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Recombinant L-Amino Acid Oxidase with Broad Substrate Spectrum for Co-substrate Recycling in (*S*)-Selective Transaminase-Catalyzed Kinetic Resolutions

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Chiral and enantiopure amines can be produced by enantioselective transaminases via kinetic resolution of amine racemates. This transamination reaction requires stoichiometric amounts of co-substrate. A dual-enzyme recycling system overcomes this limitation: L-amino acid oxidases (LAAO) recycle the accumulating co-product of (*S*)-selective transaminases in the kinetic resolution of racemic amines to produce pure (*R*)-amines. However, availability of suitable LAAOs is limited. Here we use the heterologously produced, highly active fungal hcLAAO4 with broad substrate spectrum. H_2O_2 as byproduct of hcLAAO4 is detoxified by a catalase. The final system allows using substoichiometric amounts of 1 mol% of the transaminase co-substrate as well as the initial application of L-amino acids instead of α-keto acids. With an optimized protocol, the synthetic potential of this kinetic resolution cascade was proven at the preparative scale (> 90 mg) by the synthesis of highly enantiomerically pure (*R*)-methylbenzylamine (> 99 %ee) at complete conversion (50 %).

Introduction

Chiral compounds are frequently utilized as intermediates in the industrial synthesis of pharmaceutical agents or agrochemicals.[1] Therefore, the production of non-racemic components in high quality and optical purity is of major importance. Transaminases have been widely applied for the enzymatic synthesis of chiral amines[2–4] due to important advantages in large-scale applications: (i) internal cofactor (pyridoxal-5′-phosphate, PLP) regeneration, (ii) high enantioselectivity, and (iii) a wide range of possible reactions through the broad spectrum of known transaminases which can be extended further by enzyme engineering towards broader substrate acceptance. For example, transaminases have been successfully used for the production of the herbicide L-phosphinothricin,[5] the anti-hyperglycemic drug sitagliptin[3,6] and other building blocks for subsequent synthesis reactions.[3]

Different main reaction strategies are available for the transaminase-mediated synthesis of enantiopure amines: (i) Asymmetric synthesis is based on an enantioselective amination of prochiral carbon skeletons by transaminases ((*S*)- or (*R*)-selective) leading to a theoretical yield of 100 % of the produced pure and chiral enantiomer; (ii) Kinetic resolution via enantioselective deamination by converting one enantiomer into its corresponding keto product, which leads to a theoretical yield of 50 % of the remaining amine enantiomer, (iii) Deracemization of racemic amines by coupling two transaminases with opposite stereoselectivity in a two-step process (conversion of one enantiomer to the ketone followed by asymmetric synthesis to the desired amine enantiomer in the second step) leading to a theoretical yield of 100 % of the pure enantiomer. Moreover, transaminases can be coupled with enantio-complementary amino acid oxidases (AAO). The AAOs oxidize selectively the amino acids which will be converted by the transaminase to the corresponding amines subsequently.[7] (iv) In more complex molecules, an asymmetric transamination can be coupled with a kinetic resolution, if the ketone contains a stereo center in α- or β-position. By choosing appropriate conditions that allow racemization of the ketone enantiomers, a dynamic kinetic resolution of the remote stereo center can be achieved while the amino group is installed in an asymmetric synthesis, resulting in a theoretical product yield of 100 %.[8] In some cases, despite the lower yield, kinetic resolution is the approach of choice for producing enantiopure products, for example when the amine as substrate is more easily available and cheaper than the corresponding ketone (particularly in case of unnatural amino acids[9]) or more stable (e.g. some β-amines like β-phenylalanine[10]). Also, either (*S*)- or (*R*)-selective

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transaminases are known for some chiral substrates of pharmaceutical interest, for example Apremilast[11] so that only one enantiomer is accessible by asymmetric synthesis. Moreover, asymmetric synthesis often suffers from various issues like undesired equilibrium favoring the ketone educt, product inhibition,[4,12] or formation of side products.[10]

Based on the reaction mechanism of transaminases, full conversion requires at least stoichiometric amounts of the co-substrate. This might lead to undesired side effects concerning the reaction itself and/or the products, complication of the work-up, inhibition of reaction (e.g. shown for pyruvate[12]) or high costs.[13] In this regard, enzyme cascades have moved into focus for the recycling of the co-substrate (i.e., amino acids) in transaminase-driven reactions so that catalytic amounts are sufficient to allow the reaction to take place completely. The oxidation of the amino acid to the corresponding keto acid can be performed by amino acid dehydrogenases (AADH),[14,15] deaminases (AADs)[16,17] or oxidases (AAOs).[18,19]

