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Optimization of the Kinetic Resolution of the DL-Phosphomonoesters of Threonine and Serine by Random Mutagenesis of the Acid Phosphatase from *Salmonella enterica*

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Abstract: Acid phosphatases are enzymes with a broad substrate specificity showing hydrolytic activity towards several different organic phosphate monoesters, such as nucleotides and sugar phosphates. The acid phosphatase from *Salmonella enterica* ser. *typhimurium* LT2 (PhoN-Se) is able to hydrolyze *O*-phospho-DL-threonine to yield L-threonine with a very high enantioselectivity ($E > 200$). When *O*-phospho-DL-serine was hydrolyzed by PhoN-Se, D-serine was formed, however, the *ee* values rapidly dropped to 50%. Random mutagenesis by error-prone PCR was performed on the phosphatase in order to increase its enantioselectivity in the formation of D-serine. Two variants with increased selectivity from a library of 9600 mutants have been found, N151D and V78L showing E values of 18.1 and 4.1, respectively, compared to 3.4 for the wild-type (WT) enzyme.

Keywords: acid phosphatase; directed evolution; enantioselectivity; enzyme catalysis; phosphomonoester hydrolysis; random mutagenesis

Enzymes are attractive tools in catalysis and efficiently complement (traditional) chemical methods. Some enzymes are commercially available[1,2] but the substrate specificity and enantioselectivity are not always sufficient. Finding an enzyme that shows high stereoselectivity towards a broad range of synthetically useful molecules is desirable. Bacterial non-specific acid phosphatases (NSAPs)[3,4] are a group of secreted enzymes with a broad substrate specificity that shows hydrolytic activity towards several different organic phosphate monoesters. These enzymes are also capable of transferring a phosphate group from a donor (phosphomonoester and pyrophosphate) to a wide range of acceptors (alcohols).[5,6] The acid phosphatase from *Shigella flexneri* (PhoN-Sf)[7] shows enantioselectivity in the phosphorylation of D- and L-glucose by pyrophosphate having a higher affinity for D-glucose.[6] To broaden the application range of phosphatases, we investigated the possible use of PhoN-Sf and PhoN-Se (the acid phosphatase from *Salmonella enterica* ser. *typhimurium* LT2)[8] in the kinetic resolution of phosphomonoesters and optimized the hydrolysis reaction by applying random mutagenesis.

Here we report on the formation of L-threonine and D-serine from *O*-phosphorylated DL-threonine and DL-serine by PhoN-Sf and PhoN-Se. PhoN-Se shows reasonable reaction rates in dephosphorylation of both *O*-phospho-DL-threonine and *O*-phospho-DL-serine as monitored by HPLC (Figure 1). Therefore the pH dependency of the enantioselectivity of this enzyme was investigated in more detail. Hydrolysis rates are not affected to a large extent between pH 3.9 and 5.0, but they clearly diminish at pH 6.25 and no activity is found at pH 3.3 (not shown). To our surprise, only L-threonine was detected within 5 h of incubation for pH values between 3.9 and 5.0 (~32% conversion) and *O*-phospho-D-threonine was not hydrolyzed, giving an *ee* value of 100%. [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% at 30% conversion after 5 h of reaction time.] The E value of this reaction, calculated from conversion and *ee* of the product according to Chen et al.,[9] is >200, which means that this dephosphorylation reaction can be used for preparative syn-

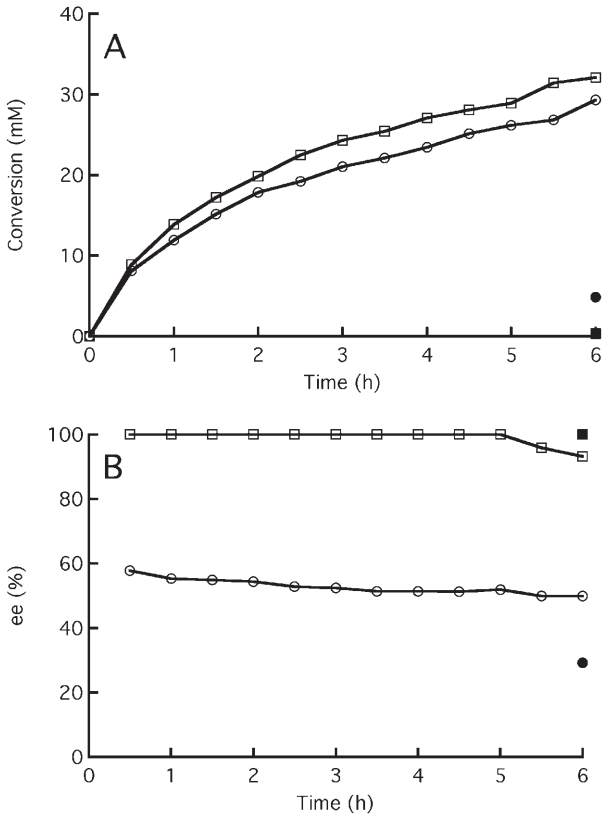

Figure 1. Time course of the dephosphorylation of 100 mM *O*-phospho-DL-threonine (squares) and *O*-phospho-DL-serine (circles) by 5 μM PhoN-Sf (closed symbols) and PhoN-Se (open symbols) at pH 5.0; Panel **A**: Extent of the reaction. Panel **B**: *ee* values of the reaction products.

