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Comparison of Three Enoate Reductases and their Potential Use for Biotransformations

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Abstract: Enoate reductases (ERs) selectively reduce carbon-carbon double bonds in α,β-unsaturated carbonyl compounds and thus can be employed to prepare enantiomerically pure aldehydes, ketones, and esters. Most known ERs, most notably Old Yellow Enzyme (OYE), are biochemically very well characterized. Some ERs have only been used in whole-cell systems, with endogenous ketoreductases often interfering with the ER activity. Not many ERs are biocatalytically characterized as to specificity and stability. Here, we cloned the genes and expressed three non-related ERs, two of them novel, in *E. coli*: XenA from *Pseudomonas putida*, KYE1 from *Kluyveromyces lactis*, and Yers-ER from *Yersinia bercovieri*. All three proteins showed broad ER specificity and broad temperature and pH optima but different specificity patterns. All three proteins prefer NADPH as cofactor over NADH and are stable up to 40 °C. By coupling Yers-ER with glucose dehydrogenase (GDH) to recycle NADP(H), conversion of $>99\,\%$ within one hour was obtained for the reduction of 2-cyclohexenone. Upon lowering the loadings of Yers-ER and GDH, we discovered rapid deactivation of either enzyme, especially of the thermostable GDH. We found that the presence of enone substrate, rather than oxygen or elevated temperature, is responsible for deactivation. In summary, we successfully demonstrate the wide specificity of enoate reductases for a range of α,β-unsaturated carbonyl compounds as well as coupling to glucose dehydrogenase for recycling of NAD(P)(H); however, the stability limitations we found need to be overcome to envision large-scale use of ERs in synthesis.

Keywords: biotransformations; enoate reductases; enzymes; Old Yellow Enzyme; redox chemistry

Introduction

Biocatalysts are increasingly used during the synthesis of enantiomerically pure compounds of industrial interest (pharmaceuticals, food, and crop protection), due to their exquisite regioselectivity and stereoselectivity. These enable them to perform difficult chemical synthesis, while circumventing the use of complicated traditional synthetic routes.[1–4] For reductive reactions, ketoreductases are already an established part of the synthetic toolbox. One of the emerging reductive chemistries is the asymmetric reduction of carbon double bonds. Enoate reductases (ERs) perform such reactions and have been recognized for decades, but the stereoselective application was only recently published.[5–7] Enoate reductase from *Saccharomyces carlsbergensis*, also known as 'Old Yellow Enzyme' (OYE), was the first isolated flavin-containing enzyme in 1933.[8] Despite the fact that OYE has been characterized thoroughly,[9] its physiological role has only recently begun to emerge.[10–12]

The OYE family has grown in recent years and a variety of different enzymes have been characterized, including different yeast OYEs,[13,14] XenA/XenB reductase,[15] bacterial morphine reductase,[16] bacterial nitrate ester reductases (pentaerythritol tetranitrate reductase),[17] YqjM from *Bacillus subtilis*,[18] plant oxophytodienoic acid reductases,[19,20] and an enzyme involved in prostaglandin synthesis in *Trypanosoma cruzi*.[21] Despite this variety in substrate specificity, the family shares a common $\alpha_8\beta_8$ barrel (TIM barrel) fold with the flavin mononucleotide (FMN) binding within the barrel near the carboxyl-terminal ends of the β-strands, however, there are significant variations in the capping subdomain.[22] Members of the OYE family also share several common functional characteristics. Although clear preferences are observed for every enzyme, they are generally able to reduce nitro

esters, nitro aromatic substrates, and simple and complex aldehydes and ketones.[23] Kinetics proceed by a ping-pong mechanism, i.e., consisting of an oxidative and reductive half reaction.[13,14,17] The proposed mechanism for OYE-catalyzed asymmetric reduction proceeds according to an *anti*-addition hydrogenation manner (*trans*-hydrogenation) and is consistent with experimental results.[27–29]

The asymmetric reduction of α,β-unsaturated carbonyl compounds creates up to two new chiral centers and is an interesting approach towards enantiomerically pure compounds. Despite the success of the homogeneous catalysis of enantioselective alkene reduction, high stereoselectivity nearly always depends on olefin proximity to highly polar functional groups such as amides, acids, and alcohols. Attempts to generalize these procedures to alkenes conjugated with less polar groups such as aldehydes or ketones have been much less successful,[6] although some exceptions have been reported.[24–26] Therefore, ERs are interesting for the hydrogenation of unsaturated aldehyde or ketone substrates, rounding off the chemist's toolbox. The stereoselective application of these enzymes has been reported recently.[6]

Currently, the use of ERs is especially limited by two main factors: i) the full biocatalytic potential of biochemically characterized ERs has not been explored, especially with respect to their specificity on enones beyond 2-cyclohexenone, and ii) until now, whole cells were generally used and these are limited by the presence of endogenous ketoreductases in the host cells, which also employ NAD(P)H to reduce the carbonyl function.

In the first part of this paper, we address the first limitation by cloning three ERs from very different sources and characterizing their substrate spectra, pH and temperature profiles. We overcome the second limitation by using an isolated ER in combination with an isolated cofactor regeneration system, here glucose dehydrogenase (GDH). For straightforward purification, we cloned a polyhistidine tag at the N-terminus of each enzyme. In summary, all these factors and findings are important for successful and economically feasible biotransformations in the future.

Results

Cloning, Overexpression, and Purification

The gene of xenobiotic reductase A (XenA) from *Pseudomonas putida*, which shows ER activity toward 2-cyclohexen-1-one,[15] was kindly provided by Brian Fox (University of Wisconsin, Madison, WI). Additionally, ER genes were isolated from the genomic DNA of *Kluyveromyces lactis* ATCC 8585 and *Yersinia bercovieri* ATCC 43970. The gene products were cloned into pET28a (+) vector (Novagen, San Diego, CA) at the following restriction sites: *Nde* I and *Xho* I for *Kluyveromyces lactis* (KYE1), and *Nde* I and *Hind* III for both XenA and *Yersinia bercovieri* (Yers-ER). These constructs enabled the expression of mature ER with an N-terminal polyhistidine tag for convenient purification.