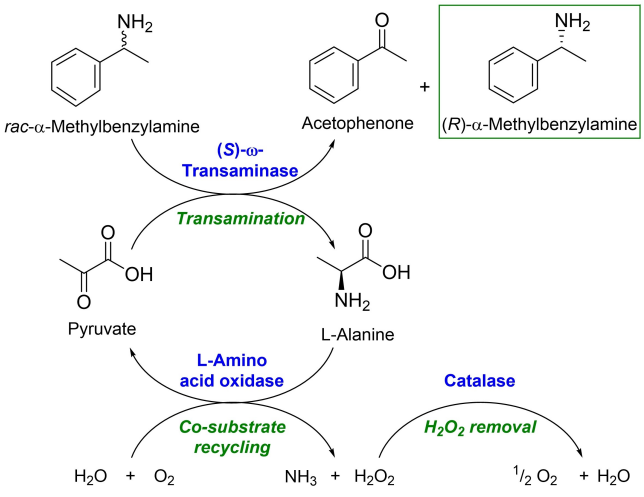
Either AADHs, AADs or AAOs selective for D-amino acids have already been coupled successfully with (*R*)-selective transaminases in enzymatic cascades for the kinetic resolution of racemic amino acid mixtures to produce L-amino acids.[20,21] To obtain D-amino acids, (*S*)-selective transaminases are usually cascaded with AADs and AADHs acting on L-amino acids.[16,18] However, AADs - that act catalytically similar to AAOs - are membrane-bound proteins which transfer electrons to reduced cofactor cytochromes while AADHs are dependent on a NADH-cofactor that needs to be regenerated externally.[14–16,22] L-amino acid oxidases (LAAOs), on the other hand, are soluble cofactor self-regenerating enzymes that are, therefore, theoretically the cascade partner of choice. However, until now, their application was limited by the hurdle of producing a recombinant enzyme in a good yield and with sufficient activity.[18,23] LAAOs isolated from snake venoms are commercially available and have been used for cascades with (*S*)-selective transaminases in a few examples.[24,25] An unspecified LAAO has been used as proof of concept after the establishment of reaction conditions with DAAOs in combination with (*R*)-selective transaminases.[20] However, as the substrate spectrum, product inhibition or activity can vary between the enzymes, the reaction conditions established with (*R*)-selective transaminases and DAAOs cannot necessarily be transferred to the enantio-complementary enzyme cascade. Recently, a recombinant highly active fungal LAAO from *Hebeloma cylindrosporum* (hcLAAO4)[26,27] was successfully expressed in *E. coli* as well as *Pichia pastoris* and purified in good amounts in our group. hcLAAO4 is able to oxidize a broad range of proteinogenic L-amino acids to the corresponding α-keto acids at the expense of molecular oxygen.[26,27] Now the possibility is open for easy and low-cost in-depth analysis of cascading (*S*)-selective transaminases with an autonomous co-substrate regenerating system without requiring further cofactor recycling.

However, one potential challenge of using AAOs is the production of H_2O_2, which is known to potentially damage enzymes or induce unwanted side reactions. To address this, we aim to apply hcLAAO4 in combination with a recombinant catalase to remove H_2O_2 efficiently. A catalase has been used successfully in combination with a DAAO.[28] Thus, this work aims to employ hcLAAO4 as the biocatalyst to recycle the consumed co-substrate of (*S*)-selective transaminases (e.g., pyruvate) in a kinetic resolution with the addition of catalase for efficient H_2O_2-removal (Scheme 1). Moreover, as the hcLAAO4 offers a broad substrate spectrum, different co-substrates worth to be analyzed as well as the establishment of sufficient enzyme and substrate concentrations to obtain optimal reaction parameters in cascades combining the LAAO with (*S*)-selective transaminases. In particular, an (*S*)-selective ω-transaminase from *Vibrio fluvialis* was used as model enzyme which is able to accept a broader range of substrates not limited to amines with carboxylic groups or amino groups in the α-position.[29]

Results and Discussion

An enzymatic cascade for co-substrate regeneration

For establishment and initial evaluation of the cascade, we used the racemic substrate α-methylbenzylamine (*rac*-α-MBA), and pyruvate as the co-substrate. In a typical transamination reaction, both substrates are required in 1:1 stoichiometry for complete enzymatic conversion. This was also verified for the (*S*)-selective ω-amino acid : pyruvate transaminase (wildtype) from *Vibrio fluvialis* (VFwt) used as the model enzyme in this study: co-substrate limited reactions only resulted in partial conversion that correspond to the initial molar percentage (mol%) of pyruvate used (Figure S1). The L-amino acid oxidase from *Hebeloma cylindrosporum* (hcLAAO4) was added to the reaction to recycle pyruvate so that only catalytic amounts have to be added initially to the reaction. As the oxidation reaction



Scheme 1. Transaminase-catalyzed kinetic resolution with co-substrate recycling. The kinetic resolution of racemic α-methylbenzylamine is performed by an (*S*)-selective ω-transaminase with pyruvate as a co-substrate, yielding the reaction products acetophenone and L-alanine by transamination and the enantiomerically pure (*R*)-α-methylbenzylamine. Pyruvate is recycled by the L-amino acid oxidase through oxidation of L-alanine, while the hydrogen peroxide generated is removed by the catalase.

catalyzed by the amino acid oxidase leads to formation of hydrogen peroxide (H_2O_2) as byproduct, recombinantly produced human catalase (hCAT) was added to remove it.[30]

We assembled reactions consisting of VFwt, hcLAAO4 and hCAT and added pyruvate in various sub-stoichiometric concentrations from 100 mol% down to 0.1 mol%. When hcLAAO4 and catalase were added, full conversion was reached with initial pyruvate concentrations of only 1 mol% (Figure 1 and Figure S1, D). This was comparable to approaches combining DAAOs and (*R*)-selective transaminases and significantly lower than other (*S*)-selective transaminase/LAAO reactions before (4-8 mol%).[20,25] However, the low co-substrate concentrations led to prolonged reaction times (12 times longer if using 1 mol% compared to 100 mol%) to complete the conversion.

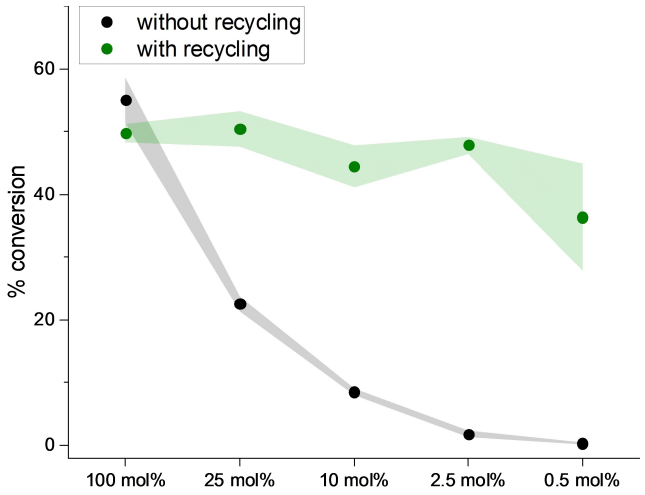


Figure 1. Conversion with varying co-substrate amounts with and without the LAAO/catalase recycling system. VFwt (0.1 mg/mL)-catalyzed reactions were performed with (green) and without (grey) the recycling system (hcLAAO4 and hCAT (both 0.15 mg/mL) and different co-substrate (pyruvate) concentrations in relation to 10 mM *rac*-α-MBA (given in mol%). The production of acetophenone was detected at 245 nm and the standard deviation of triplicates was indicated as filled area.

