WT enzyme after 3 h of incubation. The variant V78L, with a slightly improved \(E\)-value of 4.14 also shows a higher catalytic activity, comparable to the N151D mutant.

Table 1. Summary of enantioselectivity of wild type PhoN-Se and improved variants as catalysts in the hydrolytic kinetic resolution of \(O\)-phospho-DL-serine.

<table>
<thead>
<tr>
<th>Reaction Time</th>
<th>Enzyme</th>
<th>Conv. (%)</th>
<th>ee (%)</th>
<th>Conv. (%)</th>
<th>ee (%)</th>
<th>Conv. (%)</th>
<th>ee (%)</th>
<th>(E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5h</td>
<td>WT</td>
<td>8.9</td>
<td>49.6</td>
<td>30.7</td>
<td>48.0</td>
<td>40.0</td>
<td>44.5</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td></td>
<td>N151D (1)</td>
<td>33.8</td>
<td>83.0</td>
<td>61.2</td>
<td>65.8</td>
<td>68.8</td>
<td>55.0</td>
<td>18.1±4.5</td>
</tr>
<tr>
<td></td>
<td>N151D (2)</td>
<td>20.0</td>
<td>86.4</td>
<td>49.6</td>
<td>75.3</td>
<td>58.1</td>
<td>67.5</td>
<td>16.0±1.6</td>
</tr>
<tr>
<td></td>
<td>V78L</td>
<td>17.5</td>
<td>56.1</td>
<td>43.5</td>
<td>48.3</td>
<td>55.4</td>
<td>43.1</td>
<td>4.1±0.1</td>
</tr>
</tbody>
</table>

\(\text{[a]}\) The selectivity factor \(E\) reflects the ratio of the relative rates of reaction of two enantiomers.

Figure 1. Conversion vs. ee values for wild type PhoN-Se and 3 variants in the hydrolysis of \(O\)-phospho-DL-serine. Reaction mixtures contained 5 \(\mu\)M of enzyme, 100 mM \(O\)-phospho-DL-serine in 100 mM acetate buffer (pH 5.0) at 30°C. Measurements were time dependent starting at 30 minutes with 30 minutes intervals. Conversions and ee values were determined by chiral HPLC.

There were only small changes in the specific activity for the dephosphorylation of \(p\)-nitrophenylphosphate (\(p\)NPP). Specific activities of 28, 52, 24 and 45 units/mg were found for WT PhoN-Se, N151D (1), N151D(2) and V78L respectively. The higher activity of N151D (1) in both hydrolysis of \(O\)-phospho-DL-serine and \(p\)NPP can be explained by a
slightly higher purity as determined by SDS-page. In further experiments the N151D (2) variant is used for reason of comparison.

The acid phosphatases form *Salmonella enterica* ser. *typhimurium* MD6001 \(^{(12)}\) and *Escherichia blattae* \(^{(13)}\) have been crystallized and their 3D structure have been resolved. Our PhoN-Se shows 94% and 38% identity respectively, and 3D models have been build. The class of non-specific acid phosphatases to which PhoN-Se belongs share some conserved amino acid residues, which are divided into three domains \(^{(14)}\): -KX₆RP-(X₁₂₋₅₄-)PSGH-(X₃₁₋₅₄)-SRX₅HX₅D-. These conserved regions play very important roles in catalysis. They contain the active site residues that participate in the binding of the phosphate (NE2 of His 197, numbering as in PhoN-Se \(^{(15)}\)), act as a nucleophile (His 197), stabilize the pentacoordinated transition state (Lys 123, Arg 130, Ser 156, His 158 and Arg 191) and play a role in leaving group protonation (His 158). The N151D variant is situated closely to the active site, just before domain II in the primary sequence. As shown in Figure 2, N151 is positioned in the channel to the active site. It is changed from an asparagin which has an amide group as side chain into an aspartic acid, which has a carboxylic acid group as side chain. This changes not only the charge but also the volume of the residue and this may explain the observed difference in enantioselectivity as compared to the WT enzyme. Furthermore, it can be seen in Figure 3 that residue N151 is in close proximity to the residues R130 and K123 which are conserved active site residues involved in stabilizing the pentacoordinated transition state. N151 forms two hydrogen bonds with R130 and changing this residue into an aspartic acid deletes one of these hydrogen bonds. This may also result in a slightly altered active site, changing the stereoselectivity.

