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A study towards efficient L-threonine aldolase-catalyzed enantio- and diastereoselective aldol reactions of glycine with substituted benzaldehydes: biocatalyst production and process development

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ABSTRACT

The development of aldol reactions of glycine with substituted benzaldehydes in the presence of recombinant L-threonine aldolases from *Escherichia coli* or *Saccharomyces cerevisiae*, which were obtained with excellent overexpression data, has been carried out. When using glycine and *ortho*-chlorobenzaldehyde, a high conversion of >95%, an enantioselectivity of >99% ee, and a diastereoselectivity with d.r.(*syn*/*anti*) = 80:20 was obtained for the resulting β-hydroxy α-amino acid in such a biotransformation. It should be noted that this enzymatic process can be conducted at an elevated substrate concentration of 250 mM.

1. Introduction

L-Threonine aldolases have the unique capability to catalyze asymmetric aldol reactions via the formation of β-hydroxy L-α-amino acids **3** without the need to protect glycine **2**, which serves as a donor molecule in this reaction (Scheme 1).[1,2] The direct use of (non-modified) glycine **2** is an advantage over typical asymmetric chemo-catalytic aldol reactions which usually require glycine in the protected form with respect to both the amino and carboxylic acid functionalities.[3] A comparison of the 'classic' multi-step approach with a chemo-catalyst on one hand and a one-step biotransformation as described above on the other hand is given in Scheme 1. In addition, L- and D-threonine aldolases catalyze the formation of the α-carbon center with excellent stereoselectivity, thus leading to the desired products with a high enantioselectivity, typically >99% ee.[1,2] A drawback of L-threonine aldolase-catalyzed aldol reactions, however, is the low diastereoselectivity at higher conversions as a general process feature, which is due to thermodynamic reasons. Often diastereoselectivities do not exceed d.r. = 65:35, and for aromatic substrates, the highest known d.r. values are in the range of d.r. = 78:22.[1,2] In spite of this limitation, however, an intensive search is currently ongoing to gain more insight into the capabilities of known and novel threonine aldolases as catalysts for the direct enantio- and diastereoselective addition of glycine to aldehydes. A major driving force for these studies is not only the aforementioned straightforward synthetic approach of using L-threonine aldolase-catalyzed reactions to access these products **3**,[1,2] but also the pharmaceutical importance of the product class of β-hydroxy L-α-amino acids.[4]

Herein, we report our preliminary studies on the process development studies of aldol reactions of glycine **2** and several substituted benzaldehydes **1** in the presence of L-threonine aldolases from *Escherichia coli* and *Saccharomyces cerevisiae*. An efficient and technically feasible fermentation process for the production of the L-threonine aldolases from *E. coli* will be presented as well as biocatalytic processes with both enzymes, which show excellent enantioselectivity and reasonable diastereoselectivity when using *ortho*-substituted benzaldehydes. Furthermore, by using a recombinant biocatalyst synthetic processes running at attractive substrate concentrations of aldehydes **1** of up to 250 mM have been realized.

2. Results and discussion

At first, we chose as a biocatalyst a recombinant L-threonine aldolase from *E. coli*, which was developed by the Yamada et al. and Wong et al.,[5] due to its known high specific activity and favorable expression data. However, there is limited data with regard to its application range for aldol reactions with aromatic benzaldehydes.