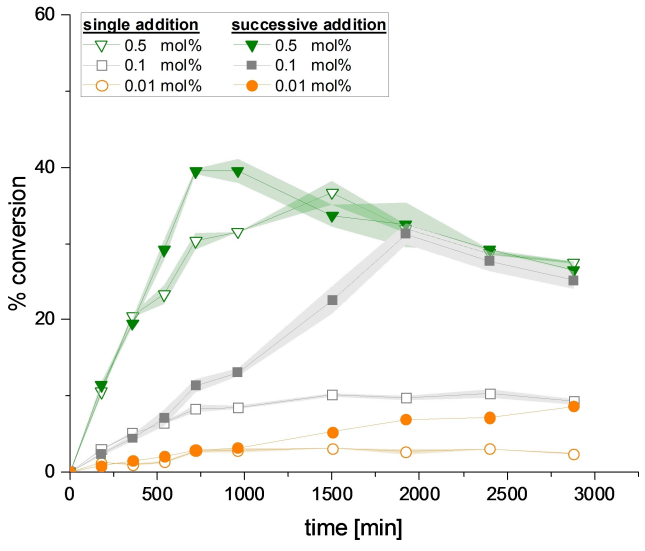


Figure 2. Time courses of conversions with low co-substrate ratios. Comparison of single (empty) vs. successive (filled bars, every 3 hours) addition of low concentrated (< 1 mol%) pyruvate as co-substrate in transaminase-catalyzed and recycling-system supported reactions. *rac*-α-MBA was kept constantly at 10 mM while pyruvate was varied (0.5 mol%: green; 0.1 mol%: grey; 0.01 mol%: orange) and the production of acetophenone was followed at different timepoints at 245 nm.

The importance of catalase supplementation was demonstrated in cascaded reactions in the absence of hCAT: the reaction did not run to completion and stopped after a short period which was dependent on the substrate ratio initially applied. However, the amount of hCAT did not influence the reaction due to its high activity (Figure S2, A–B).

Reducing the co-substrate concentration below 1 mol% was not successful, as the production of the deaminated α-MBA product acetophenone stagnated after 10 hours of reaction time (Figure S1, D). While addition of either the LAAO or the transaminase did not yield further conversion at the stagnation point, addition of fresh co-substrate led to further conversion within a short time (Figure S3). Moreover, successive addition of the co-substrate led to prolonged reactions and the stagnation could be delayed or avoided (Figure 2). This led to the assumption, that the enzymes are still active, but the co-substrate was lacking.

H_2O_2 is highly reactive with pyruvate and other α-keto acids[31,32] and acetate is being produced from pyruvate by oxidative decarboxylation within a few minutes under near-neutral or slightly basic conditions.[31] To analyze whether pyruvate was the only component sensitive to H_2O_2, the enzymes (VFwt and hcLAAO4) and all important substrates used in this study (MBA, pyruvate, L-alanine, L-norleucine) were separately pre-incubated with H_2O_2 (5 mM, 180 min, 37 °C), which was subsequently removed by catalase incubation (120 min, 37 °C). Afterwards, each H_2O_2-pretreated component was applied in independent reactions. The results show that neither the enzymes nor the amine substrates were affected by incubation with H_2O_2. However, the exposure of pyruvate to H_2O_2 led to reduced overall activity of the cascaded reaction by 90% (Figure S4). In conclusion, the stagnation of the cascade reaction is most likely due to the decarboxylation of pyruvate by H_2O_2 despite the presence of catalase. Even though the catalase has an extremely high specific activity, the catalase reaction is still diffusion limited.[33] Therefore, some of the H_2O_2 produced might not be neutralized fast enough and still decomposes pyruvate. While the loss is negligible in the presence of higher pyruvate concentrations, all pyruvate is being decarboxylated, if the transamination reaction is performed with very low sub-stoichiometric amounts of pyruvate. However, the conversion could be pushed towards completion with higher amounts of LAAO (Figure 3). In contrast, increasing amounts of transaminase were without effect at low LAAO-concentrations.

We further excluded that the catalase was inactivated over time since there was no difference between successive additions of catalase (every 90 minutes) and a single dosage of the enzyme when starting the reaction (Figure S2, C). This clearly shows that the gradual decomposition of pyruvate by

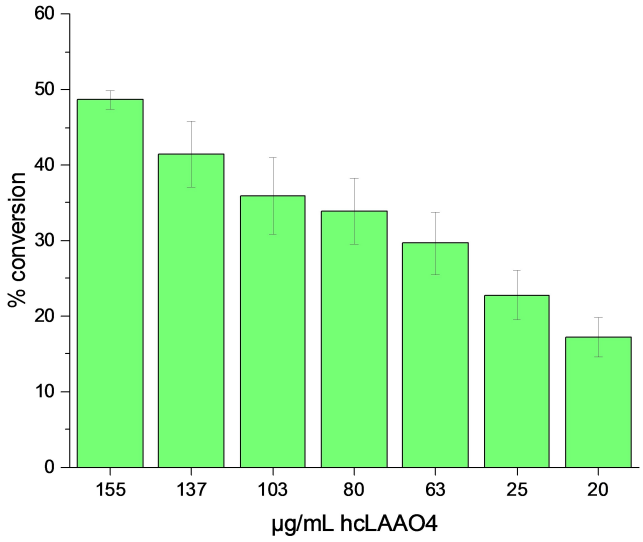


Figure 3. Application of different LAAO concentrations in the recycling system. The impact of varied hcLAAO4 concentrations on the conversion rate in the presence of the recycling system was analyzed in VFwt-catalyzed reactions. The maximum conversion (displayed as bars) was obtained within 9 h. Reactions were carried out in triplicates with 10 mM α-MBA and 0.1 mM pyruvate (1 mol%).

