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Letter

Enantioselective reductions of ethyl 2-oxo-4-phenylbutyrate by *Saccharomyces cerevisiae* dehydrogenases

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Abstract

A set of fusion proteins consisting of glutathione *S*-transferase linked to the N-terminus of putative dehydrogenases produced by baker's yeast (*Saccharomyces cerevisiae*) was screened for the reduction of ethyl 2-oxo-4-phenylbutyrate in the presence of NADH and NADPH. Two dehydrogenases—Ypr1p and Gre2p—rapidly reduced this α-ketoester, providing the (*R*)- and (*S*)-alcohol, respectively, with high stereoselectivities. The same enzymes were over-expressed in their native forms in *Escherichia coli* and growing cells of the engineered strains could also be used to carry out the reductions without the need for exogenous cofactor. These results demonstrate the power of genomic fusion protein libraries to identify appropriate biocatalysts rapidly and expedite process development.

Keywords: Glutathione; Baker's yeast; Angiotensin converting enzyme

1. Introduction

Homochiral ethyl (*R*)-2-hydroxy-4-phenylbutyrate (**2**) is a key chiral building block for the commercial synthesis of angiotensin converting enzyme (ACE) inhibitors containing the L-homophenylalanine substructure (Scheme 1). A number of routes to (*R*)-**2** have been developed, including chemical synthesis using chiral auxiliaries [1–4], catalysts [5,6] or reactants [7,8], and chiral reducing agents [9–13]. Biocatalytic strategies including lipase-mediated kinetic resolutions [14–19] and reductions [20–26] have also been explored.

While several of the above-mentioned methods offer (*R*)-**2** in ≥98% e.e. [1,7,8,10,14,15,17,19,20,24,27], the routes involve multiple steps and in the case of kinetic resolutions, a maximal 50% yield. Since α-ketoester (**1**) is an item of commerce, we sought a direct asymmetric reduction route to (*R*)-**2** as an economical alternative (Scheme 1). Given its well-known ability to reduce a variety of ketones, α- and β-ketoesters [28–32], we suspected that one or more of the dehydrogenases produced by baker's yeast (*Saccharomyces cerevisiae*) might reduce **1** with high stereoselectivity. This notion was supported by the report by Dao et al., who showed that whole yeast cells treated with phenacyl chloride reduced a series of

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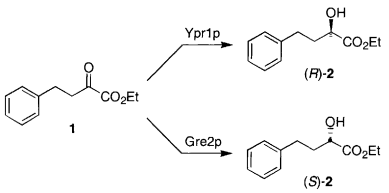

Scheme 1.

alkyl 2-oxo-4-phenylbutyrates with good to excellent stereoselectively, although a relatively high biomass: substrate ratio was required for efficient reduction (1 g yeast cells per 12 mg of **1**) [25,26]. We suspected that phenacyl chloride improved the enantiomeric purities by interfering with one or more (*S*)-selective dehydrogenases, allowing the remaining (*R*)-selective enzyme(s) to dominate. If this were the case, a more direct solution would be to identify the key *S. cerevisiae* dehydrogenase(s) participating in the whole-cell reduction, then express the individual enzyme(s) in a heterologous host and thereby avoid competition by dehydrogenases with opposing stereoselectivities. Clearly, this strategy requires a rapid method to identify the most desirable dehydrogenase(s) from the yeast genome in order to be successful.

Martzen et al. recently described a fusion protein strategy that allows one to survey the entire catalytic repertoire of the yeast genome [33]. Each of the 6144 open reading reading frames (ORFs) was fused at its N-terminus to *Schistosoma japonicum* glutathione *S*-transferase in a common *S. cerevisiae* expression vector. Individual yeast proteins could therefore be purified in a single step by affinity chromatography, then assayed for catalytic activity. This strategy has obvious applications in biocatalysis, particularly asymmetric carbonyl reductions by yeast enzymes. We used the asymmetric reduction of α-ketoester (**1**) to both (*R*)- and (*S*)-**2** as a test case for this strategy.

