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Efficient Tandem Biocatalytic Process for the Kinetic Resolution of Aromatic β-Amino Acids

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Abstract: We describe a simple and efficient enzymatic tandem reaction for the preparation of enantiomerically pure β-phenylalanine and its analogues from the corresponding racemates. In this process, phenylalanine aminomutase (PAM) catalyzes the stereoselective isomerization of (*R*)-β-phenylalanines to (*S*)-α-phenylalanines, which are *in situ* transformed to cinnamic acids by phenylalanine ammonia lyase (PAL). Preparative scale conversions are done with a mutated PAM with enhanced catalytic activity.

Keywords: β-amino acids; biotransformations; enantioselectivity; phenylalanine aminomutase; phenylalanine ammonia lyase; tandem reactions

Enantiomerically pure β-amino acids are present in a number of natural products that possess a wide range of biological activities.[1] The synthesis of β-amino acids is a challenging target for organic chemists. Thus far, a number of chemical methods have been developed for the synthesis of β-amino acids based on classical resolution, stoichiometric use of chiral auxiliaries and homologation.[1] A considerable number of catalytic asymmetric methods have also been proposed.[2] Biocatalytic routes, which would not require high catalyst loadings, could fulfill the increasing demand for environmentally friendly synthetic methods. Until recently, most of the biocatalytic approaches to prepare enantiopure β-amino acids relied on kinetic resolution with hydrolytic enzymes, usually lipases.[3] For example, Fülöp et al. reported the use of *Candida antarctica* lipase A for the kinetic resolution of aromatic aliphatic/aromatic β-amino esters,[4] heteroaromatic β-amino esters[5] and ethyl 3-amino-3-(4-cyanophenyl)propanoate.[6] Although these methods are generally of practical value, lipases with higher catalytic activity, enantioselectivity and chemoselectivity, as well as better compatibility in organic solvent, are necessary. Recently, the application of phenylalanine aminomutase (PAM) for the enzymatic synthesis of (*R*)-β-arylalanines has been reported and two catalytic routes have been developed. Walker and co-workers showed that β-phenylalanines can be obtained by PAM-catalyzed isomerization of the corresponding α-isomers[7] and our group has demonstrated that PAM can catalyze the amination of (*E*)-cinnamic acids to produce aromatic β-amino acids.[8]

PAM exhibits extremely high enantioselectivity towards (*R*)-β-amino acids. Therefore we decided to explore the use of this enzyme as a potential catalyst for the kinetic resolution of *rac*-β-phenylalanines. In this process, the (*R*)-β-phenylalanines would be converted into enantiopure (*S*)-α-phenylalanines, leaving behind enantiopure (*S*)-β-phenylalanines (Figure 1 A). The main advantage of such a procedure would be its simplicity, mild reaction conditions and cofactor independence. However, the bottleneck of this strategy is the equilibrium between the α- and β-phenylalanine (roughly 1:1) which is reached in the PAM-catalyzed isomerization reaction,[9] causing incomplete conversion of the enantiomer preferred by PAM. To shift this equilibrium, a second enzyme is required, that can effectively convert (*S*)-α-phenylalanine, the product of the isomerization reaction. Such an enzymatic