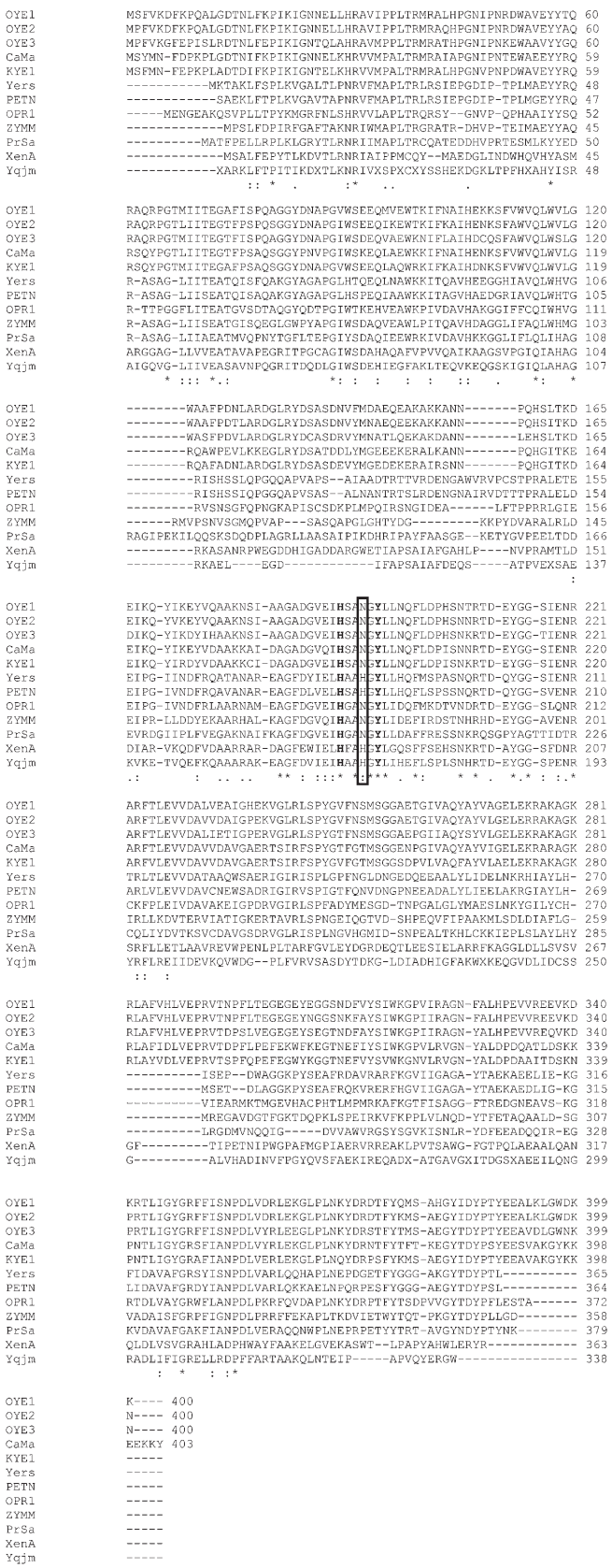
Sequence analysis of Yers-ER and XenA showed the same nucleotide sequence as derived from the NCBI databank (accession files ZP 00823209 and AAF02538), while KYE1 (P40952) revealed one difference at position 768 (t768g), leading to an amino acid change from valine to glycine (V257G).

XenA is a xenobiotic reductase from the Gram-negative, aerobic bacterium *Pseudomonas putida*. KYE1 was isolated from yeast and shares a high amino acid identity with OYE1 from the yeast *Saccharomyces cerevisiae* (71% aa identity, 84% aa similarity). KYE1 had already been mentioned in the literature,[31] but has never been characterized for its ER function. Finally, Yers-ER is from the Gram-negative, aerobic bacterium *Yersinia bercovieri* and shares high identity with the pentaerythritol tetranitrate reductase from *Enterobacter cloacae* (76% aa identity, 86% aa similarity).[17] As already expected from the wide variety of possible functions and hosts, the amino acid identities and similarities between the three cloned ER are low: KYE1 to XenA (19.0 and 35.5%, respectively); KYE1 to Yers-ER (37.0 and 47.1%, respectively); and XenA to Yers-ER (24.3 and 39.9%, respectively). The sequence alignment reveals only a few conserved residues, among them the active site residues histidine and tyrosine (Table 1 and Figure 1). Yers-ER (365 aa) and XenA (363 aa) share a similar size, in contrast to KYE1 (398 aa), but all share similar isoelectric points (calculated pI = 6.06, 6.01, and 5.99, respectively). The his-tagged ERs

Table 1. Amino acid sequence identities and similarities between the three enoate reductases with some other published proteins.[a]

	KYE1	Yers-ER	XenA
CaMa	75/85	38/54	27/45
KYE1	-	36/53	28/48
OYE1	70/82	39/54	27/44
OYE2	70/81	38/54	29/44
OYE3	67/78	39/54	28/45
Yers-ER	36/53	-	31/47
PETN	38/51	76/85	27/41
ZYMM	32/49	41/57	32/48
XenA	28/48	31/47	-
Yqjm	30/48	32/48	39/52
OPR1	36/51	44/58	28/43
PrSa	29/47	38/54	30/45

[a] Amino acid identity/similarity levels (in %); for description of ER source organisms, see caption to Figure 1.



share a slightly lower pI (Yers-ER: 5.80, KYE1: 5.61, XenA: 5.76).

