Production of (R)-Ethyl-4-Chloro-3-Hydroxybutanoate Using Saccharomyces cerevisiae YOL151W Reductase Immobilized onto Magnetic Microparticles

Jin Woo Choo and Hyung Kwoun Kim*

Department of Biotechnology, The Catholic University of Korea, Bucheon 420-743, Republic of Korea

Introduction

Various enzymes can be used to produce a variety of pharmaceutical drugs [7, 10, 17, 23]. The free enzymes used in bioconversion reactions, however, cannot be easily separated from the reaction mixtures, and the monetary cost of enzymes has therefore become the biggest obstacle in the economical production of target drugs. To solve this problem, enzymes are sometimes immobilized by various methods, including adsorption and covalent linkage to solid matrix, entrapment in gel, and encapsulation within membrane vesicles. Those immobilized enzymes can be recycled multiple times. For efficient immobilization, however, it is important to choose suitable carriers and immobilization methods [8, 9]. With the advent of nanotechnology, the usage of magnetic particles as supporting materials is presently receiving a great amount of attention [31]. Magnetic nano- or microparticles can support a maximum surface area to immobilize an enzyme and present a minimal steric hindrance to reactants; moreover, these magnetic particles can be easily separated from the reaction mixture by using an external magnet [14, 22].

In previous studies, a variety of microbial reductases were studied [1, 10, 26, 32, 34]. Some reductases exclusively converted ethyl-4-chloro-3-oxobutanoate (ECOB) into (R)-ethyl-4-chloro-3-hydroxybutanoate ((R)-ECHB) [7, 17, 23]. Among them, Saccharomyces cerevisiae YOL151W reductase...
exhibited the highest enantioselective reductase activity toward an ECOB substrate [5]. Ethyl-4-chloro-3-hydroxybutanoate (ECHB) is a useful chiral intermediate used in the synthesis of certain pharmaceuticals, and among them, the (R)-enantiomer is a precursor of L-carnitine, which is important for heart and brain function, muscle movement, and many other bodily processes [12, 18, 35].

For industrial application, reductase YOL151W should be immobilized onto some supporting material and recycled multiple times. Some literatures have reported about reductase-immobilization processes; however, most of them described the entrapment and encapsulation methods [13, 24]. There are only a few reports about covalent cross-linking between the lysine residues. Reductase can attach to carriers, but its activity becomes dramatically decreased by cross-linking of reductase to solid materials [3]. Difficulties in the immobilization of reductase enzymes seem to be caused by their unique structures. Reductase YOL151W is a short-chain carbonyl reductase (SDR), and SDR enzymes have several key amino-acid residues such as lysine and arginine in their active site pockets that interact directly with the NADPH cofactor [8, 33]. In general, the immobilization process includes the adsorption of the enzyme to carriers, and the subsequent glutaraldehyde-mediated intermolecular cross-linking between the lysine residues. Reductase can attach to carriers, but its activity becomes dramatically decreased because the NADPH can no longer interact with the immobilized enzymes.

In this study, we produced C-terminal His-tag reductase YOL151W, and we tried to immobilize the reductase onto Ni²⁺-magnetic particles using the immobilized metal-ion affinity chromatography (IMAC) method to overcome its structural factor (Fig. S1). The IMAC method is developed on the basis of the specific coordinate covalent bond of histidine to metals [15, 28]. This technique works by allowing polyhistidine-containing proteins to be retained in a column containing immobilized Ni²⁺ ions. We characterized the YOL151W immobilized reductase (Imm-Red) and performed a coupling reaction with Bacillus megaterium glucose dehydrogenase (GDH) for the production of (R)-ECHB.

Materials and Methods

Chemicals
ECOB, NADPH, and NADP⁺ were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). BcMag His-Ni Magnetic Beads were purchased from Bioclone Inc. (San Diego, CA, USA). (R)- and (S)-ECHB were acquired from Cronbio Com. (Korea). All of the other chemicals were of analytical grade.