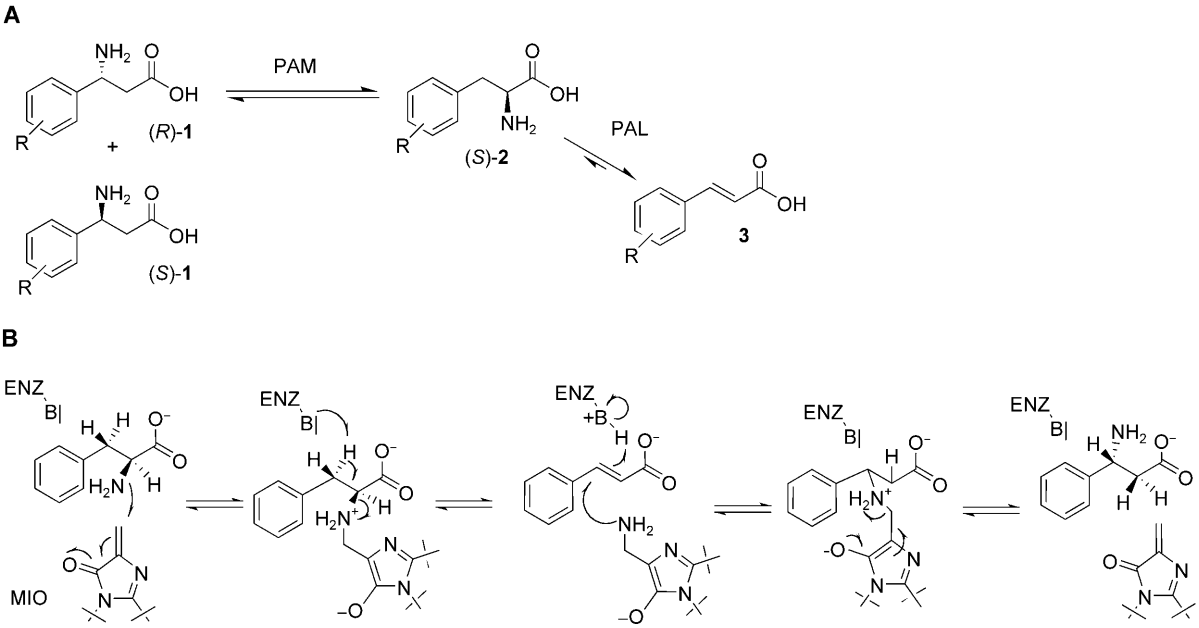

Figure 1. A) Resolution of racemic β-phenylalanines using PAM and PAL. **B**) Proposed mechanism for MIO-based enzymes. The cinnamate product will be released in a lyase-catalyzed reaction, while a mutase will catalyze the ammonia readdition as shown.

tandem reaction based on shifting an equilibrium has also been explored for preparation of enantiopure epoxide from halo ketones.[10]

Phenylalanine ammonia lyase (PAL) catalyzes the conversion of various (*S*)-α-phenylalanines to cinnamic acids.[11] Both PAL and PAM rely on a protein-derived cofactor, 4-methylideneimidazol-5-one (MIO), which is generated autocatalytically from three active site residues, Ala-Ser-Gly, forming an MIO signature motif.[12] The catalytic role of the MIO cofactor has been disputed, but most evidence suggests that it makes a covalent adduct with the ammonia and the amine substrate (Figure 1 B).[12e] The similarity in reaction conditions and complementarity in substrate specificity between PAM and PAL make the latter an ideal candidate for driving the PAM-catalyzed kinetic resolution to completion.

In this communication, we report a new approach to prepare a series of (*S*)-β-phenylalanines in which PAM is used in combination with PAL from *Rhodosporidium toruloides*. In the Scheme shown in Figure 1 A, PAM catalyzes the conversion of (*R*)-β-phenylalanines [(*R*)-**1**] to their corresponding α-isomers [(*S*)-**2**], which undergo PAL-catalyzed elimination of ammonia to form cinnamic acids **3**. The introduction of the latter enzyme should overcome the unfavourable equilibrium, since the second reaction is quasi-irreversible and the reverse reaction requires a very high concentration of ammonia (>1 M). In addition, the cinnamic acid products can be easily separated from the remaining (*S*)-β-phenylalanines.

To establish a proof of principle for this approach, the kinetic resolution of (*R,S*)-β-phenylalanine at 2 mM concentration was carried out using wild-type PAM in the absence of PAL. When approximately 27% of β-phenylalanine was converted, (*R*)-β-phenylalanine and (*S*)-α-phenylalanine reached an equilibrium and the conversion of (*R*)-β-phenylalanine stopped (Figure 2, *upper panel*). The *ee* of (*S*)-β-phenylalanine reached a value of only ~40%. In a parallel reaction, PAL was added and the reaction proceeded to ~55% substrate conversion after 48 h yielding enantiopure (*S*)-β-phenylalanine (99% *ee*) (Figure 2, *lower panel*). This result corresponds well to the expected conversion pattern and proves that PAL is needed for the complete conversion of (*R*)-β-phenylalanine.