The heterologous expression in *E. coli* BL21(DE3) (Novagen) was induced at an OD_{600} of 0.5 with 0.1 mM IPTG and incubated overnight at 37 °C. All three ERs showed activity in the supernatant. The his-tagged enzymes were purified according to the standard Ni^{2+}-NTA bead protocol from Qiagen (Valencia, CA), and purification was monitored by SDS-PAGE (Figure 2). The eluate did not contain any other bands, even while overloading the gel. The activity of a non-tagged XenA (in lysate) toward 2-cyclohexenone was measured, and these results indicated that his-tagging is not detrimental to ER activity.

Characterization

The three cloned ERs are derived from a broad spectrum of possible enzyme functions. XenA is a xenobiotic reductase, while KYE1 is a homologue of OYE1 and Yers-ER is a homologue of pentaerythritol tetranitrate reductase from *Enterobacter cloacae*. Nevertheless, the comparison of the substrate specificity on different α,β-unsaturated carbonyl compounds reveals that all three enzymes showed broad substrate specificity and were active on almost all tested substrates (Table 2). For KYE1, the specific activities are generally low ($\leq$2.5 U/mg) and did not vary much between the different substrates. The specific activity of XenA varied the most: (*R*)-carvone and 3-methyl-2-butenal were not substrates for XenA, but-3-en-2-one and maleimide yielded the highest activity levels mea-

Figure 1. Amino acid alignment (with ClustalW) of some published ERs with the three species characterized in this paper. The bold amino acids are histidine (important for substrate/ligand binding) and tyrosine (important for proton donation during the reduction of α,β-unsaturated carbonyl compounds). If the enzyme possesses two histidines in the active site (bold amino acids solid) for substrate binding, it can reduce trinitrotoluene (TNT) to the H-Meisenheimer complex, whereas the enzymes with only one histidine cannot.[32] Aligned from top to bottom are: CaMa, old yellow enzyme from *Candida macedoniensis* (BAD24850); KYE1 old yellow enzyme from *Kluyveromyces lactis* (P40952); OYE1, old yellow enzyme from *Saccharomyces carlbergensis* (Q02899); OYE2, old yellow enzyme from *Saccharomyces cerevisiae* (NP 012049); OYE3, old yellow enzyme from *Saccharomyces cerevisiae* (CAA97878); Yers-ER, ER from *Yersinia bercovieri* (ZP 00823209); PETN, pentaerythritol tetranitrate reductase from *Enterobacter cloacae* (2 ABA); ZYMM, ER from *Zymomonas mobilis* (Q5NLA1); XenA, xenobiotic reductase A from *Pseudomonas putida* (AAF02538); Xqjm from *Bacillus subtilis* (1Z48); PrSa from *Trypanosoma cruzi* (BAC24024); OPR1, 12-oxophytodienoate reductase from *Arabidopsis thaliana* (AAC78440).

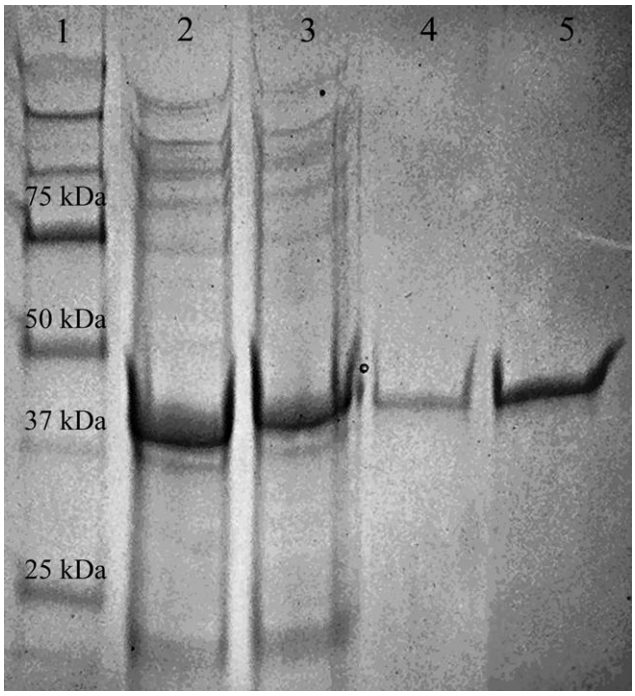


Figure 2. SDS-PAGE of each step in the Ni^{2+}-NTA purification of the ER from *Yersinia bercovieri*. The standard protocol was used from QIAGEN with Ni2+-NTA beads. Lane 1: ladder; lane 2: cell lysate, lane 3: flow through from the binding solution; lane 4: flow through from the wash buffer; lane 5: eluted fraction.

sured in our study, 22.69 and 25.82 U/mg, respectively. The Yers-ER was most consistent, showing the highest specific activity levels on eight of the twelve substrates.

The substrates can be divided into four groups: i) substrates which reacted equally well for all three enzymes, 2-cyclohexen-1-one, *trans,trans*-2,4-hexadienal, citral, and cinnamaldehyde; ii) substrates which did not react with XenA: (*R*)-carvone and 3-methyl-2-butenal; iii) substrates which show strong preference for Yers-ER, ketoisophorone and maleic anhydride, and, finally iv) substrates which show strong preference for both XenA and Yers-ER, maleimide, *N*-ethylmaleimide, but-3-en-2-one, and ethyl 3-(tetrahydrofuryl)-propanoate.

Importantly, the broad spectrum of native functions of the three proteins investigated was reflected in the broad spectrum of substrate preference and range of specific activity.

We also investigated whether reaction products of α,β-enone reduction further reacted to undesirable by-products. We chose products with two different structural features. The first group did not contain any further unsaturated double bonds (succinimide and butanone) and, as expected, showed no further reductive activity. On the other hand, KYE1 and Yers-ER still showed reductive activity on two substrates which still contain double bonds (phenylpropanal and dihydrocarvone), though in lower amounts compared to the α,β-unsaturated carbonyl substrates. Surprisingly, XenA oxidized cofactor in the presence of dihydrocarvone, but did not react with the enoate (*R*)-carvone. The tendency of these products toward further reductions is under investigation. This finding highlights the importance of controls for the biocatalytic use of ERs.