Expression and Purification of Reductase YOL151W
The recombinant Escherichia coli BL21 (DE3) harboring the reductase gene [5] was cultured at 37°C in 1 L of LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) containing 100 µg/ml ampicillin. When the OD₆₀₀ reached 0.5, 1 mM of isopropyl thio-β-D-galactoside (IPTG) was added and the cells were cultured for an additional 20 h to induce the expression. The cultured cells were harvested via centrifugation (6,000 × g, 10 min, at 4°C) and resuspended in a 1/50 volume of 50 mM potassium phosphate buffer (pH 7.0). The resuspended cells were disrupted by sonication and the cell-free extract was obtained by centrifugation (12,000 × g, 10 min, at 4°C) [11, 33].

The cell-free extract was purified as follows. First, 10 ml of the cell-free extract of E. coli BL21 (DE3)/pETR151 was loaded onto a Ni-NTA column (bed volume, 18 ml) (Qiagen GmbH, Hilden, Germany) pre-equilibrated with 50 mM of potassium phosphate and 300 mM of NaCl containing 20 mM of imidazole; unbound proteins were washed from the column with 40 ml of the same buffer. The bound recombinant reductase was then eluted from the column by the application of a 250 mM imidazole buffer, and the active fractions were then collected. Subsequently, 2.5 ml of the enzyme solution was loaded onto a PD-10 desalting column (bed volume, 8.3 ml) (GD Healthcare Bio-Sciences AB, UK) and eluted with 3.5 ml of a 50 mM potassium phosphate buffer (pH 7.0). The active reductase fractions were collected and utilized for the purpose of this research [33].

Reducase and Glucose Dehydrogenase Activity Assays
The activities of both the purified and immobilized reductases were assayed at 30°C by measuring the decrease of absorbance at 340 nm for 10 min using a spectrophotometer. The 1 ml reaction mixture for the reductase assay consisted of 1 mM ECOB (100 mM stock in DMSO), 0.2 mM NADPH, 50 mM potassium phosphate (pH 7.0) buffer, and 0.2–1 µl of cell-free extract or 2–10 µl of immobilized reductase. One unit of reductase was defined as the quantity of enzyme required to catalyze the oxidation of 1 µmol NADPH in 1 min at 30°C.

The oxidation reaction mixture (1 ml) for the GDH assay consisted of 10 mM glucose, 0.2 mM NADP⁺, 50 mM potassium phosphate (pH 7.0) buffer, and 1–5 µl of the recombinant Bacillus megaterium GDH. Using a spectrophotometer, the measurement of the reaction rate was based on the increase of absorbance at 340 nm for 10 min at 30°C. One unit of GDH was defined as the quantity required to reduce 1 µmol NADP⁺ in 1 min at 30°C.

Immobilization of Reductase YOL151W onto Ni²⁺-Microparticles (NMP)
Using a rotary shaker, NMP (300 µl, 9 mg) were mixed with 100 µl of purified reductase YOL151W (1 mg protein) and incubated at 4°C for 20 min. After washing with 50 mM potassium phosphate buffer (pH 7.0), the resulting Imm-Red was resuspended with 300 µl of 50 mM potassium phosphate buffer (pH 7.0), which included 2 mg/ml (final conc.) of bovine serum albumin to increase the enzyme stability, and was finally stored at 4°C. The immobilization yield (η) was defined as the following:

Reductase Immobilized onto Magnetic Particles 1811

Saccharomyces cerevisiae Reductase Immobilized onto Magnetic Particles

November 2015 | Vol. 25 | No. 11
\[ \eta (\%) = \frac{P_i - P_s}{P_s} \times 100 \]

where \( P_s \) is the protein amount of the reductase enzyme before immobilization and \( P_i \) is the protein amount from the supernatant after immobilization. The activity retention (A) of the reductase YOL151W was calculated using the following formula:

\[ A (\%) = \frac{U_i - U_s}{U_s} \times 100 \]

where \( U_s \), \( U_i \), and \( U_\lambda \) are the activities of the purified reductase YOL151W, the supernatant after immobilization, and the Imm-Red, respectively [14].