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*Figure 2.* Model of the WT PhoN-Se based on the acid phosphatase from *Salmonella enterica* ser. *typhimurium* MD6001, showing the conserved active site residues as sticks, the phosphate and two amino acid substitutions as balls and sticks. Molecular graphics created with YASARA (www.yasara.org) and PovRay (www.povray.org).
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In the primary structure, the other variant, V78L, is not near the conserved domains, but from the 3D structure it can be seen that it also contacts the active site. The side chain is in close proximity to H158 which is involved in leaving group protonation and a subtle conformational change due to the exchange for a leucine can cause the effect found on the stereoselectivity. Surprisingly, residue 78 has been described to be involved in the pH dependency of the dephosphorylation of pNPP by the acid phosphatase from *Salmonella enterica* ser. *typhimurium* MD6001.[15] In this protein, exchanging the isoleucine 78 for an aspartic acid resulted in a pH shift towards more basic pH. The authors explain this by an increase in pKₐ of the H158 involved in the protonation of the leaving group due to electrostatic effects from the D78, which stabilized the protonated form of H158.

Morley *et al.*, [16] performed a study to correlate the distance between the Cα of the substituted amino acid and the active site to the improvement in enzyme activity. They concluded that distant mutations (>10Å from the substrate) can be effective in enhancing the enantioselectivity. Distant mutations can propagate structural changes to the active site where they cause subtle structural changes. However, mutations closer to the active site are better than distant ones in improving the enantioselectivity. This trend is also seen with the mutations found in this study. The most improved variant, N151D, is situated 8.2 Å from the active site and V78L is at a distance of 10.5 Å from the co-crystallized phosphate.

Figure 3. Models of the active site of WT PhoN-Se (left hand panel) and mutated PhoN-Se (right hand panel) based on the acid phosphatase from *Escherichia blattae*.[13] Molecular graphics created with YASARA (www.yasara.org) and PovRay (www.povray.org).

To further improve the enantioselectivity in the hydrolysis of O-phospho-DL-serine, saturation mutagenesis of the amino acids 78 and 151 can be carried out, because random mutagenesis as carried out in this study suffers from some drawbacks. Low mutations rates are likely to change only one nucleotide per codon, but some amino acid substitutions require two nucleotide changes per codon. Changing only a single nucleotide per codon gives on average only 5.7 instead of 19 possible amino acid substitutions and therefore the optimal amino acid may not be identified.[7] In addition, the inherent bias of polymerases (preference for transitions (A→G) over transversions (C→G)) further reduces the diversity that can be accessed by epPCR. Using epPCR with a high mutation rate is also not optimal because a lot of mutations introduced are deleterious for activity, thereby nullifying the good mutations.[17] A whole range of new mutagenesis methods have been described that bypass these drawbacks of the epPCR as carried out in this study.[6-9] Combination of the
found mutations could also improve the selectivity.\textsuperscript{[17]} Further rounds of directed evolution may be carried out using the first-generation DNA as parent for the second round.

Table 2. Summary of enantioselectivity of wild-type PhoN-Se and variants in the selective dephosphorylation of O-phospho-DL-threonine.

<table>
<thead>
<tr>
<th>Reaction Time</th>
<th>3h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>Conv. (%)</td>
<td>ee (%)</td>
<td>Conv. (%)</td>
</tr>
<tr>
<td>WT</td>
<td>29.6</td>
<td>100$^{(b)}$</td>
<td>47.4</td>
</tr>
<tr>
<td>N151D</td>
<td>32.0</td>
<td>100$^{(b)}$</td>
<td>46.3</td>
</tr>
<tr>
<td>V78L</td>
<td>37.9</td>
<td>100$^{(b)}$</td>
<td>54.3</td>
</tr>
</tbody>
</table>

\textsuperscript{(a)} The selectivity factor $E$ reflects the ratio of the relative rates of reaction of two enantiomers.