Cofactor Specificity

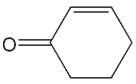
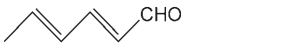
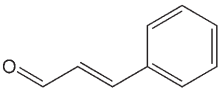
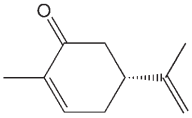
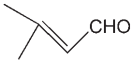
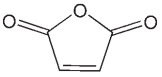
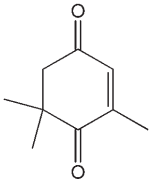
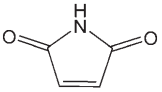
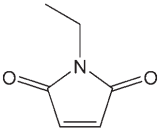
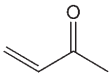
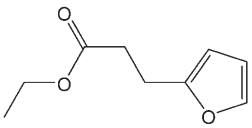
After testing substrate specificity, the cofactor preference of the three ERs was checked with four different enone substrates (Table 3). NADPH was always the preferred cofactor, and the enzymes showed very little or no activity with NADH. Interestingly, the choice of enone substrate seems to influence the NADPH/NADH specificity ratio. While NADPH did not react at all in the presence of 2-cyclohexen-1-one and rarely in presence of maleimide, all three ERs showed activity with NADH in presence of methyl vinyl ketone and ethyl-3-(2-furyl)propanoate. This phenomenon is all the more interesting, as ER is known to react according to a ping-pong mechanism,[31] i.e., the reductive (substrate) and oxidative (FMN by NADPH) half-reactions occur independently, thus, the different enone substrates should not influence cofactor specificity.

Enzyme Stability

The lack of stability of enzymes against heat and organic solvents can hamper their economical application. We investigated the thermal tolerance of all three ERs by taking samples after various incubation times at three temperatures (30, 37, and 45 °C) and measuring residual activity at 25 °C. We always found deactivation to fit a first-order rate law. Although none of the three proteins is particularly thermostable, Yers-ER at 37 °C was 25-fold and 7-fold more stable than XenA and KYE1, respectively (Table 4). Yers-ER at 45 °C in degassed solution deactivates with a half-life of 129 min, compared with 117 min in air-saturated solution, which demonstrates that oxygen has no influence on stability. The half-life of XenA and KYE1 at that temperature was less than 5 min. Since sufficient stability is an important factor for biocatalysis, we chose the Yers-ER for further characterization.

The activity-temperature profile between 10 °C and 55 °C is depicted in Figure 3. Since NADPH degrades at a significant rate at $\geq$ 45 °C, each sample beyond 45 °C was prepared as a separate tube of assay solution and NADPH was added last before starting the

Table 2. The specific activity of the three enoate reductases on different α,β-unsaturated carbonyl compounds.[a]

Molecule	Structure	Specific activity [U/mg] KYE1	XenA	Yers-ER
2-cyclohexen-1-one		1.54	2.74	4.22
trans,trans-2,4-hexadienal[&]		1.51	3.18	2.19
citral*		0.67	1.75	1.97
cinnamaldehyde[&]		2.30	1.56	2.91
(*R*)-carvone*		0.73	nd	2.54
3-methyl-2-butenal		0.45	-0.43	0.39
maleic anhydride		0.86	1.91	5.20
ketoisophorone		1.33	1.84	10.11
maleimide		2.04	25.82	18.88
N-ethylmaleimide[&]		2.46	14.87	15.81
but-3-en-2-one (methyl vinyl ketone)		2.14	22.69	14.85
ethyl 3-(tetrahydrofuran)propanoate [ethyl-3-(2-furyl)propanoate]*		2.36	9.96	12.87

[a] *Assay conditions:* 200 mM sodium phosphate, pH 7.5, 10 mM substrate, 0.2 mM NADPH, 25 °C. Extinction coefficient of NADPH of 6.22 $mM^{-1}cm^{-1}$ at 340 nm or 3.51 $mM^{-1}cm^{-1}$ at 365 nm (substrates are marked with [&]) was used in calculation of specific acitivity.[33] Compounds with an asterisk did not appear completely soluble at 10 mM, so the listed specific activities may correspond to the solubility limit. Alternate names for some of these compounds are shown in parentheses. nd = not detected.

reaction. Thermal deactivation dominates over activation at temperatures beyond 55 °C, the highest temperature investigated. A maximum specific activity of 6.15 U/mg was obtained for 2-cyclohexen-1-one at 45 °C, almost 2.5-fold higher than at room temperature (20 °C). The activation energy was calculated from an Arrhenius plot of specific activity over $1/T$ from data in the range of 10 to 45 °C; we obtained an activation energy E_a of 25.11 kJ mol^{-1} ($r^2 = 0.9937$; for the range of 10–40 °C: 24.06 kJ mol^{-1}, $r^2 = 0.9964$).

Activity-pH Profile

The activity-pH profile was measured for the purified Yers-ER (Figure 4). The enzyme showed a broad pH

Table 3. Cofactor preference for KYE1, XenA, and Yers-ER.[a]

	Substrate	Specific Activity [U/mg] NADPH	Specific Activity [U/mg] NADH
Kluyveromyces	2-cyclohexen-1-one	1.54	nd
	maleimide	2.04	nd
	methyl vinyl ketone	2.14	0.30
	ethyl 3-(2-furyl)-propanoate	2.36	0.56
XenA	2-cyclohexen-1-one	2.74	nd
	maleimide	25.82	1.50
	methyl vinyl ketone	22.69	1.43
	ethyl 3-(2-furyl)-propanoate	9.96	1.08
Yersinia	2-cyclohexen-1-one	4.22	nd
	maleimide	18.88	nd
	methyl vinyl ketone	14.85	0.69
	ethyl 3-(2-furyl)-propanoate	12.87	0.56

[a] *Assay conditions:* 200 mM phosphate, pH 7.5, 10 mM substrate, 0.2 mM cofactor, 25 °C. Detection limit: 0.1 U/mg. nd = not detected.