Physical Characterization of Imm-Red

The size and morphology of Imm-Red were investigated using SEM. Additionally, its size was measured using zeta potential measurement, and its magnetic properties were investigated at room temperature using a magnetic property measurement system (MPMS).

Biochemical Characterization of Imm-Red

The effects of temperature and pH on the Imm-Red were evaluated and compared with the free reductase. The reaction rates were measured at various temperatures (10°C to 60°C). The enzyme was pre-incubated for 30 min at 10°C to 50°C, and the remaining activity was assayed at 30°C to evaluate its temperature stability. The following buffers (50 mM) were used to assess the effects of pH: pH 4.0 to 6.5, sodium acetate/acetate acid; pH 6.5 to 8.0, KH₂PO₄/K₂HPO₄; pH 7.5 to 9.0, Tris-Cl; pH 9.0 to 11.0, glycine-KCl-KOH, and pH 11.0 to 12.0, K₃HPO₄/K₂PO₄. To confirm the pH stability, the enzyme was pre-incubated for 30 min in these pH buffers on ice and then adjusted to pH 7.0, followed by an evaluation of the residual activity of the enzyme.

Cloning and Expression of Glucose Dehydrogenase Gene

_Bacillus megaterium_ QM B1551 was purchased from the Korean Collection for Type Culture (KCTC) and chromosomal DNA was isolated using a HiGene Genomic DNA Prep Kit (Biofact, Korea). The PCR primer set for cloning the GDH gene was designed on the basis of nucleotide sequence information from GenBank (ID CP001983.1, locus_tag BMQ_0838): primer F, 5'-CGC GGA TCC G ATG TAT AAA GAT TTA GAA-3'; primer R, 5'-ATA AAG CTT TTA TCC TGC TGC-3'. The PCR was performed under the following conditions: 30 cycles of 95°C for 1 min, 46°C for 1 min, and 72°C for 1 min. The PCR product was ligated into the pGEM-T Vector (Promega Corp., Madison, WI, USA) and transformed into _E. coli_ XL1-Blue. The resulting plasmid was digested with BamHI and HindIII, and the GDH gene was ligated downstream of the first T7 promoter of the pACYC duet-1 vector. Finally, the recombinant plasmid pACNHisBMGDH was constructed. The plasmid was transformed into _E. coli_ BL21 (DE3), and the _E. coli_ cells were cultured at 20°C in 1 L of LB medium containing 170 μg/ml chloramphenicol. When the cells reached an optical density at 600 nm of 0.5, expression was induced by adding 1 mM IPTG. Cultivation was continued for an additional 20 h, and the cells were harvested by centrifugation at 6,000 ×g for 10 min at 4°C and suspended in a 1/50 volume of 50 mM potassium phosphate (pH 7.0) buffer. The cells were disrupted by sonication, and crude extracts were obtained by centrifugation at 12,000 ×g for 10 min at 4°C.

Enzymatic Coupling Reaction Using Imm-Red

The conversion rate and enantiomeric excess values of the enzymatic reduction of ECOB were evaluated as follows. For the coupling reactions, 20 mM ECOB, 45 mM D-glucose, 1 mM NADP⁺, 10 U of Imm-Red, and 40 U of GDH were mixed in 10 ml of potassium phosphate buffer (50 mM, pH 7.0). The mixture was put into a titration vessel with thermostat jacket (Metrom AG) and incubated at 25°C with stirring at 400 rpm (728 Magnetic stirrer, Metrom). The pH of the reaction mixture was monitored with a pH meter (Mettler Toledo), and it was maintained at 6.5 to 7.0 during the reaction period by adding 1 M NaOH. ECHB was extracted with ethyl acetate and dried via evaporation. After the acetylation and washing steps [11], the organic phase was subsequently analyzed using a gas chromatography system equipped with a Chirasil-dex column (Varian, Palo Alto, CA, USA). The column temperature was increased from 70°C to 180°C at a rate of 5°C/min, and maintained for 2 min at 180°C. The total product quantities in the reaction mixtures were calculated by comparing the retention times and peak areas of the standard (R)- and (S)-alcohol peaks. The retention times of the (R)- and (S)-alcohols were 14.97 min and 15.08 min, respectively.