H_2O_2 is a slow process and does not have much effect if the reaction completes fast enough.

As the addition of catalase to the reaction cascade proved to be insufficient to completely prevent the negative effect of H_2O_2 at pyruvate concentrations of less than 1 mol%, we looked for other possibilities to remove H_2O_2 (Figure S5).

H_2O_2 self-decomposition has been described in literature to occur at high pH or high temperatures.[34] However, altered pH-values and temperatures are known to negatively affect the enzymatic reactions. Indeed, increasing the pH to 9.0 as well as increasing the reaction temperature to 50 °C both resulted in a decreased conversion, as hcLAAO4 and hCAT display a lower activity at basic pH,[26,27,35,36] and VFwt and hCAT have low stability at higher temperatures.[35,37] Alternatively, H_2O_2 could be scavenged by small molecules (e.g. PLP)[38] or salts (e.g. Cu, Mn)[39] as additives. In case of DTT and PLP (each 1 mM) no positive or negative effect was observed whereas $CuSO_4$ (1 mM) severely decreased the conversion.

Overall, we were not able to counteract the negative influence of H_2O_2 to pyruvate by additives or altering the reaction conditions so far. However, the adverse impact of H_2O_2 was only notable at co-substrate concentrations of $\leq$0.5 mol% which could be counteracted to a certain extent by successive addition of co-substrate (Figure 2).

For large-scale applications, the cost effectiveness of enzymatic cascades is of major importance, and the amount of product in limited volumes should be as high as possible. Transaminases are known to be inhibited by even low to moderate concentrations of acetophenone.[12,40] Therefore, reactions were performed with different initial concentrations of the substrate (*rac*-α-MBA) and the maximum conversion was determined to analyze the maximal possible concentration of *rac*-α-MBA that can be applied in the reactions. Product inhibition was already observed with concentrations above 8–10 mM (Figure S6). High conversion was observed using 10–20 mM *rac*-α-MBA (48 and 45%, respectively) which yielded up to 8–10 mM acetophenone. Increasing the substrate concentration resulted in higher acetophenone concentrations (up to 16 mM), but on the other hand only in partial enzymatic conversion. Therefore, a maximum of 20 mM *rac*-α-MBA was applied to further reactions.

As LAAOs are O_2-dependent,[18] sufficient oxygen has to be supplied to maintain hcLAAO4 reactivity. Therefore, the ratio between reaction volume and vessel volume was varied in different reactions with 40 mM *rac*-α-MBA: Using a larger volume ratio yielded slightly higher conversion (Table S1).

Another crucial factor was the stability of the enzymes used. A decrease of soluble protein due to protein denaturation was observed in different reactions (either visual in larger reactions or by Bradford quantification) which led to lowered reaction rate or an aborted reaction. To analyze if the substrate (*rac*-α-MBA) or product (acetophenone) showed an impact on protein stability, incubations were performed with the proteins and *rac*-α-MBA or acetophenone at 37 °C for 6 h (Figure S7). hcLAAO4 was not affected by acetophenone and α-MBA, whereas VFwt was affected negatively by α-MBA exposure. This could be explained by amine-induced cofactor- and thus dimer-dissociation leading to inactive transaminases highlighting the well-known operational instability of transaminases in amine-rich solutions.[29,41] However, the effect in this study was negligible. In contrast, hCAT was less stable and precipitated upon addition of acetophenone (40%) and α-MBA (38% precipitated protein). Remarkably, incubating all proteins together, major loss of protein could be detected with and without substrates (65–68%). The high aggregation potential seemed to be caused by the instability of catalase in the presence of substrates since application of the minimal concentration of hCAT (Figure S2) reduced the protein precipitation to a certain extent (Figure S7) and the conversion could be increased (Table S1). Consequently, the concentration of the catalase should be kept low (0.02 mg/mL) which is further beneficial to save costs.

Interestingly, the co-substrate itself was able to affect the reaction. Instead of pyruvate, L-norleucine was used in cascaded reactions, which was oxidized by hcLAAO4 to its keto acid counterpart 2-oxohexanoic acid used by the transaminase. The concentration of hcLAAO4 required for complete conversion could be reduced to one-fifth (34 µg/mL, Figure S8) compared to pyruvate (155 µg/mL, Figure 3). This may be explained by either the higher affinity of hcLAAO4 towards L-norleucine compared to L-alanine (Michaelis constant K_M of 0.32 mM vs. 7.15 mM, respectively, data not shown) or a possibly lower H_2O_2 reactivity towards 2-oxohexanoic acid compared to pyruvate. This underlines the necessity to study suitable co-substrates for the artificial cascade with minimal co-substrate and enzyme amounts. However, further reduction of the concentration of hcLAAO4 (Figure S8) or L-norleucine below 1 mol% (data not shown) also resulted in incomplete conversion, leading to the suggestion that the prolonged reactions result in loss of this keto acid as well.