\textsuperscript{(b)} The detection limit of D-threonine in this system is 0.75 mM. In this case, no D-threonine detected means that less than 1 mM of D-threonine is formed. This leads to an ee value of > 97%.

To evaluate the effect of the mutations on other stereoselective hydrolysis, the variants were also tested in other reactions. The wild type enzyme already shows a selectivity factor of $E>200$ when hydrolyzing O-phospho-DL-threonine in favour of L-threonine (Chapter 5). This is useful in the resolution of O-phospho-DL-threonine. To check whether the introduced amino acid substitutions influence the stereoselectivity of this reaction, the mutants were also screened for their selectivity in dephosphorylation of O-phospho-DL-threonine (Table 2). The variants did not show significant difference in the stereoselective hydrolysis compared to the WT enzyme. The rate of hydrolysis by the variants is slightly higher compared to the WT enzyme (Figure 4), but this difference in rate is not as obvious as observed in the hydrolysis of O-phospho-DL-serine. What can be seen is that the V78L is the fastest variant and continues to hydrolyze O-phospho-D-threonine (the slower reacting enantiomer) with a higher rate compared to the WT enzyme. In contrast, the N151D variant was slightly faster in hydrolyzing the L-isomer, but is slower in hydrolyzing the D-isomer compared to the WT enzyme. The enzymes were still active after 200 h of incubation as was determined by the addition of a new portion of O-phospho-DL-threonine.

To evaluate the effect of the mutations on other stereoselective hydrolysis, the variants were also tested in other reactions. The wild type enzyme already shows a selectivity factor of $E>200$ when hydrolyzing O-phospho-DL-threonine in favour of L-threonine (Chapter 5).

![Figure 4](image-url) Time course of O-phospho-DL-threonine hydrolysis by WT PhoN-Se and variants. Reaction mixture contained 2 μM of enzyme, 100 mM O-phospho-DL-serine in 100 mM acetate buffer (pH 5.0) at 30ºC. Conversions were determined by chiral HPLC. Insert shows the initial part of the reaction.

The phosphatase shows similarity in the active site with vanadium haloperoxidases.\textsuperscript{[18]} When vanadate is bound to the active site of the acid phosphatase, it can function as a vanadium haloperoxidase and perform an enantioselective sulfoxidation. The WT PhoN-Se catalyzes the sulfoxidation of thioanisole towards the (S)-enantiomer with a selectivity of
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36%. The PhoN-Se variants with a higher enantioselectivity in the hydrolysis of O-phospho-DL-serine were therefore also investigated for their enantioselectivity in the sulfoxidation of thioanisole. Surprisingly, the variant N151D and V78L showed increased ee value of 47% and 42%, respectively. The phosphatases show higher conversion rates (≥ ± 11 min⁻¹) compared to the vanadium bromoperoxidase from Ascophyllum nodosum (1 min⁻¹).[19] Although it is common knowledge in directed evolution research that ‘you get what you screen for’, the variants found can also act as better catalysts in other reactions as searched for, as shown by the improved sulfoxidation of phenylmethyl sulfide. Apparently, the active site of the acid phosphatase can be optimized for other (enantioselective) conversions than phosphorylation/dephosphorylation reactions, showing the promiscuity of the enzyme.

Conclusions
We have used a directed evolution approach to improve the stereoselectivity of the Salmonella enterica ser. typhimurium LT2 acid phosphatase in the hydrolysis of O-phospho-DL-serine. Two variants with improved stereoselectivity have been found, both characterized by a one amino acid replacement located just outside the active site; N151D and V78L showing E-values of 18.1 and 4.1, respectively, compared to 3.4 for the WT enzyme. Further saturation mutagenesis and error prone PCR can improve the stereoselectivity still more. The variants found were also tested in the hydrolysis of O-phospho-DL-threonine. No significant differences were observed compared to the WT enzyme which is already very specific in this reaction (E>200). Both variants showed slightly higher enantioselectivity in the sulfoxidation of thioanisole compared to the WT enzyme.

