Process Study on the Enzyme-Catalyzed Preparation of Key Chiral Intermediates for Saxagliptin

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Abstract

Saxagliptin is a therapeutic drug for diabetes. The key synthesis process of the drug involves catalyzing 2-(3-hydroxy-1-adamantyl)-2-oxoacetic acid (A) into (S)-3-hydroxyadamantane glycine (B), during which enzymes phenylalanine dehydrogenase mutant from Thermoactinomyces intermedius (TiPDHm) and formate dehydrogenase (FDH) were most often used for biocatalysis. However, the process was limited due to difficulty in enzyme preparation and a low conversion rate. This study focuses on co-expression of TiPDHm and FDH in recombinant Escherichia coli, cell homogenate clarification, enzyme concentration as well as the optimized conditions of enzyme-catalyzed reaction. Our data showed that the wet weight density of bacteria reached 300 g/L, and the yields of TiPDHm and FDH were 7674.24 and 2042.52 U/L, respectively. The combination of ammonium formate and polyethyleneimine favors the clarification of the bacteria homogenate. The clarified enzyme solution obtained can be concentrated by ultrafiltration and directly used in a reductive amination reaction in a high concentration of keto acid A. The reaction time was only 12 hours and the conversion rate reached 95%. Therefore, this process could provide a reference for enzyme-catalyzed preparation of saxagliptin on an industrial scale.

Keywords

► saxagliptin
► phenylalanine dehydrogenase
► formate dehydrogenase
► clarification
► biocatalysis
► reductive amination

Introduction

As a potent dipeptidyl peptidase IV inhibitor, saxagliptin is developed by Bristol Myers Squibb and AstraZeneca, and has shown a great success in treating type II diabetes in adults.1,2 Currently, there are many key chiral intermediates in the synthesis of saxagliptin, and approaches involved chemical resolution, asymmetric catalytic synthesis, and biocatalytic semi-synthesis.3–6 The latter is environmentally friendly, efficient, and represents the most advanced method. In 2007, Hanson R et al reported co-expression of PDH mutant from Thermoactinomyces intermedius (TiPDHm) and the FDH gene sequence from Pichia pastoris (PpFDH) in Escherichia coli, which was used to prepare an amino acid intermediate for saxagliptin, namely (S)-3-hydroxyadamantylglycine (C).5 As illustrated in Fig. 1, the approach mainly solves the synthesis of the key chiral intermediate of saxagliptin (C), which is prepared by the reductive amination of 2-(3-hydroxy-1-adamantyl)-2-oxoacetic acid (A) with PDH mutant from TiPDHm.7 TiPDHm is a hexameric protein and belongs to the class of dehydrogenases. TiPDHm participates in the reductive amination reaction, in which the reduced coenzyme I (nicotinamide adenine dinucleotide, NADH) is required.8 Formate dehydrogenase (FDH) is a mainly

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NAD\(^+\)-dependent enzyme, and could reconvert the oxidized coenzyme to a reduced form.\(^9\)\(^{10}\) The use of FDH may be associated with the most productive manner to provide NADH in TiPDHm-mediated reductive amination. However, the preparation of enzymes also faces many technical challenges, including higher demands on the yield, activity, and low process costs.

Generation of recombinant enzymes from engineered bacterium \textit{E. coli}, followed by extraction and isolation, is the most common process in industrial production. However, during the extraction process, due to the influence of cell debris, liposomes, nucleic acids, and host cell proteins, the homogenate solution tends to contain considerable turbidity and viscosity, which has significant impact on subsequent purification methods such as ion exchange and ultrafiltration concentration. In addition, the purification methods for biological enzymes are more restricted than other proteins, as they are required to maintain enzymatic activity.

The aim of this study was to improve recombinant expression and pretreatment, and to enhance conversion efficiency, TiPDHm and \textit{PpFDH} were co-expressed in \textit{E. coli} according to a reported study.\(^5\) Then high-density fermentation process, clarification method, reaction conditions, and other aspects are optimized to improve the catalytic efficiency. The preparation process provides a reference for producing biological enzymes and saxagliptin intermediates on an industrial scale.

