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Enantioselective scavenging using homogenate of *Rhodotorula graminis*: a facile preparation of D-amino acid derivatives in enantiopure form

Zizhang Zhang *

Department of Chemistry, Zhejiang University, 38 Zheda Road, Hangzhou 310027, PR China

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ABSTRACT

An enantioselective scavenger (ES) comprised homogenate of *Rhodotorula graminis* containing multiple enzymes can enantioselectively remove L-enantiomer in a racemic mixture of amino acid derivatives (AADs), yielding D-enantiomer in high ee. Thirteen non-proteinogenic AADs were produced in enantiopure D form. The method appears to be an efficient cleaning and preparative strategy which can be applied to the production of D-AADs in high ee by enantioselectively scavenging the 'L-contaminants'.

One hundred and fifty years ago, soon after the discovery of molecular chirality, Pasteur showed that *Penicillium glaucum* specifically fermented the D form of ammonium tartrate, leaving the L form unaffected.[1] This work should be considered as the earliest example of an enantioselective scavenger (ES), although the terminology has not been adopted. With this as our inspiration, we recently tried to explore an ES strategy aimed at discovering new biocatalytic processes, new biocatalysts (enzymes or strains) and their reaction types regarding substrate specificity and enantioselectivity. Ultimately, the ES strategy is expected to provide us with the tools necessary to gain more insight into nature's rich realm of enzymatic catalysis, addressing fundamental questions such as the scope and usefulness of the radical mechanism in enzymes—a topic revitalized by the recent findings from Kim et al.[2] A typical ES process contains at least one step of kinetic resolution, followed by cascade reactions of the fast reacted enantiomer, resulting in one enantiomer of the racemic (*rac*) substrate deeply transformed and another un-reacted enantiomer in high ee. The un-reacted enantiomer serves as a 'scale' of the degree of the reaction, and the form of the product(s) gives the indication of types of biotransformations as well as of the responsible enzymes. But unlike a simple kinetic resolution, the ES process is irreversible and may involve an individual or a series of enzymes acting in sequence and/or in competition. Interestingly, in the course of our study, it appears that the ES strategy may serve as an efficient method for the preparation of AADs in high ee.

In this Letter, we wish to report our preliminary results on the exploration of an L-ES, a cell free homogenate of the yeast *Rhodotorula graminis* (*R. g.*), which contains a series of L-selective enzymes. Using an L-ES strategy, a series of non-proteinogenic AADs were prepared in D form in high ee.

The L-AA metabolizing enzymes exist in a variety of animals, plants, and microorganisms, and have been well documented.[3] For instance, L-Tryptophan (L-Trp) can be transformed by a series of enzymes. The transformation can be initiated through the cleavage of 2,3-double bonds by dioxygenase (EC 1.13.11.11 and EC 1.13.11.52),[4] through the replacement of an amino group by transaminase (EC 1.4.3.2 and EC 2.6.1.27),[5] or through the removal of a carboxyl group by Trp 2-monooxygenase (EC 1.13.12.3) or 2′-dioxygenase (EC 1.13.99.3).[6] It can also be initiated through 5-hydroxylation on the aromatic ring by hydroxylase (EC 1.14.16.4),[7] or through the lesser known 7-chlorination by 7-Halogenase (PrnA).[8] Each of these reactions triggers a distinct cascade pathway and produces a wide array of intermediates (Fig. 1). Similarly, distinct enzyme pathways exist for all proteinogenic AAs.

R. g. (*R. g.,* ATCC 10804) is an yeast reported to contain L-mandelate dehydrogenase[9] and L-phenylalanine ammonia lyase (PAL, EC 4.3.1.5).[10] To the best of our knowledge, it is not known to contain any Trp metabolizing enzymes. However, when *rac*-Trp was administered to the resting cell of *R. g.* under anaerobic conditions, the L-Trp was gradually enantioselectively scavenged (ES'ed). The reaction was notably faster under aeration, indicating the use of molecular oxygen. Furthermore, the ES system performed even better under aeration in the form of a homogenate made from *R. g.* through ultra-sonication. The L-Trp was steadily transformed to indole acetic acid (IAA) also known as Auxin, a phytohormone, whose role in plant growth regulation was revealed recently.[11] Bio-oxygenation processes which use molecular oxygen are typically not done in vitro (with no supplements of cofactors) because the mechanisms for cofactor regeneration are lost once the cells are broken. The unusual feature of this ES attracted us to further investigate its use in the transformation of non-natural substrate, and non-proteinogenic AADs such as phenylalanine derivatives. The homogenate has been the preferred form of ES used in the entire course of this work except for a few cases where both forms were used for comparison or confirmation.