Acknowledgements
We gratefully acknowledge Dr. W. Hemrika and Dr. V. Dumay for providing us the plasmid harbouring the Salmonella enterica ser. typhimurium LT2 PhoN gene. Dr. A. L. Duchateau, DSM fine chemicals, Geleen, is greatly appreciated for providing the HPLC method for separation of the amino acids and OPA derivatization. Dr. G. Schafternaar, University of Nijmegen, is greatly appreciated for building the PhoN-Se models. This work was supported by the Dutch National Research School Combination Catalysis (NRSC-Catalysis).

Experimental section
Random mutagenesis. The protein sequence space was explored by using the error-prone polymerase chain reaction (epPCR) as the mutagenesis method, using the GeneMorph™ PCR mutagenesis kit (Stratagene). EpPCR was performed on a 0.7 kbp DNA fragment. The primers used were the forward primer 5'-ATCGCCATGGAAATACATCACGACGAGAAACAGTGC-3' (NcoI site underlined; first residue of mature PhoN-Se: bold) and the reverse primer 5'-GGGAAAGCTTTACCTTTACCTTTAGTAATTTGGG-3' (HindIII site underlined; original TGA stop is changed for TAA: bold). An average frequency of 2.6 base changes per kilobase was verified by DNA sequencing (MWG Biotech AG) of 20 independent colonies. The amplified DNA was subcloned into the NcoI-HindIII digested pBAD/gIIIA and transformed back in E. coli TOP10 cells. From an estimated library size of 126000, 9600 colonies were robotically picked and transferred to the 96-well format and grown in LB to prepare cells for glycerol stocks.

High-throughput expression of PhoN-Se mutants. Using a 96-pin colony replicator, frozen cells were transferred to LB-agar plates containing 100 μg/mL ampicillin and grown overnight at 37°C. Cell growth and enzyme expression were performed in 96-well format, 2 mL volume polypropylene deep well plates (Greiner Bio-One). Using the 96-pin colony replicator, cells were transferred to the deep well plates containing 1 mL liquid LB-medium with 100 μg/mL ampicillin and 0.02% L-arabinose and covered with Breath Seal porous films (Greiner Bio-One). The cells were grown using an INFORS Microtron plate shaker for 24 hours at 25°C and 560 rpm. The plates were chilled to 4°C and centrifuged at 3500 rpm (Beckmann S5700). The growthmedia were removed and
These PhoN-Se mutants were grown on 0.5 L scale and purified as previously described. Plasmid DNA was using a reaction mixture containing 100 mM

Enantioselective hydrolysis by promising variants was followed in time

Secondary phosphatase activity assay. Primary phosphatase activity assay. The assay conditions used for the initial screening were 10 mM D- and L-O-phosphoserine (Bachem and Sigma respectively) in 100 mM acetate buffer (pH 6.0) in separate wells, but on a single plate to keep conditions equal. Enzyme dilutions and transfer for assay were performed by a Roboseq SE automated liquid handling system (MWG Biotech AG). Aliquots of enzyme lysates (2 x 20 μL) were transformed to flat-bottom microtiter plates containing 180 μL D- or L-O-phospho-serine, mixed and allowed to react for 24 h at room temperature. The amount of P was determined by a spectrophotometric assay based on the method of Saheki et al.[23] μL of reaction mixture was transferred to a fresh flatbottom polystyrene 96-wells microtiterplate and 125 μL of reagent mixture was added as fast as possible and mixed. After 30 minutes of incubation at 30°C the absorbance at 620 nm was measured using a 96 wells plate reader (Fluostar Galaxy, BMG Labtech). Ratios of the amount of P, formed from the D- and L-substrate were used to select mutants with changed dephosphorylation behavior.