thesis of L-threonine. In principle, the remaining *O*-phospho-D-threonine can be hydrolyzed and subjected to a threonine racemase[10,11] and phosphorylated again for further resolution and higher yields. For *O*-phospho-DL-serine, the rate of the pH dependent dephosphorylation was equal to that seen with *O*-phospho-DL-threonine. However, after 0.5 h of incubation the *ee* values were already significantly lower (Figure 1) and further slowly decreased to about 50% at 24 h. The formation of D-serine was faster compared to its L counterpart. This results in an E value of 3.7 for the hydrolysis of *O*-phospho-DL-serine. Thus, PhoN-Se shows a slight preference for the hydrolysis of the D-isomer of serine, but surprisingly a high preference for the L-isomer of threonine. The same specificity has been observed for wheat germ acid phosphatase.[12] Since it is known that the *ee* of an enantioselective reaction may strongly depend on the temperature, the temperature dependency of the *O*-phospho-DL-threonine dephosphorylation by PhoN-Se was investigated ($T = 5$–75 °C). However, no effect was seen on the enantioselectivity of the reaction.

PhoN-Sf was virtually unable to dephosphorylate *O*-phospho-DL-threonine as shown by a very low conversion of 0.3% after 5.5 h at pH 5.0 (Figure 1). The observed *ee* value of 100% in formation of L-threonine is therefore not representative for the whole reaction. *O*-phospho-DL-serine conversion by PhoN-Sf after 24 h at pH 5.0 was 10% and at this low conversion, the *ee* value had already dropped to 13.7% in favour of the D-isomer. Considering this result, this enzyme was not studied further.

To improve the enantioselectivity in hydrolysis of *O*-phospho-DL-serine, error prone PCR mutagenesis was used to create a PhoN-Se mutant library. High-throughput screening of the mutants was performed in which the enzymatic hydrolysis of both D- and L-*O*-phosphoserine was followed in separate incubations using identical enzyme lysates and subsequent assay for inorganic phosphate to identify variant enzymes with changed stereospecific hydrolysis. After the first screening of 9600 mutants, 61 variants were selected and screened with a more sensitive Biomol Green™ P_i assay. The three most promising variants were grown in larger amounts, purified and assayed in time-dependent reactions and their stereoselectivity was determined 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.

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 2. This variant appears to be quite active, reaching a conversion of 61% compared to 31% for the WT

Table 1. Summary of enantioselectivity of wild-type PhoN-Se and improved variants as catalysts in the formation of D-serine from *O*-phospho-DL-serine.

	0.5 h		3 h		6 h		
Enzyme	Conversion [%]	ee_p [%]	Conversion [%]	ee_p [%]	Conversion [%]	ee_p [%]	E value[a]
WT	8.9	49.6	30.7	48.0	40.0	44.5	3.4 ± 0.1
N151D (1)	33.8	83.0	61.2	65.8	68.8	55.0	18.1 ± 4.5
N151D (2)	20.0	86.4	49.6	75.3	58.1	67.5	16.0 ± 1.6
V78L	17.5	56.1	43.5	48.3	55.4	43.1	4.1 ± 0.1

[a] The selectivity factor *E* reflects the ratio of the relative rates of reaction of two enantiomers.

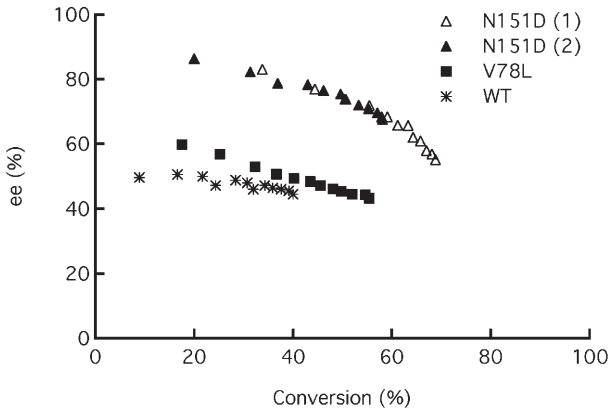

Figure 2. Conversion vs. *ee* values for wild type PhoN-Se and 3 variants in the hydrolysis of *O*-phospho-DL-serine. Reaction mixture contained 5 μM of enzyme, 100 mM *O*-phospho-DL-serine in 100 mM acetate buffer (pH 5.0) at 30 °C. Measurements were time dependent starting at 30 min with 30 min intervals. Conversions and *ee* values were determined by chiral HPLC.