To investigate the scope of the catalytic system, a variety of compounds were tested. In order to accelerate the reaction, a PAM mutant Q319M, which possesses higher activity towards β-amino acids and was prepared on the basis of the similarity of PAM to PAL[13] and tyrosine aminomutase,[14,15] was used instead of wild-type PAM. We found that several β-phenylalanines can be converted by PAM Q319M with a reasonable rate and their corresponding (*S*)-α-isomers are good substrates of PAL. Hence, these compounds could be processed by the tandem reaction described here (Figure 3).

The kinetic resolution of compounds **1** was performed on an analytical scale. For compound **1a**, less PAL was added since it is the natural substrate. The conversion and *ee* were determined by chiral HPLC

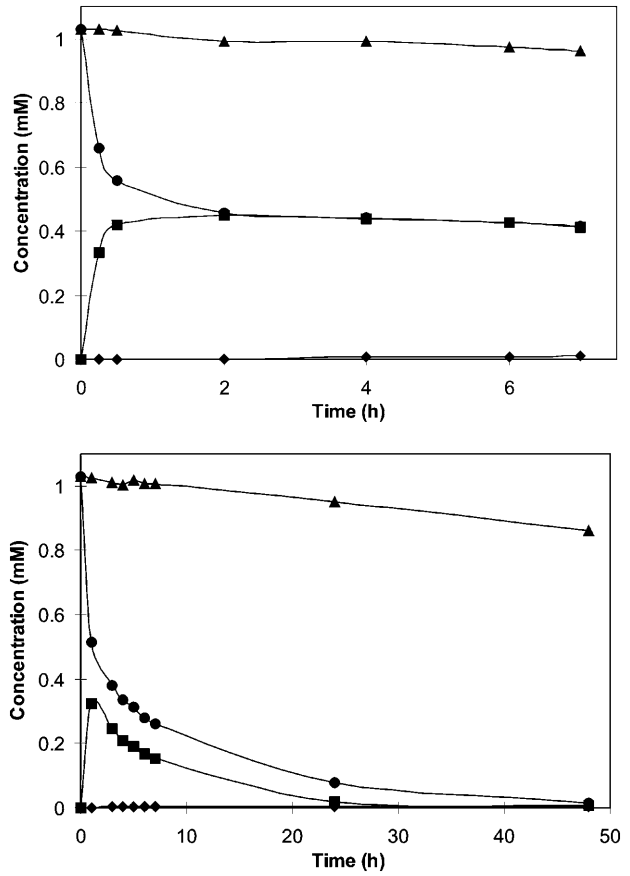

Figure 2. PAM-catalyzed resolution of *rac*-β-phenylalanine (2 mM), without (*upper panel*) and with (*lower panel*) PAL. ◆, (*R*)-α-phenylalanine; ■, (*S*)-α-phenylalanine; ●, (*R*)-β-phenylalanine; ▲, (*S*)-β-phenylalanine.

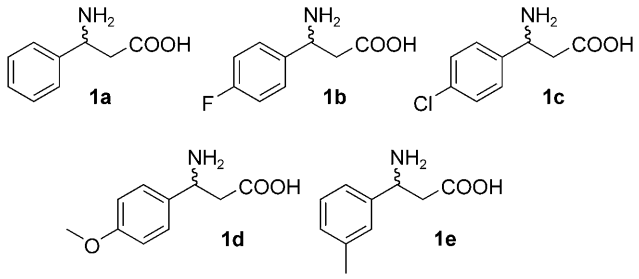

Figure 3. Substrates tested for the PAM/PAL system.

with 24 h time intervals. All five compounds were successfully resolved, giving the corresponding enantiopure (*S*)-β-phenylalanines at approximately 50% conversion (Table 1). The *E*-values that were observed with all the remaining enantiomers are sufficiently high to be practically useful.

Having found excellent enantioselectivity and good conversion, we set up a preparative experiment to kinetically resolve racemic β-phenylalanine **1a**. The preparative scale reaction was performed by incubating 50 mg of racemic β-phenylalanine with 7 mg of PAM Q319M (0.03 mol%) and 0.5 mg of PAL (0.002 mol%) in 30 mL buffer. To simplify product isolation, phosphate buffer was used, instead of Tris·HCl buffer. After 72 h, the (*R*)-β-phenylalanine was completely transformed. The product was purified on a Dowex 50W X8 cation exchange resin column, to give 48% isolated yield of pure (*S*)-β-phenylalanine [(*S*)-**1a**] with an excellent *ee* of >99%.