Results and Discussion

Immobilization of Reductase YOL151W onto NMP

Recombinant reductase YOL151W was overexpressed in _E. coli_ BL21 (DE3) cells harboring pETR151 plasmid [11, 33]. The reductase activity of the cell-free extract was measured as 14.48 U/mg protein. As this enzyme had a His-tag at its C-terminus, it was easily purified with an Ni-NTA column; the purified reductase showed a specific activity of 25.40 U/mg. Then, the His-tag was also used for the immobilization of reductase YOL151W onto Ni²⁺-magnetic particles. The specific coordinate covalent bonds between the nitrogen atoms of the imidazole ring and the Ni²⁺ metal ion of NMP made the enzyme bind tightly onto the surface of the NMP as an active form (Fig S1). According to the homology model [11], the C-terminal part of the enzyme is far apart from its active site. This seemed to be the reason why YOL151W reductase maintained an active form after immobilization. Even after two washing steps, the bound enzymes were not detached from the...
NMP. The enzymes remained attached to the NMP, unless a high concentration of competing material such as imidazole was added or the pH condition was changed.

The protein amounts of the enzyme solutions before (P_A) and after (P_R) immobilization were assayed to calculate the immobilization yield (Table 1). In addition, the reductase activity of the enzyme solution before (U_A) and after (U_R) immobilization and the Imm-Red (U_I) were assayed to calculate the activity retention. The immobilization yield (η) was 99.27%, and the reductase activity and activity retention (A) of Imm-Red was 9.52 U/9 mg bead and 36.37%, respectively. This decreased activity retention seemed to be caused by an external diffusion limitation of the substrates [29].

To evaluate the practical immobilization efficiency, we calculated the number of reductase molecules that attached to the NMP. The calculation was performed as follows, whereby the number of NMP beads (N) used in our experiment was

\[
N = 9 \text{ mg} \times (1.7 \times 10^9 \text{ beads/mg}) = 1.53 \times 10^8 \text{ beads}
\]  (1)

The total protein amount (y) for immobilization was 1 mg, whereby the average number (B) of reductase YOL151W immobilized onto the NMP was

\[
B = \left( \frac{\sum \text{molecules}}{M} \right) = 9.88 \times 10^6 \text{ molecules/bead}
\]  (2)

where M is the molecular mass of reductase YOL151W (39,825 Da). This result shows that the yield of this immobilization method using the Ni^{2+}-His-tag coordinate covalent bonds is similar to that of the glutaraldehyde-cross-linker method [14].

### Analysis of Imm-Red Properties

The physical properties of Imm-Red were analyzed using SEM, the zeta potential measurement, and the MPMS. The SEM analysis showed that the Imm-Red and raw NMP were irregular aggregates of 1-µm-size particles. The

---

**Table 1.** Immobilization yield and activity retention of Imm-Red.

<table>
<thead>
<tr>
<th>Immobilization time (min)</th>
<th>Reductase activity in supernatant (U)</th>
<th>Protein amount in supernatant (mg)</th>
<th>Immobilization yield (η, %)</th>
<th>Immobilized enzyme activity (U)</th>
<th>Activity retention (R, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.83 ± 4.12</td>
<td>1.00</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>20</td>
<td>0.23 ± 0.05</td>
<td>0.0097 ± 0.0033</td>
<td>99.04 ± 0.33</td>
<td>8.84 ± 0.97</td>
<td>31.44 ±7.85</td>
</tr>
</tbody>
</table>

NMP (300 µl, 9 mg) was mixed with 100 µl of reductase (1 mg protein). After immobilization, Imm-Red was resuspended with 300 µl of 50 mM potassium phosphate buffer (pH 7.0).

N/D, not determined.