Table 4. Half-lives of enoate reductase (Yers-ER) and glucose dehydrogenase (GDH-103).

Enzyme	Temperature [°C]	Half-life [min]
XenA	30	600
XenA	37	99
XenA	45	<5
KYE1	30	1146
KYE1	37	210
KYE1	45	<5
Yers-ER	30	>2160
Yers-ER	37	1488
Yers-ER	45	117
Yers-ER[a]	30	78
Yers-ER[b]	30	2880
GDH-103[c]	30	~7800
GDH-103[a]	30	1.6
GDH-103[b]	30	564

[a] In the presence of 25 g L^{-1} of 2-cyclohexen-1-one.
[b] In the presence of 25 g L^{-1} of 2-cyclohexanone.
[c] Literature value from Pollard et al.[37]

optimum between pH 6.0–8.5 with a maximum specific activity (3.46 U/mg) around pH 7.0. The activity dropped off sharply between pH 5–6 and pH 8.5–9.5, with inflection points of the activity-pH profile

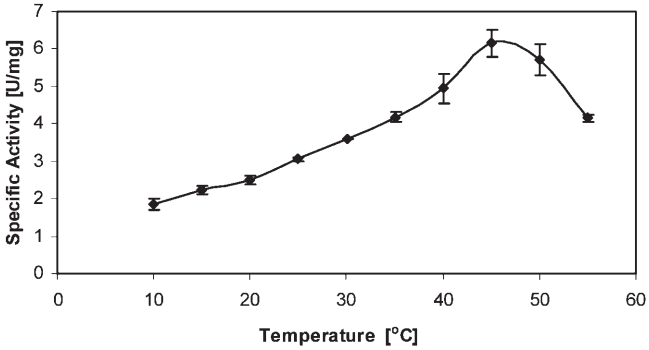


Figure 3. Activity-temperature profile of Yers-ER. The samples were heated immediately before the assay and contained extra NADPH (250 μM) to ensure saturation with reduced enzyme at the start of the run. The buffer contained 200 mM sodium phosphate buffer at pH 7.5, 5 mM 2-cyclohexen-1-one and 200 μM NADPH.

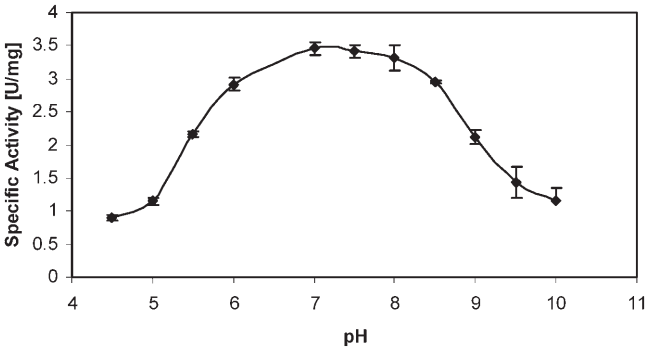


Figure 4. Activity-pH profile of Yers-ER. The measurements were conducted in buffered solution (see Experimental Section) at 25 °C with 5 mM 2-cyclohexen-1-one and 200 μM NADPH.

around pH 5.5 and 8.8, possibly corresponding to the pK_a values of the catalytic histidine182 (pH 6.04) but far from the typical pK_a value of tyrosines of 10.13 (tyrosine 188 is crucial for binding).

Inhibition Patterns

Substrate and product inhibition can significantly reduce the biocatalytic usefulness of enzymes. Therefore, the specific activity of Yers-ER was measured at 25 °C in 200 mM phosphate (pH 7.5) with 200 μM NADPH and various concentrations of 2-cyclohexenone (substrate) and 2-cyclohexanone (product). For the substrate inhibition study, the concentration of 2-cyclohexenone was varied from 5–20 mM and no activity loss was detected. To test for product inhibition, the concentration of 2-cyclohexanone was varied from 0–100 mM in a reaction solution containing 10 mM substrate. No decrease in activity was observed and

product inhibition was ruled out for the tested conditions.

Finally, kinetic data were measured for the Yers-ER at 25 °C in 200 mM phosphate buffer (pH 7.5) and varying 2-cyclohexenone (substrate) concentrations. The assay solution contained 200 μM NADPH, a concentration at which the enzyme was very close to saturation with respect to NADPH (Figure 5). At

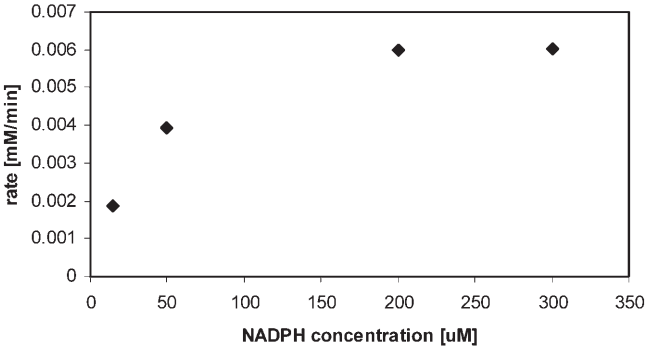


Figure 5. Determining the saturation concentration of NADPH for Yers-ER. *Assay conditions:* 25 °C, pH 7.5, 10 mM 2-cyclohexen-1-one.