2. Results

While the original GST-fusion protein library covered all 6144 ORFs in the *S. cerevisiae* genome, the complete collection is unnecessary for identifying dehydrogenases with desirable stereoselectivities. In an earlier analysis of the yeast genome, we identified a group of 49 ORFs likely to encompass nearly all proteins capable of reducing carbonyl compounds of synthetic interest [34]. We further narrowed the scope of potentially useful dehydrogenases by eliminating those known to have narrow substrate specificities, leaving 24 candidate ORFs. GST-fusion proteins from these 24 strains were therefore expressed in *S. cerevisiae* and purified as reported earlier [33]. Each purified fusion protein was screened separately for the ability to reduce **1** in the presence of NADH and NADPH. Unfortunately, it was difficult to obtain reproducible results by this approach. The problem was traced to variable protein yields from the yeast over-expression systems coupled with our need to prepare macroscopic quantities of reaction products. We, therefore, expressed the GST-fusion proteins in *E. coli* under control of the IPTG-inducible T7 system [35], which provided much greater quantities of the purified fusion proteins. Plasmids were constructed by standard techniques and used to transform *E. coli* BL21(DE3). Based on our earlier results, two of these—Ypr1p and Gre2p—were selected for further study.

Both the purified Ypr1p and Gre2p fusion proteins were used to reduce **1** in the presence of NADPH, which was supplied by glucose-6-phosphate and glucose-6-phosphate dehydrogenase.

2.1. Preparation of GST-fusion proteins

A 1 ml culture of the appropriate over-expression strain grown overnight in LB medium containing 25 μg/ml kanamycin was diluted into 100 ml of the same medium. The culture was shaken at 37 °C until the optical density at 600 nm reached 0.50, then IPTG was added to a final concentration of 0.10 mM and the culture was shaken overnight at room temperature. Cells were collected by centrifugation, washed twice with cold water, re-suspended in 5 ml of 0.10 M KP_i, pH = 7.0 containing 20% glycerol and lysed by sonication (3 × 30 s). Debris was removed by centrifugation (12,000 × *g* for 10 min at 4 °C), then the supernatant was mixed with equal volume of 50 mM Tris–Cl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, pH = 7.5 (purification buffer) and 0.50 ml of glutathione–agarose resin (Clontech, #8912-1). After mixing gently for 3 h at 4 °C, the resin was washed with two 1 ml portions of purification buffer supplemented with 500 mM NaCl. The

GST-fusion protein was eluted by adding 2 ml of purification buffer supplemented with 20 mM NaOH and 25 mM glutathione, then mixing gently at 4 °C for ≥40 min. The resin was discarded and the supernatant was dialyzed against 20 mM Tris–Cl, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 55 mM NaCl, 50% glycerol. Purified proteins were stored in aliquots at −80 °C.

2.2. Reduction of **1** by GST-fusion proteins

Reaction mixtures contained $NADP^+$ (0.20 μmol, 0.15 mg), glucose-6-phosphate (14 μmol, 4.25 mg), baker's yeast glucose-6-phosphate dehydrogenase (1.5 U, 5 μg), hydroxypropyl-β-cyclodextrin (ca. 5 μmol, 7.5 mg), **1** (5.0 μmol, 0.95 μl) and 200 μl of purified GST-fusion protein in a final volume of 1.0 ml in 100 mM KP_i, pH = 7.0. Reaction mixtures were incubated at 30 °C. A control reaction in which glutathione *S*-transferase was substituted for the GST-fusion protein showed no reduction of **1**, as expected.

2.3. Analytical methods

Time courses for reductions of **1** were determined by mixing equal volumes of reaction mixture and hexanes by vortexing, then analyzing an aliquot of the organic phase by normal-phase HPLC (4.6 mm × 250 mm column) using 0.5% *iso*-propanol in hexanes (1 ml/min) as the mobile phase and UV detection at 254 nm. The absolute configurations and optical purities of **2** produced by enzymatic reductions were determined by chiral-phase GC (25 m Chirasil-Dex CB column, oven program 100–180 °C at 1 °C/min with initial and final times of 2 and 5 min, respectively).

2.4. Stereoselectivities of reductions

Neither enzyme showed significant activity in the presence of NADH. Under these conditions, GST-Ypr1p afforded (*R*)-**2** in 97% e.e. and GST-Gre2p provided (*S*)-**2** in 90% e.e. The absolute configurations were assigned by chiral-phase GC analysis using authentic (*R*)-**2** as a standard under conditions that gave near-baseline resolution of the enantiomers (Fig. 1).

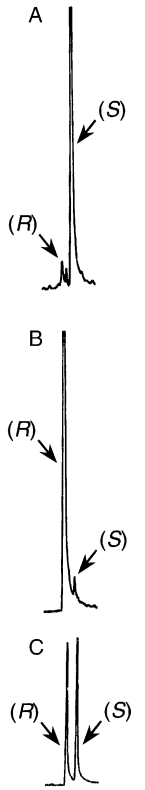

Fig. 1. Chiral-phase GC analysis of α-ketoester reduction products. Samples were analyzed on a 20 m Chirasil-Dex CB column as described in Section 2.3. (A) (*S*)-**2** prepared by GST-Gre2p reduction of **1**; (B) (*R*)-**2** prepared by GST-Ypr1p reduction of **1**; (C) Racemic **2** prepared by $NaBH_4$ reduction of **1**.