Taken together, the experiments proved that the LAAO/catalase recycling system can be utilized in (*S*)-transaminase-catalyzed reactions to recycle the co-substrate and thereby reduce the pyruvate requirement for full substrate conversion from stoichiometric to catalytic amounts down to 1 mol% pyruvate. Furthermore, we were able to show the importance of the enzyme concentrations which was consequently lower compared to other studies with high and non-optimal concentrations (e.g., 1 mg/mL of the LAAO).[20] This saves material costs and further avoids negative effects to the reaction (e.g. by precipitation).

Transferability of the recycling system

Next, the transferability of the novel recycling system to other (*S*)-transaminases was investigated. Beside VFwt, four other well known (*S*)-transaminases with various substrate scopes were studied: VFH3RA, an engineered VFwt transaminase enabling the efficient conversion of bulkier substrates compared to the wildtype;[37] 3HMU, an aminotransferase from *Ruegeria pomeroyi*;[42] 3FCR/4M, an engineered variant with enlarged acceptance of bulkier substrates compared to the wildtype transaminase (3FCR) from *Ruegeria* sp.TM1040,[42,43] and CVwt, a wildtype transaminase from *Chromobacterium violaceum*. Using α-MBA as model substrate and pyruvate as model co-substrate, the reactions catalyzed by each transaminase (except 3FCR/4M) reached near-complete conversion (Table 1, Figure S9) revealing that the recycling system can be applied as an autonomous system to different (*S*)-selective transaminase-catalyzed reactions. Thus, the transferability of the recycling system was shown for five (*S*)-selective transaminases using the same amine substrate in all reactions since either the substrate or product could have major impact to the proteins (e.g. precipitation or inhibition). This supports previously published results combining snake venom LAAOs with specific (*S*)-selective transaminases.[25] This offers a broad range of possible kinetic resolution reactions using various transaminases with different substrate scopes making a large variety of potential products accessible, like aliphatic, small, bulky, or unnatural amines even with only catalytic amounts of co-substrate (1 mol%) supplied. As (*S*)-selective transaminases are studied over decades, the enzymes as well as their substrate spectrum is well known, and many kinetic resolutions of interesting and important racemic amines have been shown already. Therefore, the focus of this study was on the co-substrates rather than the amine substrates, as this is a crucial factor for the two enzymes (transaminase and LAAO) and hence for the successful and efficient applicability of the overall cascaded reaction. Moreover, only the co-substrate is converted by the recycling system (hcLAAO4 and hCAT) motivating our focus on the co-substrates in the following experiments.

The hcLAAO4 is able to convert many different L-amino acids selectively to their α-keto acid counterparts.[26,27] Consequently, L-amino acids instead of α-keto acids could be initially applied as co-substrates to the reaction mixture. Therefore, 9 different L-amino acids, which are known to be converted by hcLAAO4[26,27] were added with *rac*-α-MBA to different transamination reactions. With four of them, a high conversion was achieved, which qualified them as usable co-substrates (Table 2, Figure S9). Since the hcLAAO4 exhibits a broad substrate spectrum it becomes possible to adapt the co-substrate to the properties of the transaminase used in order to find a suitable co-substrate for each transaminase. The importance of the co-substrate used in dependence of the transaminase could be demonstrated in case of the 3FCR/4M transaminase (highlighted in Figure S9, E). Therefore, the establishment and choice of an optimal co-substrate for both enzymes ((*S*)-selective transaminase and LAAO) gets crucial for a sufficient kinetic resolution with minimal input of enzymes as shown in this study. In addition, the use of L-amino acids offers the advantages that some substrates are more readily available than their keto acid counterparts and are usually cheaper (especially in the case of unnatural variants),[9] since they are obtained on a large scale from protein rich organisms[44] or

Table 1. Transferability of the recycling system to other transaminase/co-substrate systems. Reactions with 1 mol% pyruvate, 10 mM *rac*-α-MBA and the recycling system were performed with the listed (*S*)-selective transaminases. The conversion was followed by detection of acetophenone at 245 nm at different times and the corresponding time courses are shown in Figure S9.

(*S*)-Transaminase	Conversion[a] [%]	Time[b] [h]
VFwt	48	6
VFH3RA	49	6
3HMU	46	7.5
CVwt	46	4.5
3FCR/4 M	34	6

[a] Maximal conversion whereafter stagnation was observed. [b] Timepoint at which the maximal conversion was reached.

Table 2. Transferability of the recycling system to other transaminase/co-substrate systems. Different (*S*)-selective transaminases and various co-substrates (with a substrate ratio to α-MBA of 1 mol%) were applied in reactions with the recycling system. The conversion was followed by detection of acetophenone at 245 nm at different times and the corresponding time courses are shown in Figure S9.

(*S*)-Transaminase	Co-substrate[a]	Conversion[b] [%]	Time[c] [min]
VFwt	L-alanine	50	270
	L-norleucine	43	360
VFH3RA	L-alanine	49	360
	L-norleucine	50	180
	L-methionine	45	270
	L-leucine	47	270
3HMU	L-alanine	45	360
CVwt	L-alanine	50	270
	L-norleucine	48	180
3FCR/4M	L-alanine	40	360
	L-norleucine	20	270
	L-methionine	26	450

[a] In total 9 different co-substrates (L-phenylglycine, L-phenylalanine, L-alanine, L-arginine, L-isoleucine, L-leucine, L-norleucine, L-methionine, L-glutamine) were analyzed; if not listed, the conversion yielded ≤10% after 600 minutes. [b] Maximal conversion whereafter stagnation was observed. [c] Timepoint at which the maximal conversion was reached.