The absence of contaminating phosphatase activity in the E. coli TOP10 expression system was established by assaying the empty vector control under the same conditions.

Secondary phosphatase activity assay. 61 Interesting mutants were screened again using Biomol Green™ (Biomol) as a more sensitive P assay which is a modification of the malachite green method. 2 μL of the assay mixture as described above were robotically diluted 10 times. From this dilution, 10 μL were diluted 10 times and 100 μL of the Biomol reagent was added. After incubation for 30 minutes, absorbance at 620 nm was measured using a 96 wells plate reader. Again ratios were determined and 5 mutants were selected for further investigation. These PhoN-Se mutants were grown on 0.5 L scale and purified as previously described.[19] Plasmid DNA was isolated using the QIAprep spin Miniprep kit (Qiagen) and used for sequence determinations (MWG Biotech AG).

Specific phosphatase activity. The phosphatase activity was measured by hydrolysis of 10 mM p-nitrophenyl phosphate as a substrate in 100 mM sodium acetate (pH 6.0). The reaction mixtures were quenched with 0.5 M NaOH to change the pH to 12, and the production of p-nitrophenol was measured at 410 nm (extinction coefficient: 16.6 mM-1 cm-1). One unit is defined as 1 μmol p-nitrophenyl phosphate hydrolyzed per minute.

Stereoselective dephosphorylation. Enantioselective hydrolysis by promising variants was followed in time using a reaction mixture containing 100 mM O-phospho-DL-serine (Sigma) or O-phospho-DL-threonine (Sigma) in 100 mM acetate buffer pH 5.0. Reactions were started by the addition of 2.0 or 5.0 μM mutant enzyme. The amounts of D- and L-amino acids were determined by chiral HPLC using a Daicel crownpak CR column (0.4 cm x 15 cm) at -5°C, equipped with a Dionex 580 LPG pump and Dionex UV-D340/Shodex RI-101 detector. The column was eluted with 100 mM H2OCl at a flow rate of 0.5 mL min-1, o-Phthalaldehyde (OPA) post-column derivatization to detect the amino acids was used. An LKB Bromma 2150 HPLC pump at 0.5 mL min-1 was used to pump the OPA reagent to the reaction loop (0.38 mm x 12 m). The HPLC effluent was monitored at 340 nm. The Chromeleon software program (Dionex) was used for HPLC data acquisition and evaluation. Samples were diluted 50 times before injection.

Sulfoxidation. The enantioselective sulfoxidation by the mutant phosphatases was demonstrated using thioanisole as a substrate. Methyl phenyl sulfoxide (thioanisole, 2 mM) was incubated with H2O2 (2 mM), vanadate (100 μM) and enzyme (0.5 μM) in 100 mM acetate buffer (pH 5.0) at 30°C in 0.5 mL sealed glass vials to prevent evaporation of the substrate. After 6 and 24 h, the reaction mixtures were extracted with 1 mL of CH2Cl2, concentrated by nitrogen-flush to 25 μL and dissolved in 500 μL hexane/isopropanol (IPA) 4:1. The amounts (R)- and (S)-sulfoxide were determined by chiral HPLC using a Chiralcel OD column (0.46 cm x 25 cm) equipped with a Pharmacia LKB HPLC Pump 2248 and a LKB Bromma optical unit 2140 Rapid Spectral Detector. The column was eluted with hexane/IPA 4:1 at a flow rate of 1 mL min-1. The HPLC effluent was monitored at 254 nm. The Borwin software program was used for HPLC data acquisition and evaluation. The retention times for the (R)- and (S)-enantiomers were 6.6 and 7.4 min., respectively.

Docking. The X-ray structure of PhoN from Salmonella enterica ser. typhimurium MD6001[12] (PDB: 2A96) and Escherichia blattae[15] (PDB: 1E0J) were used to prepare the models of PhoN-Se[12] using the Whiraff program.[12]
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