---

**Fig. 1.** Particle size and appearance analysis by electron microscopy and zeta potential measurement. (A) Raw NMP was analyzed using SEM. (B) Imm-Red was analyzed using SEM. (C) The particle sizes of NMP before (●) and after (○) immobilization were measured using the zeta potential.
1814 Choo and Kim


Fig. 2. The magnetic properties of raw NMP and Imm-Red. (A) The hysteresis loop of raw NMP (▲) and Imm-Red (○) were analyzed using the magnetic property measurement system. (B) Neodymium magnet was used for Imm-Red separation.

differences of the size and appearance of the Imm-Red and raw NMP were not observed clearly through the SEM analysis (Figs. 1A–1B); therefore, the sizes of the Imm-Red and raw NMP were analyzed using the zeta potential measurement, whereby the average sizes of Imm-Red and raw NMP were 1.21 \( \mu m \) and 1.15 \( \mu m \), respectively (Fig. 1C). After the immobilization reaction, the average particle size was increased by approximately 60 nm, and this size difference seemed to be caused by the binding of the enzyme onto the NMP.

The magnetic properties of the Imm-Red and raw NMP were measured using the MPMS (Fig. 2A). After the enzyme immobilization, the magnetization value of the NMP was increased slightly from 29.36 emu/g to 31.76 emu/g. Before the binding of the enzyme, the Ni\(^{2+}\) ions slightly inhibited the magnetization value of the magnetic particles. After immobilization, the Ni\(^{2+}\) ions were covered with enzymes, and the magnetization value of Imm-Red was therefore slightly higher than that of the raw NMP. The Imm-Red was easily separated from the reaction mixture by the application of an external neodymium magnet (D30 x T3, 4000 Gauss) (Fig. 2B).

Characterization of Imm-Red

The biochemical characteristics of Imm-Red were determined by assaying its reductase activity, and they were subsequently compared with those of the free reductase. The optimum temperature of the Imm-Red was 45\(^{\circ}\)C, and its activity was reduced as the temperature increased to 50\(^{\circ}\)C; this was also the case with the free reductase (Fig. 3A). When the free reductase was pre-incubated at various temperatures for 30 min, it was stable up to 35\(^{\circ}\)C; however, its stability was dramatically decreased when the temperature increased above 35\(^{\circ}\)C, whereas the stability of Imm-Red was decreased when the temperature increased above 30\(^{\circ}\)C (Fig. 3B). Protein stability has a tendency to decrease when the protein concentration becomes less than 1 mg/ml [2, 4, 30]; therefore, we added 2 mg/ml of bovine serum albumin to the resuspension buffer to maintain its stability and performed our enzyme conversion reaction at 25\(^{\circ}\)C.

The optimum pH was 6.0 for both Imm-Red and the free enzyme (Fig. 3C), and the former was stable from pH 4 to pH 11 during a 30 min incubation. Compared with the free enzyme, Imm-Red was more stable at pH 11 (Fig. 3D).

These results demonstrate that the biochemical properties of the investigated enzyme were not dramatically changed after it was bound to magnetic particles. In fact, the homology model of the reductase YOL151W structure has been reported [8, 33]; according to the structural model, the C-terminal part is remote from the active site in the tertiary structure (Fig. S1).

Recovery Yield of Imm-Red

To reuse Imm-Red for a bioconversion reaction, it should be separated easily from the reaction mixture. The reductase YOL151W immobilized onto the NMP was easily separated from the reaction mixture within 2 min by using the neodymium magnet (Fig. 2B). The residual reductase activity was measured after repeated cycles of the recovery process to confirm the recovery yield of the Imm-Red (Fig. 4). Imm-Red (100 \( \mu l \), 3 mg) was used for this experiment,
and the reductase activity was maintained at almost 90% until five cycles of the repeated recovery process were completed. When the recovery cycle was repeated 10 times, the residual activity was approximately 70%.