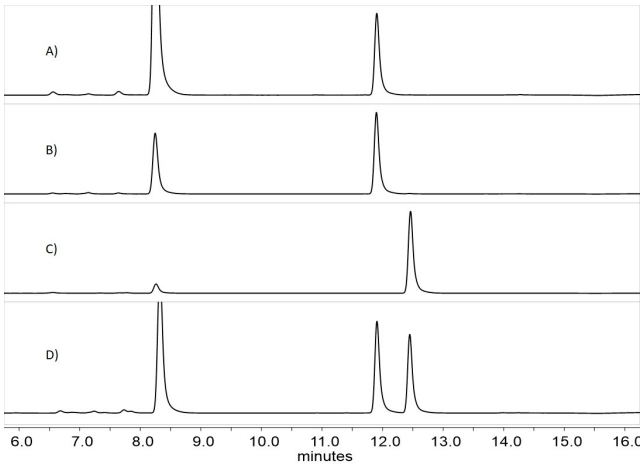


Figure 4. Marfey's test to confirm enantiomeric purity. To confirm the enantiomeric excess (%ee) of the deracemized product in the up-scaled kinetic resolution, α-methylbenzylamine (α-MBA) was extracted, purified and analyzed by Marfey's test using FDAA as reagent (A). As control, purchased enantiopure (*R*)-α-MBA (B), (*S*)-α-MBA (C) and racemic α-MBA (D) was analyzed as well. The signal with a retention time of 8.26 min is the corresponding FDAA and the spectra were recorded at 340 nm.

other methods. This is also true for some non-proteinogenic amino acids, as for example L-norleucine, the preferred amino acid in this study compared to L-alanine, is much less expensive (≈200–900 times) than its keto acid counterpart 2-oxohexanoic acid.

Up-scaled kinetic resolution with the triple enzyme cascade

In order to prove the potential for biotechnological applications of the soluble triple enzyme cascade in a one-pot approach, preparative scale reactions were performed. Therefore, an increased volume of 90 mL (50 mM tris buffer, 0.5 %DMSO, pH 8.0) was prepared with the enzymes (VFwt (0.12 mg/mL), hcLAAO4 (0.15 mg/mL) hCAT (0.02 mg/mL) and substrates (20 mM *rac*-α-MBA, 0.2 mM pyruvate) in a 500 mL vessel. Subsequent HPLC-analysis (Figure S10) confirmed complete conversion (50 %) after 16 hours reaction time at 37 °C and 130 rpm leading to a product concentration of about 10 mM corresponding to about 100 mg of (*R*)-α-MBA.

To further verify the product as successfully enantiopure, (*R*)-α-MBA was isolated as a solid TFA salt (94 mg lyophilizate). The enantiomeric purity of the isolated compound was analyzed by NMR-spectroscopy (Figure S11) as well as Marfey's test using FDAA (1-fluoro-2-4-dinitrophenyl-5-L-alanine amide).[45] Commercial (*S*)-, (*R*)- and *rac*-α-MBA served as reference compounds. It is known that VFwt features an excellent enantioselectivity and thus, the determined enantiomeric excess of >99 %ee further demonstrates that the reactive enantiomer has been converted completely (Figure 4, Figure S12).

Conclusions

In this study, the recycling system for (*S*)-selective transaminase-catalyzed reactions consisting of a recombinant L-amino acid oxidase (hcLAAO4) and catalase for the oxidation of the co-product (i.e., L-amino acids) to the co-substrate (i.e., α-keto acids) was established, thus opening the possibility of biotechnological applications. Only catalytic (up to 1 mol%) instead of stoichiometric quantities of co-substrate were sufficient to complete reactions. The co-substrate amount could be lowered further compared to previous studies (8 mol%[20] and 4 mol%[25]). Additionally, the system offers the utilization of L-amino acids as co-substrates instead of α-keto acids as cheaper or more practicable co-substrates. In this context, it became clear that the choice of the co-substrate is crucial for a complete reaction and also depends on the transaminase used, as demonstrated for some proteinogenic L-amino acids. The recycling system could further be transferred to other (*S*)-selective transaminase catalyzed reactions while the broad substrate spectrum accepted by hcLAAO4 allowed to adjust the co-substrate to the preferences of the applied transaminases. In contrast to previously published cascades, the concentrations of the enzymes involved were analyzed and it was found that enzyme amounts could be reduced and they can affect the stability of the cascade if they are too highly concentrated. Furthermore, the preparative scale reaction confirmed excellent applicability of the system for synthesis and yielded enantiomerically pure (*R*)-α-methylbenzylamine (>99 %ee). This proof of concept offers the opportunity for further optimization of the cascade system, i.e., via immobilization of the enzymes.

Experimental Section

General information: Chemicals and solvents were mainly purchased from commercial distributors with analytical grade: Merck ((Darmstadt, Germany), VWR (Hannover, Germany), Carl Roth (Karlsruhe, Germany) or Thermo Fisher Scientific (Waltham, USA). DNAse I was used from AppliChem, lysozyme from Fluka Analytical and KAPA HiFi HotStart ReadyMix PCR Kit from Roche. *E. coli* BL21 (DE3) was purchased from New England Biolabs.

Cloning of human catalase: The gene for human catalase was purchased from Sino Biological (Plasmid: pCMV3-CAT-His, Catalog Number HG12084-CH, NCBI NM 001752.3). The catalase sequence was amplified using the primers *Nde*I-hCAT1-fwd (GGGGAATTCCA TATGGCTGACA GCCGGGATCCC) and hCAT1-*Not*I-rev (TTTTCCTTTT GCGGCCGCTC ACAGATTTGC CTTCTCCCT), adding a N-terminal *Nde*I and C-terminal *Not*I restriction site. This was followed by a restriction digest with the corresponding enzymes. As target vector for the CAT sequence served pSBL14,[26] a modified pET28b containing the hcLAAO4 sequence, an upstream PreScission protease cleavage site as well as an N-terminal His_6-tag and the CTPSR tag. The hcLAAO4 sequence was cut out using *Nde*I and *Not*I, the plasmid backbone gel purified and ligated with the digested hCAT insert, yielding pFHP-hCAT.