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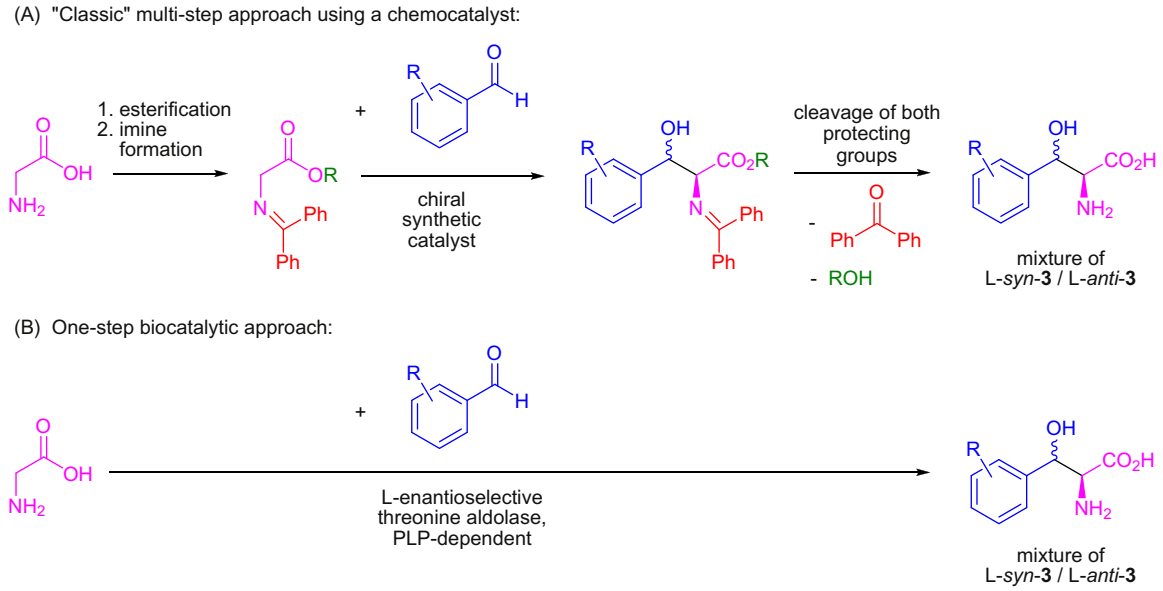


Scheme 1. Comparison of chemocatalytic versus biocatalytic approaches toward β-hydroxy L-α-amino acids L-**3**.

The isolation and cloning of the gene (*ltaE*) of this enzyme in *E. coli* BL21(DE3) led to an excellent overexpression in shaking flask experiments. This is underlined by the corresponding SDS–PAGE gel shown in Figure 1. A volumetric activity of the crude protein extract with recombinant L-TA from *E. coli* of 35 U/mL was obtained, corresponding to a specific activity of 1.4 U/mg of crude protein extract. Notably, the corresponding recombinant *E. coli* strain also has been successfully produced in high cell density fermentation on a 5 L scale, reaching a volumetric activity of 15 U/mL and a specific activity of 0.8 U/mg of crude protein extract. Although the volumetric activity was somewhat lower compared with shaking flask conditions, this recombinant L-TA from *E. coli* still represents an attractive economical and technically feasible biocatalyst.

As a second biocatalyst, we prepared the L-threonine aldolase from *S. cerevisiae* on a shaking flask scale according to a literature protocol.[6] The resulting crude protein extract showed an activity of 25 U/mL for this L-TA, corresponding to a specific activity of 1.25 U/mg of crude protein extract (Fig. 1).

Next we used the recombinant L-threonine aldolase (L-TA) from *E. coli* as a biocatalyst for the aldol reaction of glycine with different