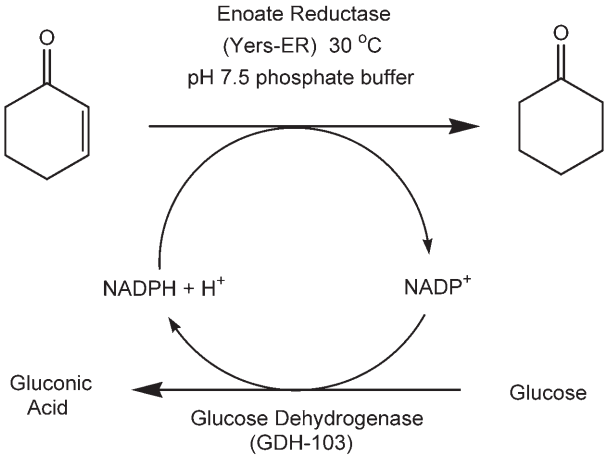
10 mM substrate concentration, the K_M value for NADPH is calculated to be 40 ± 5 μM. The catalytic constant k_{cat} ($118.2 \pm 0.6\ min^{-1}$), a Michaelis constant K_M (1.24 ± 0.01 mM) and specificity ratio k_{cat}/K_M ($95.32 \pm 0.71\ mM^{-1} min^{-1}$) values were determined.

Coupling of the Enoate Reductase with the Cofactor Recycling System

The coupling of Yers-ER with GDH was performed in the batch mode (Scheme 1).

All reactions were run at 30 °C in 200 mM phosphate buffer (pH 7.5) containing 25 g/L (260 mM) of the substrate 2-cyclohexen-1-one, 53.8 g/L (299 mM, 15% molar excess) of glucose, 1 g/L (1.2 mM) of cofactor $NADP^+$ and both enzymes at varying ratios of kU/L. Four reactions were run at these conditions at 8:16, 8:8, 4:8, and 2:4 kU/L of Yers-ER to GDH-103, respectively (Figure 6).

It is evident that enzyme loading had a significant effect on reaction rate. At 8:16 kU/L of Yers-ER to GDH-103, the reaction reached >99% conversion within an hour (217 turnovers), while at a loading of 8:8 kU/L the reaction required 6 h for the same degree of conversion (>99%) (Figure 6). This difference in reaction rate might be explained by the increased supply of reduced cofactor achieved at a higher loading of GDH-103. At more diluted levels of Yers-ER and GDH-103, 4:8 kU/L and 2:4 kU/L,



Scheme 1. Enzyme-coupled cofactor regeneration system with enoate reductase from *Yersinia bercovieri* and glucose dehydrogenase (GDH-103).

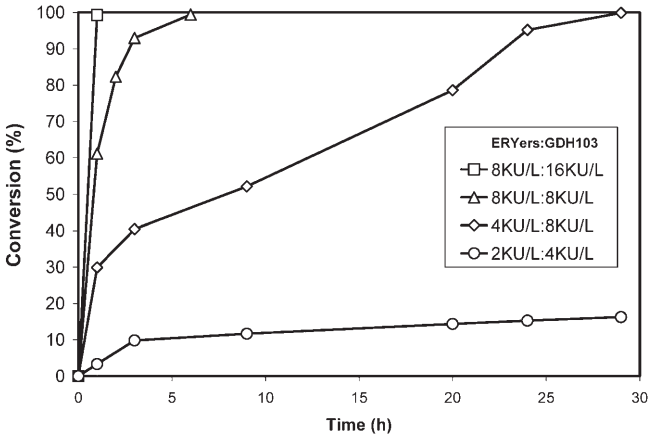


Figure 6. Enzymatic oxidation of 25 g/L of 2-cyclohexen-1-one to 2-cyclohexanone at 30 °C in 200 mM phosphate pH 7.5.

>99% and >16% conversion were measured after 29 h, respectively. Such a dramatic difference in reaction rate between the 8:16 kU/L and the 4:8 kU/L enzyme loadings for Yers-ER and GDH-103 suggests significant enzyme deactivation at the operating conditions. To test this hypothesis, the residual activities of Yers-ER and GDH-103 were monitored separately in batch experiments in the presence of reactant or product at the operating conditions (30 °C, 200 mM phosphate pH 7.5 and 25 g/L of 2-cyclohexen-1-one or 2-cyclohexanone) in the absence of cofactor. The residual activity was measured by monitoring NADP(H) depletion (Yers-ER) or formation (GDH-103). Assuming first-order deactivation (corroborated above for ER and independently verified for GDH from various organisms[40]), half-lives were estimated

for Yers-ER to be 78 min and 48 h in the presence of reactant (2-cyclohexen-1-one) and product (2-cyclohexanone), respectively. For GDH-103, the half-lives were estimated to be 1.6 min and 9.4 h in the presence of reactant and product, respectively. The addition of 260 mM 2-cyclohexen-1-one leads to a more than 30-fold decrease of the half-life at 30 °C from >2160 min to 78 min for Yers-ER. A high degree of precipitation was observed in both enzyme samples which contained 260 mM 2-cyclohexen-1-one.

Discussion

For the synthesis of enantiomerically pure compounds, the reduction of α,β-unsaturated carbonyl compounds is of great interest. Homogeneous chemical catalysts have dominated *cis*-hydrogenation,[44] while *trans*-hydrogenation seems to be more challenging for this type of catalyst. The asymmetric reduction with OYE was found to be consistent with an *anti*-addition mechanism (*trans*-hydrogenation).[27–30] This means that ERs are an important addition to the chemist's toolbox at many process scales, but their application has thus far encountered two major limitations.

First, isolated enzymes have not been characterized for their potential use in biotransformation. Our work demonstrates that ERs from three different organisms showed, as expected, significant differences in their substrate preferences: i) xenobiotic reductase, XenA, from *Pseudomonas putida* was known to react with cyclohexenone but no further substrates had been tested;[15] ii) KYE1 from *Kluyveromyces lactis* had been described in the literature but never tested for its reductive activity;[31] finally, iii) the ER from *Yersinia bercovieri* had not been described in the literature at all, but its kinetic parameters on 2-cyclohexenone are similar to the ones of its homologue, pentaerythritol tetranitrate reductase (PETN).[17] Based on the differences in substrate spectrum, the isolation and characterization of additional enzymes would be advantageous. Their development towards large-scale use would be improved with the establishment of a high-throughput screening system, which already exists for ketoreductases.[36] Regarding the specificity of these enzymes, tests with selected products of the ER reaction revealed that, while the carbonyl group was not reduced, additionally present double bonds could be further reduced and form unwanted side products.