Expression and purification of transaminases, human catalase and hcLAAO4

Expression: For heterologous protein production, *E. coli* BL21 (DE3) was transformed with pET24b-VFwt, pET24b-VHH3RA, pET22b-3HMU, pET22b-3FCR/4 M, pET28b-CVwt or pFHP-CAT. Expression cultures were inoculated by overnight cultures to an optical density at 600 nm (OD_{600}) of 0.1. Protein expression was induced at an OD_{600} of 0.6-0.7 with 0.1 mM (transaminases) or 0.25 mM (catalase) Isopropyl ß-D-1-thiogalactopyranoside (IPTG) and performed for 22 h at 30 °C (transaminases) or 22 °C (catalase). Afterwards, cells were harvested by centrifugation for 20 min at 7000×g and 4 °C, washed with buffer and stored at −20 °C. Expression of hcLAAO4 was done in *E. coli* Arctic Express (DE3), as described in ref. [26].

Purification: The cell pellets were resuspended in lysis buffer (10 mL/g cell wet weight; 50 mM Tris-HCl pH 8.0, 15 mM imidazole, 150 mM NaCl, DNAse I, 1 mM AEBSF, 0.1 mg/mL lysozyme, 0.5 mM PLP) and incubated at room temperature for 30 and at 4 °C for 15 minutes. After disruption by sonication (3×10 min; 4 °C; Branson Sonifier cell-disruptor B15) the suspension was centrifuged (30 min, 18500×g, 4 °C), the supernatant was filtered (0.22 μm filter) and then applied onto HisTrap™ HP columns (Ni^{2+}-NTA affinity chromatography) using the ÄKTA (GE Healthcare) or NGC (BioRad) system. Elution was performed by step gradients (10, 15, 70, 100 %) with an elution buffer (50 mM Tris-HCl pH 8.0, 300 mM imidazole, 150 mM NaCl). Both proteins were eluted at 210 mM imidazole in 15 mL and directly dialyzed in several steps using dialysis buffer (25 mM TRIS, 50 mM NaCl, pH 9.0). If needed, protein solutions were concentrated via vacuum at 40 °C (vacuum concentrator RVC 2–18) and stored at −20 °C. The optimized expression and purification yielded in about 50–80 mg of transaminases and 40 mg catalase per liter expression medium (5 g wet weight). For purification of hcLAAO4, the cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.4) and disrupted by French press. After centrifugation (40 min, 18500×g, 4 °C), the filtered (0.22 μm) supernatant was loaded onto HisTrap™ HP columns using the ÄKTA (GE Healthcare) or NGC (BioRad) system. Unbound components were washed out with 20 mL lysis buffer, followed by a wash step with 13 % and the elution of hcLAAO4 with 50 % elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 7.4). According to Bloess et al.,[26] the eluted enzyme was activated by dilution with 4 volumes of citric acid buffer pH 3. After short incubation at acidic pH, the enzyme was dialyzed against HEPES buffer (100 mM, 150 mM NaCl, pH 7.0) for long-term storage. The optimized expression and purification of hcLAAO4 yielded in about 20 mg protein per liter expression medium. Purification of enzymes was continuously monitored at 280 nm (aromatic amino acids) and additionally at 388 nm (free PLP) and 425 nm (imine between PLP and enzyme-lysine) in case of transaminases, 405 nm in case of the catalase (Figure S13) and 450 nm in case of the hcLAAO4. Chromatograms and SDS-gels are visualized in Figure S14.

Activity assay of transaminases: All activity assays were done in sealed 1.5 mL reaction vessels to avoid evaporation of acetophenone, the product of the activity assays which can be monitored at 245 nm specifically.[46] First the enzymes were submitted and prewarmed to appropriate temperatures (standard: 37 °C). Addition of reaction solution (standard: 50 mM Tris-HCl pH 8.0, 0.1 mM PLP) with respective amounts of substrates[1] started the reaction (standard: 300 μL reaction volume). The production of acetophenone was periodically determined by adding 7 μL of each enzymatic reaction sample to 205 μL water within 96-well UV-microtiter plates (UV-Star®, Greiner Bio-One), which was always done in duplicates. The extinction at the start served as blank. The amount of acetophenone was calculated using an extinction coefficient of 12 $mM^{-1}cm^{-1}$. The stated co-substrates were supplied in the indicated substrate ratios (mol %; ratio between *rac*-α-MBA and co-substrate). The upscaling-approaches were achieved in sealed 2.0 mL reaction vessels and the kinetic resolution of (*R*)-α-MBA in larger reaction vessels with varied reaction volumes analogous to the above-mentioned procedures. Here, the product yield was additionally determined by HPLC-measurements.

Determination of protein concentration: The protein concentration was determined by the Bradford assay using bovine serum albumin as standard.[47]

SDS-PAGE: Protein samples were prepared with SDS buffer (50 mM Tris-HCl pH 6.8, 12 % glycerol, 4 % SDS, 0.01 % bromophenol blue) and incubated at 95 °C for 5 minutes. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 4 % stacking gels and 15 % running gels were used with bisacrylamide as crosslinker and the electrophoresis was conducted with 30 mA per gel. After completion, the gels were washed with water, stained with colloidal Coomassie staining solution (20 % ethanol, 10 % ammonium sulphate, 3 % orthophosphoric acid, 0.1 % Coomassie G-250) and destained with water.