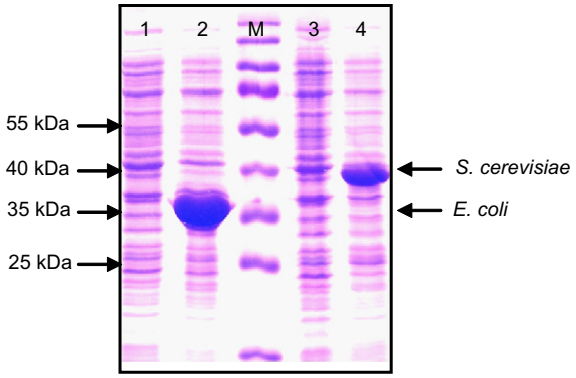
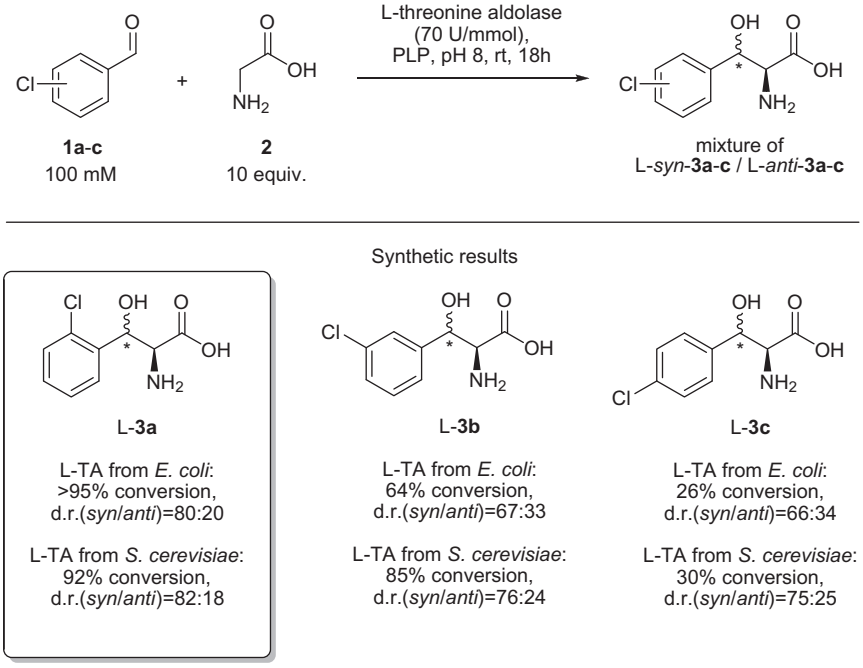


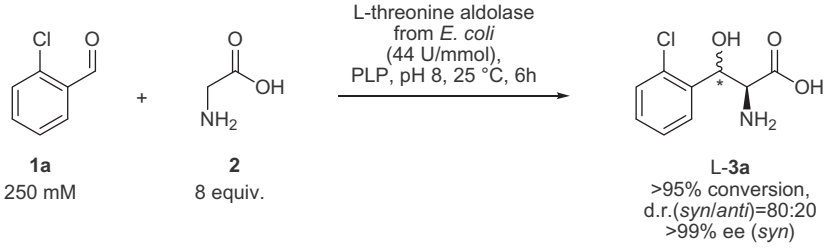
Figure 1. SDS–PAGE gel of crude protein extracts of the recombinant L-TA from *E. coli* (runs 1 and 2), recombinant L-TA from *S. cerevisiae* (runs 3 and 4), and a protein marker (run M). Runs 1 and 3 refer to the crude protein extracts obtained prior to induction, runs 2 and 4 refer to the crude protein extracts obtained after induction of the recombinant strains with IPTG.

types of aromatic aldehydes. Initially, we were in particular interested in studying the influence of the position of the substituents on the aromatic moiety on the diastereoselective course of the aldol reaction. This study was carried out with the different regioisomers of chloro-substituted benzaldehyde (Scheme 2), and the experiments were conducted at a synthetically useful substrate concentration of 100 mM of aldehydes **1a**–**c**. When using both *meta*- and *para*-chlorobenzaldehydes **1b** and **1c**, the diastereoselectivity was in the non-satisfactory range of only d.r.(*syn*/*anti*) = 67:33 and d.r.(*syn*/*anti*) = 66:34 at conversions of 64% and 26%, respectively. However, we observed a significant increase in the diastereoselectivity toward d.r.(*syn*/*anti*) = 80:20 when using the *ortho*-substituted 2-chlorobenzaldehyde **1a** as a substrate. In addition, an excellent conversion of >95% was achieved in this experiment. The same tendency and high diastereoselectivity in the case of *ortho*-chlorobenzaldehyde **1a** (2-chlorobenzaldehyde) was also found when using the recombinant L-threonine aldolase (L-TA) from *S. cerevisiae* as a biocatalyst, leading to a high conversion of 92% and are a diastereomeric ratio of d.r.(*syn*/*anti*) = 82:18 (Scheme 2).