The second limitation results from the hitherto dominant use of whole cells for enoate reductases, since the specific reduction of the unsaturated double bond is limited by the presence of endogenous ketoreductases in the host cells. The latter employ the same cofactor, NAD(P)H, to reduce the carbonyl function, which can lower selectivity and produce additional impurities.[36,37] The use of isolated enzymes can circumvent this problem but still requires a cofactor regeneration system. Importantly, both ER and the regenerating enzyme have to be active at the same pH range. As maximum activity was measured for Yers-ER in the pH range of 6.0–8.5, we chose a commercially available and stabilized glucose dehydrogenase (GDH-103); GDH-103 has been reported to lose its activity only above pH 9.[39,41] The coupled system revealed that conversions of >99% can be easily obtained when using high amount of enzymes and short incubation times. This confirms the advantage of employing isolated enzymes. Another advantage is that isolated enzyme systems show higher productivity and are less tedious to optimize than whole-cell systems.[36,37] Lastly, the expression and purification of his-tagged, isolated enzymes is straightforward, does not seem to influence activity very much, and allows easy assessment of new activities and reactions without interference from side reactions.

Conversion dropped off more rapidly than expected upon longer incubation time. Therefore, we measured the residual activity at different incubation times in the presence of 25 g/L of the substrate 2-cyclohexenone or the product 2-cyclohexanone. We obtained the following two unanticipated results.

i) The kinetic stability of the thermostable GDH-103[37] was lower than that for the thermolabile Yers-ER. In many cases, thermal stability correlates with the stability against other denaturing factors, such as organic solvents.[42,43] For GDH-103 compared to Yers-ER this is not the case, as we measured a half-life of 117 min for Yers-ER and around 4800 min for GDH-103 at pH 7.0 and 45 °C.[37]

ii) The presence of the substrate 2-cyclohexenone causes rapid loss of activity in both Yers-ER and GDH-103 (Table 4): half-life decreased about 30-fold to 78 min for Yers-ER but a drastic 6300-fold to 1.7 min for GDH-103. In comparison, the presence of the product cyclohexanone caused no deactivation at all in Yers-ER and an 18-fold reduction of half-life to 564 min for GDH-103. This difference in deactivation behavior is astonishing, given that one double bond constitutes the only structural difference between the two. This finding highlights the importance, but also the unpredictability, of measuring enzyme stability in the presence of substrates and products, not just in aqueous buffer at the same pH value and temperature.

Different strategies, such as chemical modification, lyophilization, immobilization, additives, or protein engineering, can be used to stabilize enzymes, as low stability is a known problem.[34,35] The choice of strategy is case-dependent, since the main target variable, e.g., costs or time of process development, can vary substantially.

Conclusions

In the present study we have characterized the substrate spectrum of three ERs, XenA from *Pseudomonas putida*, KYE1 from *Kluyveromyces lactis*, and ER from *Yersinia bercovieri*, to a complex set of *α,β*-unsaturated carbonyl compounds. We demonstrated that all three ERs feature broad substrate specificity but different substrate preferences. These enzymes can be part of a future screening set of enzymes, which can be used to identify ERs for the synthesis of enantiomerically pure compounds.

Furthermore, we demonstrate the feasibility of cell-free biocatalytic reduction of α,β-unsaturated carbonyl compounds and have addressed the major limitation of the *in vitro* application of ERs. By employing glucose dehydrogenase as a cofactor regeneration system, we eliminate the need for whole-cell catalysis, which is often limited by side reactions and low activity. The coupled enzyme system Yers-ER/GDH-103 is quickly deactivated by 2-cyclohexenone, our substrate, much more so than by heat or by the presence of oxygen or cyclohexanone, our reaction product.

Overall, we have shown that biocatalytic reduction of α,β-unsaturated carbonyl compounds with recombinant, isolated ERs can straightforwardly be applied as a tool for biochemical synthesis from enones.

Experimental Section

Bacterial Strains, Media and Growth Conditions

The xenobiotic reductase A (XenA) from *Pseudomonas putida* was kindly provided by Brian Fox.[15] The genomic DNA of *Kluyveromyces lactis* (ATCC 8585D-5) and strain *Yersinia bercovieri* (ATCC 43970) were obtained from ATCC and grown in trypticase soy media (30 g/L trypticase soy broth, BD 211768) under aerobic conditions at 26 °C.

Primer Sequence and Cloning

The DNA sequences were identified using a search of the NCBI Genebank (Accession files AAF02538 for XenA, ZP 00823209 for *Yersinia bercovieri* and P40952 for *Kluyveromyces lactis*). The corresponding specific 5′- and 3′-primers were synthesized at MWG Biotech (High Point, NC).

The primers contain a gene-encoding segment (italic), a restriction site (underlined), and an overhang. All 5′-primers introduced a NdeI restriction site into the PCR fragment. (XenA: 5′-AAC CAA CCA A<u>CA T</u>*ATG TCC GCA CTG TTC GAA CCC TAC ACC C-3′*; KYE1: 5′-AAC CAA CCA A<u>CA T</u>*AT GTC GTT TAT GAA CTT TGA ACC AAA GCC ATT GGC-3′*; *Yersinia bercovieri: 5′*-AAC CAA CCA A<u>CA T</u>*AT GAA GAC TGC TAA ACT GTT CTC TCC TTT GAA GGT TGG-3′*) The 3′-primers introduced a *Hin*dIII or a *Xho*I restriction site into the PCR fragment (XenA: 5′-ATA ATA ATA A<u>AA GCT T</u>*TT AGC GAT AGC GCT CAA GCC AGT GCG C-3′*; KYE1: 5′-ATA ATA ATA A<u>CT CGA G</u>*CT ATT TCT TGT AAC CCT TGG CAA CAG CTT CCT CG-3′*; *Yersinia bercovieri*: 5′-ATA ATA ATA A<u>AA GCT T</u>*TT ACA GCG TTG GGT AAT CAG TGT AGC CCT TAG CAC CG*C-3′).