Once appropriate *S. cerevisiae* dehydrogenases had been identified, we used whole cells of *E. coli* strains that over-expressed the enzymes in their native forms to carry out preparative-scale reactions. The *YPR1* and *GRE2* genes were placed downstream from T7 promoters in these over-expression plasmids, which were used to transform *E. coli* BL21(DE3). Bioconversions were carried out with growing cells of the engineered strains and α-ketoester (**1**) was added to a final concentration of 10 mM shortly after dehydrogenase expression had been induced by adding IPTG. One equivalent of hydroxypropyl-β-cyclodextrin was also included to solubilize the hydrophobic ketone [36]. After all of the ketone substrate had been consumed, the alcohol was isolated by solvent extraction and purified by flash chromatography.

2.5. Reduction of **1** by whole *E. coli* cells

Preparative-scale reactions with growing cells were carried out by diluting an overnight culture of the appropriate strain into 100 ml of LB medium containing 25 μg/ml kanamycin, then shaking at 30 °C until the optical density at 600 nm reached 0.30. IPTG was added to a final concentration of 0.10 mM and the culture was shaken at room temperature for an additional 30 min. Neat **1** (1.0 mmol, 189 μl) was added along with hydroxypropyl-β-cyclodextrin (1.0 mmol, 1.5 g) and the culture was shaken at room temperature and sampled periodically for HPLC analysis. After all of the α-ketoester had been consumed, the entire mixture was extracted with hexanes (4 × 100 ml). The combined organics were dried with $MgSO_4$ and concentrated by rotary evaporator. The alcohol was purified by flash chromatography on a 1 cm × 18 cm silica column using 1:1 ether hexanes as the eluant. Activated charcoal was used to remove colored impurities remaining after chromatographic purification. All yields reported were determined from quantities of isolated and purified products.

Using these conditions, an *E. coli* strain overexpressing Ypr1p [1] afforded (*R*)-**2** in 46% yield and with 87% e.e. The same reaction was also successfully carried out on a 10 mmol scale, although in slightly lower yield (32%). The (*S*)-alcohol was prepared in 58% yield and 91% e.e. by using an *E. coli* strain that overproduced Gre2p. [2]

3. Discussion

Our success in identifying yeast enzymes that reduce **1** to (*R*)- or (*S*)-**2** with high stereoselectivities underscores the power of genome sequence data to solve problems in biocatalysis quickly. While an earlier study had demonstrated that *S. cerevisiae* produced at least one enzyme capable of reducing **1** [25,26], it could not identify the participant(s). By contrast, screening a collection of fusion proteins that covers the entire genome of an organism reveals all of the enzymes with a desired catalytic activity. Some of these might be missed by screening whole cells of the native organism, either because of low expression levels or because other, competing catalysts, dominate the reaction. Purified fusion proteins are very convenient for catalyst identification, however, they are poorly-suited to preparative-scale reactions since any necessary cofactors must be supplied exogenously. On the other hand, whole cells of engineered *E. coli* expressing a single heterologous protein can be used for redox biotransformations under growing [37–40] or non-growing conditions [41–43]. We have, therefore, developed a collection of *E. coli* over-expression strains that over-express the same yeast proteins as in our GST-fusion protein collection, thereby facilitating rapid scale-up as soon as the most appropriate enzyme has been identified by small-scale screening.

The process conditions for preparative-scale reduction of **1** by whole *E. coli* cells were not optimized in this study. While use of cyclodextrin was not mandatory, we found that it improved reaction rates and product recovery. Solid polymeric resins might be a more economical substitute for large-scale reductions [44,45]. The small, but reproducible, decrease in enantioselectivity when **1** was reduced by engineered *E. coli* strains rather than by purified yeast proteins likely reflects a small contribution by one or more host dehydrogenases. Since the *E. coli* genome sequence has been completely sequenced, it should be a relatively simple matter to use a similar fusion protein library strategy to identify the *E. coli* enzyme(s) responsible for reducing **1** and eliminate their contribution by knockout mutagenesis.

[1] *E. coli* BL21(DE3) harboring pAA2.
[2] *E. coli* BL21(DE3) harboring pAA3.

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