We next focused on the process development, in particular on a further increase of the substrate concentration of aldehydes **1**. To the best of our knowledge, threonine aldolase-catalyzed aldol reactions were carried out at substrate concentrations of the aldehyde **1** of 100 mM as the highest.[3] Thus, processes running at elevated substrate concentrations of >100 mM still represent a challenge and are a prerequisite for the process to be attractive for technical purposes. During process development of the aldol reaction of glycine **2** with *ortho*-chlorobenzaldehyde **1a** catalyzed by L-TA from *E. coli*, we found that the reaction proceeds efficiently at such a desired elevated substrate concentration of 250 mM, leading to the formation of the desired β-hydroxy L-α-amino acid L-**3a** with still excellent conversion of >95% and an unchanged diastereoselectivity of d.r.(*syn*/*anti*) = 80:20 (Scheme 3). In addition, the desired major *syn*-diastereomer in the product L-**3a** was formed with an excellent enantioselectivity of >99% ee. Thus, compared to the experiment at a substrate concentration of 100 mM, the conversion as well as diastereoselectivity remained unchanged. To the best of our knowledge, this is the highest substrate concentration, which has been successfully applied so far in threonine aldolase-catalyzed diastereo- and enantioselective aldol reactions. At a

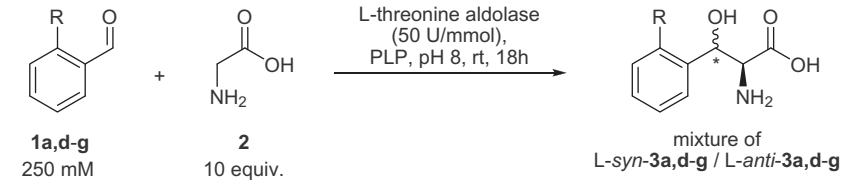


Scheme 2. Influence of the position of the chloro-substituent in **1** on the conversion and diastereoselectivity.



Scheme 3. Optimized process for the aldol reaction of *ortho*-chlorobenzaldehyde with glycine catalyzed by L-TA from *E. coli*.

Table 1
Overview about biotransformations with different types of *ortho*-substituted benzaldehydes **1a,d–g**



Entry[a]	Product	R	Type of L-TA[d]	Conversion[e] (%)	d.r.[f] (*syn*/*anti*)
1[b]	L-**3a**	Cl	L-TA from *E. coli*	81	82:18
2[c]	L-**3a**	Cl	L-TA from *S. cerevisiae*	93	80:20
3	L-**3d**	Br	L-TA from *E. coli*	51	73:27
4	L-**3e**	CH_3	L-TA from *E. coli*	24	78:22
5	L-**3f**	OCH_3	L-TA from *E. coli*	69	75:25
6	L-**3g**	OH	L-TA from *E. coli*	9	n.d.[g]

[a] The reactions were carried out in analogy to the representative biotransformation protocol given in the Section 4, however, using the reaction conditions indicated in the reaction scheme shown above; for the derivatization of the products **3** for analytical purpose 1.5 equiv of benzoylchloride were used in these experiments.
[b] In this experiment 8 equiv of glycine and a catalytic amount of the L-TA of 30 U/mmol were used; the reaction time was 6 h and the reaction was carried out at 25 °C.
[c] In this experiment, a catalytic amount of the L-TA of 70 U/mmol was used.
[d] The enzymes were used in non-purified, recombinant form as a crude extract.
[e] The conversion was determined from the crude product; for the determination of the diastereomeric ratio, see Section 4.
[f] d.r. = diastereomeric ratio; the d.r. values were determined from the crude product; for the determination of the diastereomeric ratio, see Section 4.
[g] n.d. = not determined.

lower catalytic amount of the L-TA from *E. coli* of 30 U/mmol, we saw a decrease in conversion to 81%, but still a high diastereoselectivity of d.r.(*syn*/*anti*) = 82:18 was obtained for the desired product L-**3a** (Table 1, entry 1). This type of aldol reaction also runs successfully when using an L-TA from *S. cerevisiae*, leading to L-**3a** with a high conversion of 93% and a diastereoselectivity of d.r.(*syn*/*anti*) = 80:20 (entry 2).