HPLC measurements: The quantification of α-MBA and acetophenone was performed by HPLC-measurements. Therefore, samples of reaction mixtures were taken, acidified to pH 2.0 with HCl and incubated at 95 °C for 5 minutes to denature proteins. The solutions were centrifuged twice, and the supernatants were diluted with HPLC buffer containing 0.2 % TFA to obtain 0.5-1.0 mM solutions. Solutions were transferred to glass vials with screw caps to avoid evaporation of the products (see Figure S15) and analyzed via analytical HPLC on a Shimadzu NexeraXR 20 A system with an autosampler, degasser, column oven, diode array detector and a Phenomenex Luna C18 column (2.9 μm, 50×2.1 mm) with a gradient (in 5.5 min from 5 % to 95 % B, 0.5 min 95 % B and back in 0.1 min to 5 % B with a total runtime of 9 min) of eluent A (0.1 % TFA in water) and eluent B (0.1 % TFA in acetonitrile) 10 μL were injected with a flowrate of 650 μL/min (column oven temperature 40 °C) and the runs were monitored at 245 nm (acetophenone) or 254 nm (α-MBA). Quantification of products were done using propiophenone as internal standard and standard curves of acetophenone and α-MBA.

Extraction of α-MBA: For the extraction of α-MBA from enzymatic reaction mixtures, the solution was initially acidified with HCl to pH 2.0. After the addition of dichloromethane (DCM) in a 1:1 volume ratio, the solution was briefly incubated and then centrifuged to separate the phases. The organic phase was withdrawn (without protein precipitates within the interphase) while the aqueous phase was further washed twice with DCM in a 1:1.5 volume ratio and afterwards basified with NaOH to pH 12.0. *n*-Hexane was used for the extraction of α-MBA and supplied in a 1:2.5 volume ratio. After brief incubation the solution was centrifuged whereupon the aqueous phase was further washed twice with n-hexane (1:2.5 volume ratio). The organic fractions were pooled, dried with anhydrous $MgSO_4$, filtered (0.22 μm filter) and finally the solvent was removed under reduced pressure (335 mbar). The yellow and oily product was resuspended in *n*-hexane and distributed in glass-vials (screw caps) whereafter *n*-hexane was evaporated under an argon stream and dried in vacuum.

Marfey's test: The enantiopurities of the purchased and synthesized compounds were determined using the chiral agent 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA). Therefore, a solution of FDAA (100 μL, 15 mM, 1.5 eq.) in acetonitrile was added to a 1:2 water/acetonitrile solution (300 μL) containing the corresponding

[1] Racemic α-methylbenzylamine (*rac*-α-MBA) was used as main substrate in varied concentrations (standard: 10 mM).

α-MBA (3.33 mM, 1 eq.) and sodium hydrogen carbonate (0.1 M, 30 eq.). The mixture was incubated for 60 min at 40 °C, neutralized with aq. HCl (50 μL, 0.2 M) and diluted with 1:1 water/acetonitrile (450 μL). The resulting solution was analyzed using LC-MS.

LC-MS: The analysis was performed with an Agilent 6220 TOF-MS with a Dual ESI-source, 1200 HPLC system (Agilent) with autosampler, degasser, binary pump, column oven, diode array detector and a Hypersil Gold C18 column (1.9 μm, 50×2.1 mm). The gradient started with 100% eluent A (water/ACN/formic acid, 94.9:5:0.1), during 12.5 min the percentage of eluent B (ACN/water/formic acid, 94.9:5:0.1) increases from 0% to 98% B and returned to 0% B in 0.5 min. Total run time was 18.5 min at a flow rate of 0.3 mL/min and column oven temperature of 40 °C. After separation via the 1200 HPLC system ESI mass spectra were recorded in extended dynamic range mode equipped with a Dual-ESI source, operating with a spray voltage of 2.5 kV. The same system was used for high resolution mass spectroscopy.

NMR measurements: NMR spectra were recorded on a Bruker Avance III 500 HD (500 MHz for ^{1}H, 126 MHz for ^{13}C) in deuterated methanol whereat the chemical shift δ is reported in [ppm] relative to the residual proton signal of the solvent (CD_3OD (δ 3.31 ppm (^{1}H NMR), δ 49.0 ppm (^{13}C NMR). 1D spectra were confirmed by executing 2D experiments (HMQC, HMBC). α-MBA: **^{1}H-NMR** (600 MHz, Methanol-d_4) δ [ppm] = 8.37 (s, 3H, –N**H_3**), 7.48 (d, ^{3}J = 7.6 Hz, 2H, Ph-2/2'-**H**), 7.43 (dd, ^{3}J = 7.6 Hz, ^{3}J = 7.1 Hz, 2H, Ph-3/3'-**H**), 7.38 (t, ^{3}J = 7.1 Hz, 1H, Ph-4-**H**), 4.41 (q, ^{3}J = 6.8 Hz, 1H, Ph-C**H**(NH_3)-CH_3), 1.50 (d, ^{3}J = 6.7 Hz, 3H, Ph-CH(NH_3)-C**H_3**). **$^{13}C\{^{1}H\}$-NMR** (151 MHz, Methanol-d_4) δ [ppm] = 139.2 (Ph-**C**1), 128.7 (Ph-**C**3/3'), 128.4 (Ph-**C**4), 126.7 (Ph-**C**2/2'), 49.9 (Ph-**C**H(NH_3)-CH_3), 20.7 (Ph-CH(NH_3)-**C**H_3).

Author Contributions

T.H. performed all experiments and practical work expect the work mentioned below and wrote mainly the paper under the supervision of N.S., M.H., U.T.B and G.F.v.M. J.P. performed the preparative purification via HPLC, the determination of enantiomeric excess and the NMR-measurements. S.K. worked on the expression and purification of hcLAAO4. T.B. cloned the expression plasmid of hCAT.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: biocatalysis · co-substrate recycling · enantiopure (*R*)-amines · kinetic resolution · L-amino acid oxidase (LAAO) · transaminases

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