Production of (R)-ECHB by Imm-Red and GDH Coupling Reaction

For a continuous carbonyl-reduction reaction, a plentiful supply of NADPH cofactor is used (Scheme 1) [16, 19, 21]; however, because NADPH is a fairly expensive compound, an NADPH-regeneration system is indispensable [6, 21]. In general, cofactors can be regenerated by both the “coupled-substrate” [1, 27] method and the “coupled-enzyme” [6, 12, 20] method. In the “coupled-enzyme” method, one enzyme is used for the desired bioconversion and the other enzyme is used to regenerate the cofactor. In this study, GDH was used to regenerate the NADPH.

We performed the cloning and overexpression of the GDH gene using the Bacillus megaterium QM B1551 strain (Fig. 5A). At first, we tried to produce the GDH enzyme with C-terminal His-tag (281 amino acids, 30,329 Da); the
enzyme was produced successfully (Fig. 5A) but its activity was very low (0.017 U/mg) (Fig. 5B). It is actually known that the Bacillus GDH forms a tetramer structure as a functional unit, in which the C-terminal parts come together in the center of the tetramer [25]; however, the addition of the C-terminal His-tag seemed to prevent the formation of the typical tetramer structure. We then tried to produce the GDH enzyme with an N-terminal His tag (275 amino acids, 29,712 Da), whereby GDH was produced as a soluble active form (Fig. 5A) and the GDH activity of the cell-free extract was measured as 6.4 U/mg (Fig. 5B); seemingly, the N-terminal His-tag did not prevent the formation of the tetramer structure. Then, we performed the immobilization of the N-terminal His-tag GDH onto the Ni$^{2+}$-magnetic particles; however, after binding, the GDH activity decreased dramatically (data not shown), whereby it seemed that the tetrameric structure was separated during the binding of the enzyme to the magnetic particles. We therefore used the free N-terminal His-tag GDH for the subsequent coupling reactions, as follows.

The optimum pH of the Imm-Red was 6.0 and that of the B. megaterium GDH was 7.0 (data not shown). During the coupling reaction, gluconic acid was produced and accumulated continuously as a by-product; as a result, the pH of the reaction mixture was decreased. In this research, the pH of the solution was therefore maintained at between 6.5 and 7.0 by the intermittent addition of 1 M NaOH solution (Fig. 6B).

The reaction products of the Imm-Red/GDH coupling reaction were taken with time course and analyzed using a GC system equipped with a chiral column. Evidently, only the target product (R)-ECHB was detected, with a retention time of 14.97 min (Fig. 6A), whereas the (S)-ECHB (retention time of 15.08 min) was not detected. When the coupling reaction was performed with an initial substrate concentration of 20 mM, approximately 99% of the ECOB was converted within 120 min (Fig. 6B), implying that the NADPH was recycled approximately 20 times by the GDH. The (R)-ECHB was noticeably produced with an enantiomeric excess value of around 98%, exhibiting the efficient biocatalytic capability of Imm-Red.

**Scheme 1.** Schematic coupling reaction using Imm-Red and GDH.

Reducase YOL151W immobilized onto Ni$^{2+}$-microparticles (NMP) catalyzes the enantioselective reduction of ECOB to (R)-ECHB. GDH catalyzes NADPH regeneration.

**Fig. 5.** Overexpression of His-tagged B. megaterium GDH from E. coli cells.

(A) SDS-PAGE analysis was conducted for the two GDH enzymes containing a histidine tag at their N- or C-terminal. (B) Specific GDH activities were assayed for the two GDH enzymes.
In this study, we successfully immobilized yeast reductase YOL151W onto magnetic particles, using the coordination bond between the His-tag and the Ni$^{2+}$ ion. The immobilized enzyme had biochemical properties that are similar to the free enzyme, it was recovered easily using a magnet, and it maintained its activity after repeated recoveries. The Imm-Red and *Bacillus megaterium* GDH recycled the NADPH and converted the ECOB into (R)-ECHB, suggesting that Imm-Red can be used for the efficient production of (R)-ECHB.

**Acknowledgments**

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIP) (2014R1A2A2A01006978).

**References**

1804: 1841-1849.