* Tel.: +86 571 87953086; fax: +86 571 87951895.
E-mail address: zhangzz@zju.edu.cn

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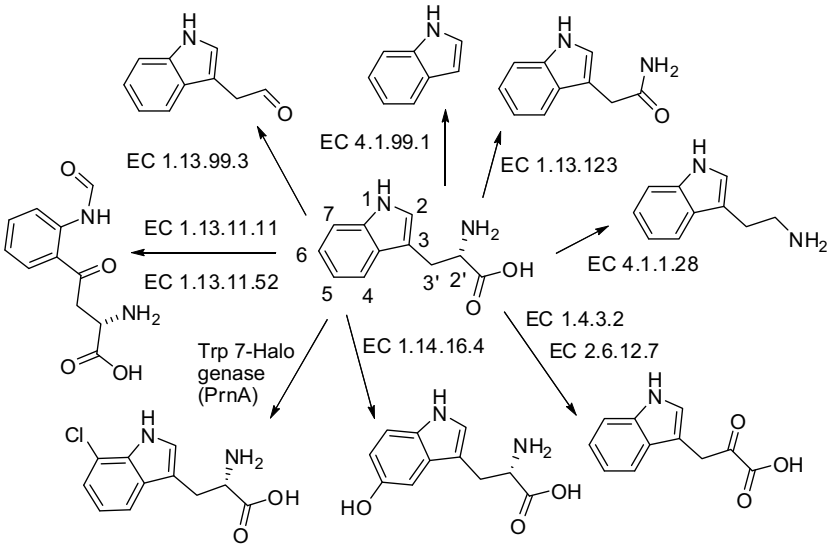
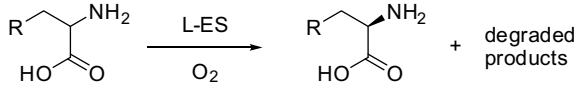


Figure 1. Tryptophan metabolic pathways and the enzymes involved.

All of our experiments were carried out repeatedly as to minimize the variations of reproducibility. A typical experimental procedure of the bioconversion is as follows: The yeast *R. g.* was inoculated under sterilized conditions in a 500 mL flask containing 100 mL of growth media (yeast extract 1.0%, peptone 1.0%, K_2HPO_4 0.5%, and L-phenylalanine 0.5%) (the pH was not adjusted after sterilization), and incubated under 30 °C for 24–26 h. The culture was then harvested, centrifuged, washed with ice water, and the supernatant was decanted. The culture cake was ultra-sonicated with ice for 40 min to break the cells (in the experiment using whole cells, the culture cake was put back after washing with no sonication). The homogenate was put back into a shaking flask. The substrate (50 mg) was put into the flask after being dissolved in dilute sodium hydroxide. The pH was quickly adjusted to 7.3, and the total volume was adjusted to 50 mL by tris-buffer. The flask was aerated before being closed by an air permeable stopper. The flask was then set in the shaker with a controlled speed (200 r/min) and temperature (30 °C). The progress of the bioconversion was monitored by regular sampling and HPLC analysis. The reaction mixture was left to react for 24–48 h, and extended to 53 h in some cases depending on the conversion. For the samplings, 1 mL of reaction mixture was removed to an Eppendorff vial and centrifuged. The cell debris was decanted, and the supernatant was filtered. The filtrate was diluted by distilled water and used for injection into HPLC column for analysis. The HPLC system was equipped with a chiral column Crownpak CR (+) (Daicel, Japan). The standard conditions of elution for *rac*-Phe suggested by the column supplier were modified by adding 10% of methanol to isocratically elute all the AADs. In all cases, the clear (or baseline) separation of the two enantiomers was achieved. The detection wavelength was set at 200 nm. For the isolation of products, the experiment was carried out in duplicates (maximum in parallel with 28 flasks). At the end of transformation, the mixture was collected and centrifuged. The cell debris was decanted, and the supernatant was loaded onto Resin column and eluted by buffer. The elution conditions differ from one substrate to another with no optimization. The fractions were also analyzed by HPLC. D-AADs (and IAA, in case of Trp) were collected in high purity after concentration. The results are shown in Table 1.

The ES systems demonstrated excellent enantioselectivity toward Phe derivatives substituted with methyl-, mono- and di-chloro-, bromo-, and nitro- groups, in addition to Tyr and homo-Phe. As shown in Table 1, the L-ES selectively scavenged L-isomer from all these AADs in their corresponding racemates, leaving the remaining D-isomer in 36–50% yield and >99% ee in less than 24 h (entries 1–12) with the exception of homo-Phe (entry 11), which took two days and yielded slightly lower ee (98%). Particularly, in the case of 2-chloro-Phe, the process was completed within 1 h (entry 9). A set of chromatograms traced from the experiments are shown in Figure 2.

Table 1
Preparation of enantiopure AADs by enantioselective scavengers[a]



Entry	Substrate racemate	Product (D)		
		T (h)	Yield	ee
1	2-Br–Phe	2	36	>99
2	4-Cl–Phe	3	44	>99
3	4-Me–Phe	24	42	>99
4	4-Br–Phe	5	38	>99
5	3,4-DiCl–Phe	22	45	>99
6	2,4-DiCl–Phe	3	49	>99
7	Phe	5	38	>99
8	4-NO2–Phe	3	49	>99
9	2-Cl–Phe	1	50	>99
10	4-F–Phe	3	47	>99
11	Homo-Phe	46	38	98
12	Tyr	1	47	>99
13	Trp	8	48	>99

[a] ees are from HPLC analysis of the last sample. *E* values which describe the enantioselectivity of kinetic resolution were not calculated because the exact measure of conversions could not be performed in this range to meet the sensitivity of Sih's equation.