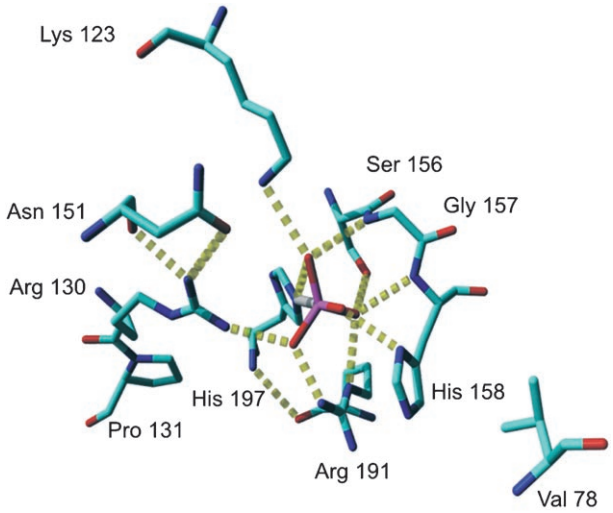

Figure 3. Model of the active site of WT PhoN-Se based on the acid phosphatase from *E. blattae*. Molecular graphics created with YASARA (www.yasara.org) and PovRay (www.povray.org).

enzyme after 3 h of incubation. The variant V78 L, with a slightly improved *E* value of 4.14 also shows a higher catalytic activity, comparable to the N151D mutant. Figure 2 fits well with simulations described by Chen et al.[9]

The acid phosphatases form *Salmonella enterica* ser. *typhimurium* MD6001[13] and *Escherichia blattae*[14] have been crystallized and their 3D structures have been resolved. Our PhoN-Se shows 94 % and 38 % identity, respectively, and 3D models have been build. The class of NSAPs to which PhoN-Se belongs share some conserved amino acid residues, which are divided into three domains.[3] These conserved regions play very important roles in catalysis. They contain the active site residues that participate in the binding of the phosphate, act as a nucleophile, stabilize the pentacoordinated transition state and play a role in leaving group protonation. The N151D variant is situated closely to the active site. It is changed from an asparagine 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. It can be seen in Figure 3 that residue N151 is in close proximity to the residues R130 and K123 which are 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. The other variant, V78L, 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 may change the stereoselectivity. Surprisingly, residue 78 has been described to be involved in the pH dependency of the dephosphorylation of *p*NPP by the acid phosphatase form *Salmonella enterica* ser. *typhymurium* MD6001.[13] 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 pK_a 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.

The effect of the mutations on the hydrolysis of *O*-phospho-DL-threonine was also tested. The wild type enzyme already shows a selectivity factor of $E > 200$ in the formation of L-threonine. The variants did not show significant difference in the stereoselective hydrolysis compared to the WT enzyme.

In conclusion, we investigated the applicability of PhoN-Se in the kinetic resolution of *O*-phospho-DL-threonine and serine. We show that PhoN-Se may be used for the preparative synthesis of enantiomerically pure L-threonine. The *ee* value was 100 % for L-threonine up to 60 % conversion of *O*-phospho-L-threonine. It is shown that the enantioselectivity for hydrolysis of *O*-phospho-DL-serine by PhoN-Se is much less and reversed; D-serine is formed preferentially. The other optical antipodes may be obtained by hydrolyzing the remaining phosphate esters using an unspecific alkaline phosphatase after the resolution and subsequent separation. We have used a directed evolution approach and obtained two variants with improved stereoselectivity in the hydrolysis of *O*-phospho-DL-serine. A better E value for the synthesis of D-serine may be obtained by saturation mutagenesis of the

N151 and V78, or a further round of random mutagenesis using the mutants found as parent strains.

Experimental Section

General Information

Expression and purification of recombinant *Shigella flexneri* PhoN (PhoN-Sf)[5] and *Salmonella enterica* ser. *typhimurium* LT2 PhoN (PhoN-Se)[15] were as described. All chemicals and other enzymes were purchased from commercial suppliers and used without purification.

Dephosphorylation *O*-Phospho-DL-threonine and *O*-Phospho-DL-serine

A mixture containing *O*-phospho-DL-threonine (100 mM) or *O*-phospho-DL-serine (100 mM) in acetate buffer (100 mM) at 30 °C was completed with either PhoN-Sf (5 μM) or PhoN-Se (5 μM). pH-dependent dephosphorylation was carried out at pH 3.3, 3.9, 4.3, 5.0 and 6.25. Reaction rates were determined by time course HPLC measurements of the released phosphate. Chiral HPLC was used to determine the *ee* values of the released amino acids.

Supporting Information

For analytical procedures, random mutagenesis, high-throughput expression and high-throughput screening assays, see the Supporting Information.

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