In summary, a novel system was developed for the kinetic resolution of racemic β-amino acids using an aminomutase (PAM) coupled with an ammonia lyase (PAL). Various aromatic β-amino acids were resolved in high yields and with excellent enantioselectivities. The synthetic applications of PAM to obtain enantiopure (*R*)-β-phenylalanines have been explored previously. By introducing PAL into the reaction system, PAM can also be used for the preparation of (*S*)-β-amino acids. Since different PALs possess varying substrate spectra,[17] the substrate range of this tandem process can be broadened by using other PALs (e.g., PAL from *Petroselinum crispum*).[13] Considering the mild reaction conditions, the fact that no expensive cofactors are required and the simple downstream-processing, this newly introduced shift-moving enzymatic system provides an attractive method for the preparation of useful chiral building blocks.

Experimental Section

Expression and Purification of PAM and PAL

The genes coding for PAM (*T. chinensis*) and PAL (*R. toruloides*) with optimized codon usage for *E. coli* were synthe-

Table 1. PAM Q319M/PAL-catalyzed kinetic resolution of substrates **1**.[a]

Substrate	Time	PAM Q319M (mol%)	PAL (mol%)	Configuration of **1**	Conversion	*ee* of (*S*)-**1**	*E*
1a	72 h	0.03	0.002	*S*	52%	99%	116
1b	72 h	0.03	0.02	*S*	51%	99%	>200
1c	48 h	0.03	0.02	*S*	51%	99%	>200
1d	48 h	0.06	0.02	*S*	52%	98%	91
1e	96 h	0.09	0.02	*S*	50%	97%	>200

[a] 30 μmol of racemic β-phenylalanines were incubated with a suitable amount of purified PAM Q319M and PAL[16] in 3 mL Tris·Cl buffer (20 mM, pH 8.8, with 3% glycerol) at 37 °C.

sized by DNA 2.0. The construction of the PAM expression vector was described before.[8a] Similarly, the PAL gene was cloned into the pBAD-His vector (Invitrogen) at the same cloning sites (NdeI/HindIII) as PAM. The wild-type PAM, the Q319M mutant and PAL were expressed in *E. coli* TOP10 as N-terminal hexahistidine fused proteins. The purification of the protein was achieved by one step Ni-based metal affinity chromatography. After purification, the enzyme was desalted into a storage buffer (20 mM Tris·HCl, 1 mM DTT, 25% glycerol, pH 8.8) and stored at −80 °C. The purity of the protein was analyzed by SDS-PAGE and the protein concentration was determined by the Bradford assay.

Preparative Scale Kinetic Resolution of Racemic β-Phenylalanine

The preparative scale reaction was performed by incubating 50 mg of racemic *β*-phenylalanine with 7 mg of PAM Q319M (0.03 mol%) and 0.5 mg of PAL (0.002 mol%) in 30 mL phosphate buffer (20 mM, pH 8.8, with 3% glycerol). The reaction mixture was filter-sterilized after the addition of the enzyme, and kept at 37 °C. Every 24 h, samples were taken from the reaction mixture to examine the conversion of the (*R*)-*β*-phenylalanine. After 72 h, the (*R*)-*β*-phenylalanine was completely transformed and the reaction was stopped by adjusting the pH to 1.5. The reaction mixture was then applied to a Dowex 50W X8 cation exchange resin column, pre-conditioned by washing with 2 M aqueous ammonia, 1 M aqueous HCl and water. The column was washed with H_2O to remove the undesired compounds, such as cinnamic acid, buffer components and glycerol. (*S*)-*β*-Phenylalanine was eluted with 2 M ammonia solution. After lyophilization, (*S*)-*β*-phenylalanine was obtained as white solid; yield: 24 mg.

Supporting Information

A list of used compounds and stereochemical analysis of the products are presented in the Supporting Information.

Acknowledgements

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