As seen from the chromatograms, although the reaction rates differed from one substrate to another, the ES processes progressed in the same pattern. In all cases, the L-enantiomers which eluted slower on HPLC column decreased gradually as the reaction progressed until it was completely scavenged at the end of the reaction. The D-isomers were left on the other hand in enantiopure form. The ES process required aerobic conditions, indicating clearly the use of molecular oxygen. This also indicates a potential cascade oxidative reaction where multiple enzymes might be involved.

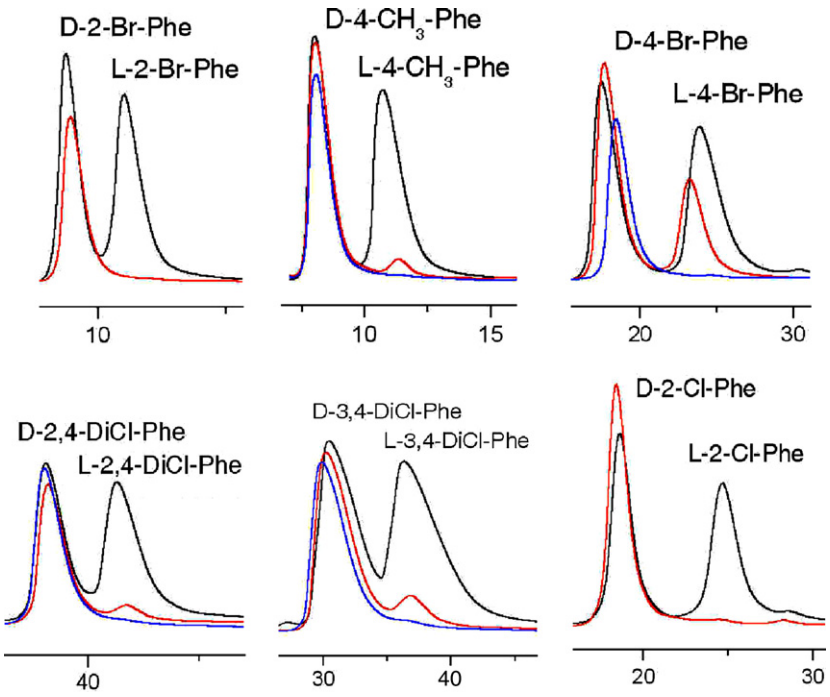


Figure 2. Selected chromatograms of the ES processes corresponding to entries 1,3,4,5,6, and 9 are shown. The black, red, and blue lines are of the samples at the beginning, the middle, and the end of the reaction, respectively. In the case of 2-Br–Phe and 2-Cl–Phe, since the reactions were completed before the second sampling (red), the blue line is absent. The retention times (min) varied from one substrate to another. The L-enantiomer which eluted slower was scavenged. The elution conditions were the same for all the experiments.

Structurally, the substrates used in this study are all derivatives of Phe with diverse substitutions except for Trp and homo-Phe, which is a 2-amino 4-phenylbutyric acid. It is possible for them to share a common pathway and enzymes. However, different substrates might be transformed by different sets of enzymes. The isolation of IAA from the experiment with *rac*-Trp clearly indicates the involvement of Trp's IAA pathway. This implies the decarboxylation by either Trp 2-monooxygenase (EC 1.13.12.3) or 2′-dioxygenase (EC 1.13.99.3) or both. At this point, it is not clear if these two enzymes were solely responsible for the scavenging process. Nevertheless, it seems that the ES recognizes the stereogenic center regardless of the structural diversity of the substrates. Even though the substitutions on the aromatic ring of the substrates might affect the reaction rate of the enzyme through changes in their substrates' solubility and velocity of penetration/binding to the enzyme's active site, they did not dramatically alter its enantioselectivity.

It remains to be seen which enzymes are responsible for the L-ES system. Nevertheless, this work uncovers a rich realm of discoveries. The practical usefulness of this work provides an invaluable target for further studies in sequencing, cloning, expression, genetic engineering, enzymology, et cetera so as to identify and investigate the potential of these biocatalysts in synthetic applications.

In summary, we demonstrated an L-ES strategy for the preparation of enantiopure D-AADs from racemic or non-racemic mixtures using *R. g.* homogenate. The ES demonstrated excellent enantioselectivity and unprecedented substrate scope. A series of D-AADs were prepared in high yield and high ee. Obviously, the work presented herewith may be better used as a cleaning method. Nevertheless, this work opens doors to many new possibilities in the area of biocatalysis. We anticipate that our results and the concept of ES could be used in many scientific fields, due to its simplicity and usefulness. Ongoing work is being done in identifying the enzymes responsible for the reaction in this ES system.

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