Having demonstrated the suitability of the L-TA from *E. coli* to catalyze aldol reactions with *ortho*-chlorobenzaldehyde **1a** with good diastereoselectivities and excellent conversions at elevated substrate concentrations, we became interested in studying other types of *ortho*-substituted benzaldehydes **1d**–**g**. Again an elevated substrate concentration of 250 mM was used in all of these experiments. The results of these preliminary studies, which were done in the presence of L-TA from *E. coli* as a catalyst, are summarized in Table 1. Using *ortho*-bromobenzaldehyde **1d** as a substrate led to the formation of the desired product L-**3d** with 51% conversion and a diastereomeric ratio of d.r.(*syn*/*anti*) = 73:27 (entry 3). Diastereoselectivities of d.r.(*syn*/*anti*) = 78:22 and d.r.(*syn*/*anti*) = 75:25 were found when using *ortho*-methylbenzaldehyde **1e** and *ortho*-methoxybenzaldehyde **1f**, respectively, as substrates (entries 4 and 5). When using *ortho*-hydroxybenzaldehyde **1g**, however, the reaction proceeds with a low conversion of 9% only (entry 6).

3. Conclusion

In conclusion, a process development of the aldol reactions of glycine with substituted benzaldehydes in the presence of a recombinant L-threonine aldolase from *E. coli* or *S. cerevisiae* as a biocatalyst has been reported in addition to an efficient fermentation process, which also turned out to be suitable on a large scale, for production of the L-threonine aldolase from *E. coli*. This recombinant biocatalyst was obtained with excellent overexpression data. When using glycine and *ortho*-chlorobenzaldehyde **1a** as a model substrate, a high yield of >95%, an excellent enantioselectivity of >99% ee, and a sufficient diastereoselectivity with d.r.(*syn*/*anti*) = 80:20 were obtained in the biotransformation. It should be noted that this process can be conducted at an elevated substrate concentration of 250 mM, which to the best of our knowledge represents the highest substrate concentration reported so far for threonine aldolase-catalyzed aldol reactions. A remaining challenge to make this route synthetically more attractive for the diastereo- and enantioselective preparation of *ortho*-substituted phenylserines is in particular the further improvement of the diastereoselectivity.

4. Experimental

4.1. Procedure for the preparation of crude protein extracts containing a recombinant L-TA from *E. coli* and *S. cerevisiae*, respectively.

The preparation of cell-free crude protein extracts, containing recombinant threonine aldolases from *E. coli* (*ltaE*) and from *Saccharomyces cerevisiae* (*GLY1*) in overexpressed form, was carried out according to the procedure described in the literature,[5a,6] respectively, followed by a subsequent sterile filtration step. A host organism *E. coli* (BL21(DE3)) was used for overexpression of both enzymes. The enzyme activities (in U) were determined according to the rate of the retro-aldol reaction with L-threonine as a substrate (Unit [U] definition: 1U corresponds to the formation of 1 μmol of acetaldehyde from L-threonine per minute at 30 °C according to the previously reported assay.[5,6]

4.2. Procedure for the L-TA-catalyzed aldol reaction of glycine with aromatic aldehydes (exemplified for the biotransformation shown in Scheme 3).

A crude protein extract with a recombinant L-TA from *E. coli* [500 μl, 44 U/(mmol of **1a**)] was added to glycine **2** (1 mmol), *ortho*-chlorobenzaldehyde **1a** (2-chlorobenzaldehyde, 125 μmol) and pyridoxal-5-phosphate (50 μM). The reaction mixture was stirred at 25 °C for 6 h. For the determination of the conversion as well as the diastereo- and enantioselectivity, a derivatization of the reaction mixture was carried out on the basis of a protocol reported in the literature[7] as follows: to the reaction mixture we added an aqueous solution of NaOH (5 M, 700 μl) and benzoylchloride (1.7 equiv, 1.69 mmol) were added and the resulting mixture was stirred for 2 h. Next, the pH was adjusted to pH 1 by the addition of HCl (5 M), and after extraction with ethyl acetate (3 × 5 mL), the combined organic layers were dried over $MgSO_4$. Removal of the solvent gave the resulting crude product, which was directly analyzed without further purification by ^{1}H NMR spectroscopy as well as chiral HPLC analysis [Daicel Chiralcel® column OJ-H, eluent: hexane/isopropanol/formic acid (95:5:0.1), flow: 0.8 mL min^{-1}, 230 nm, retention times: 63.05 min (L-*syn*-**3a**), 68.07 min (L-*anti*-**3a**)].

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