Amplification of the target DNA was performed using a standard PCR protocol with Pfu polymerase from New England Biolabs (Marlborough, MA) and PCR buffer from Stratagene (La Jolla, CA). Setting up the PCR reactions involved final DNA concentration of 100 ng (XenA, KYE1, or Yers-ER), 200 μM of each dNTP, 10 μM of each primer and 1 U of Pfu polymerase in a final volume of 45 μL. To each of the reactions, 5 μL of the Pfu buffer were added. DNA was amplified successfully for 30 cycles in an Eppendorf Gradient Thermocyler (Eppendorf, Hamburg, Germany) using the following conditions: each cycle involved a denaturation step at 1 min 95 °C, an annealing step at 1 min at 50 °C, 55 °C or 60 °C, and an extension step at 3.5 min at 68 °C. Of the final reaction mixture of 50 μL aliquots were analyzed on 1 % agarose gels. Prior to any further use, These PCR products were gel purified using a gel extraction kit (Qiagen, Valencia, CA).

The PCR products were digested with the adequate restriction enzymes (XenA: *Nde*I/*Hind*III; KYE1: *Nde*I/*Xho*I; Yers-ER: *Nde*I/*Hind*III), ligated into pET28a (+) (Novagen, San Diego) and transformed into competent *E. coli* XL1-Blue cells. The sequence of the ER genes was determined by sequencing with an Applied Biosystems 3100 Genetic Analyzer (Perkin–Elmer-AB, Boston, MA).

Expression and Purification of the Enoate Reductase Genes

Heterologous expression of the ER genes in *E. coli* BL21 (DE3) was performed as follows: 5 mL starter LB_{Kan} cultures were inoculated with aliquots from frozen stock culture and grown overnight at 37 °C. These starter cultures were each used to inoculate a 500 mL culture (1 % v/v), which was vigorously aerated until OD_{600} reached 0.5–0.6, at which point the cultures were incubated with 0.1 mM IPTG (final concentration) and protein expression was performed over night. Cells were harvest and pellets frozen away at −80 °C or used directly for the protein purification.

The cell pellets were resuspended in lysis buffer (5 mL/g wet weight; 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole) and sonicated on ice. The supernatant was separated after centrifugation and the purification was performed with Ni^{2+}-NTA beads according to the standard protocol of Qiagen (Valencia, CA).

Enzyme Assay

Apparent steady-state kinetic constants were recorded by monitoring the oxidation of NADPH at 340 nm using a molar absorption coefficient of $6.22\ mM^{-1}cm^{-1}$ or $3.51\ mM^{-1}cm^{-1}$ for 365 nm, while systematically varying the substrate concentration. Reactions were initiated by addition of enzyme and monitored for 2 min. All experiments were conduced in triplicate and results were averaged. One unit of enzyme activity was defined as the oxidation of 1 μmol NADPH per min at 25 °C in the assay buffer. Since members of the OYE family catalyze the slow oxidation of nicotinamide cofactor in the absence of substrate, we determined this background oxidase activity and subtracted it from the specific activities. The enzyme assays were mea-

sured at 25 °C in 200 mM phosphate, pH 7.5, 200 μM NADPH, 42.9 nM Yers-ER), where 2-cyclohexen-1-one was used as oxidative substrate.

Activity-pH Profile

The pH profile was obtained for the Ni^{2+}-NTA eluted fractions. All samples were tested in triplicate in 1.5 mL methyl acrylate disposable cuvettes. An appropriate buffer was used for each different pH range: acetate (5.0–5.5), phosphate (6.0–7.5), Tris (8.0–9.0) and carbonate (9.5–10.0), all at 200 mM. The rate of change of absorbance at 340 nm was measured at 25 °C and an initial 2-cyclohexenone concentration of 5 mM.

Protein Gel Analysis

Prior to SDS-PAGE, protein samples were diluted to the desired concentration in deionized water if the initial concentration was above 200 ng/μL. The 50 μL diluted samples were then mixed with 50 μL of 2X sample buffer composed of 125 mM Tris-HCl, pH 6.8, 4 % SDS, 50 % glycerols, 0.02 % bromophenol blue, and 10 % 2-mercaptoethanol. Mixed samples were incubated at 100 °C for 5 min and then placed on ice. 10 to 20 μL of the samples were loaded onto a 12 % PAGE™ Gold precast gel and run in a BioRad Mini Protean 3 cell chamber (BioRad, Hercules, CA) at 125 V for 45 min (running buffer: 25 mM Tris base, 192 mM glycine, 0.1 % SDS). Molecular weight standard BioRad Precision Plus protein ladder from BioRad was added to lanes immediately adjacent to the sample lanes. Coomassie Blue from Pierce (Rockford, IL) was used in staining.

Determination of Protein Concentration

Protein concentrations were determined by the Bradford method utilizing Coomassie Plus Protein assay reagent and pre-diluted protein assay standards – BSA (Pierce Chemical) for the calibration curve.

Coupling of the Enoate Reductase with the Cofactor Recycling System

Enoate reductase from *Yersinia bercovieri* and GDH-103 (a glucose dehydrogenase from Biocatalytics, Pasadena, CA) were added in different ratios to a 200 mM phosphate buffer of pH 7.5, 299 mM glucose and 1.2 mM of $NADP^+$. The reaction was performed in the batch mode at 30 °C and substrate cyclohexen-1-one was added to a final concentration of 260 mM. The work was carried out at 125 mg scale (5 mL total volume), the temperature was maintained at 30 °C using a water bath, pH was adjusted manually using 2 M NaOH and agitation was achieved using a magnetic stirrer at 200 rpm. The conversion was determined via gas chromatography (GC) by measuring the substrate and product content of a reaction sample extracted with ethyl acetate using an Agilent GC-FID (model 6890) with a DB17 column (Agilent, Palo Alto, CA).

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