Materials and Methods

Construction and Verification

TiPDHm and \textit{PpFDH} were translated into DNA sequences according to codon bias of \textit{E. coli} BL21 (DE3). The GCG-GAAAAAAA sequence was inserted between \textit{PpFDH} and TiPDHm, thus constituting the target gene sequence \textit{PpFDH-TiPDHm} and expressed under the control of the same promoter. Restriction sites Ncol (CCATGG) and EcoRI (GAATTTC) were added to the 5’ and 3’ ends of the sequence. The sequence was then cloned into Ncol-EcoRI sites of the expression vector pET28a, and the plasmid pET28a-\textit{PpFDH-TiPDHm} was generated finally. The plasmid was transferred into \textit{E. coli} BL21(DE3), and the recombinant strain \textit{E. coli} BL21(DE3)/pET28a-\textit{PpFDH-TiPDHm}, namely DMR532, was obtained.

After expansion in Luria–Bertani (LB) media, 1% inoculum at 37°C and 220 rpm for 6 hours. The culture temperature was lowered to 25°C. Cells were induced by adding 15 μL of isopropyl-\textit{D}-1-thiogalactopyranoside (IPTG, Sangon Biotech, Shanghai) at a final concentration of 0.1 mmol/L, and then cultured for 18 hours. The fermentation broth (4 mL) was centrifuged at 12,000 × g for 5 minutes to obtain wet cells, which were resuspended in 1 mL of Tris-HCl (25 mmol/L, pH = 8.0), then fragmented by ultrasonication, and centrifuged at 12,000 × g for 10 minutes. The supernatant was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Materials for electrophoresis were obtained from commercial kits (Shanghai Epizyme Biotech Co., Ltd.). The samples were denatured and added to the gels for separation. After that, the gels were stained with Coomassie brilliant blue R250 and decolorized with acetic acid solution until the protein bands were visualized.

Enzyme Activities Assay

Since NADH has a maximum absorption at 340 nm,\(^1\) the absorbance values of different molar concentrations of NADH at 340 nm were measured at 30°C using the enzyme-labeled Assay instrument (SpectraMax i3X Microplate reader, Molecular Devices Company, Austria), and a concentration–OD\(^340\) standard curve was plotted. The activity of TiPDHm was measured in a medium containing NADH (4 mmol/L), sodium phenylpyruvate (5 mmol/L), and ammonium chloride buffer (0.75 mmol/L), with pH = 9.0 adjusted with ammonium hydroxide. The decrease in absorption values was monitored. The activity assay of \textit{PpFDH} was measured in a medium containing oxidized coenzyme I (NAD\(^+\), 4 mmol/L), ammonium formate (20 mmol/L), and glycine buffer (50 mmol/L), with pH = 10.4 adjusted with potassium hydroxide.\(^3\)\(^1\) The increase in absorption values was monitored. “Enzyme activity unit (U)” was defined as the amount of enzyme required to consume or generate 1 μmol/L NADH per minute in the buffer system at a temperature of 30°C.

Analysis and Identification of Compounds A, B, and C

High-performance liquid chromatography (HPLC) was used to monitor peak areas of keto acid \textit{A} (Tianjin Minxiang Biomedical Co., Tianjin) and amino acid \textit{B} using an evaporative light scattering detector (ELSD; Agilent 1260 HPLC-ELSD system, Agilent Technologies, United States). There were some difficulties in making a standard curve for \textit{A} and \textit{B} by the ELSD-HPLC method. Therefore, the method of peak
area normalization was used to define the efficiency of the reaction. The conversion of the product (B) was calculated by Equation (1).

\[
\text{Conversion efficiency (\%) = \frac{\text{Peak area of B}}{\text{Total peak areas of A and B}} \times 100}
\]

HPLC was performed on a Kromasil 100–3.5-C18 (4.6 × 150 mm) column at 40°C. UV absorption at 214 nm was recorded. Mobile phases A and B were 0.1% formic acid solution in water and 90% methanol solution, respectively. ELSD parameters were set as follows: the temperature for both evaporator and nebulizer were 35°C; the gas flow rate was 1.6 standard liter per minute. Other parameters were kept at their default values. Peak time for compound B is 12 minutes and for compound A is 16 to 17 minutes.

The chiral column CHIRALPAK MA(+) (0.46 cm I.D. × 5 cm L × 3 μm) was used to identify B and C with a mobile phase of TFA/MeOH (90:10). The peak time of B was 14.1 minutes. With trifluoro acetic acid treatment, the BOC protecting group of the standard intermediate C (Tianjin Minxiang Biomedical Co., Tianjin) was removed to obtain standard B.

High-Density Fermentation at a 300 L Scale

The fermentation process of DMR532 strain was first explored and optimized using a 5 L fermenter. A scale-up production was performed at a 300 L fermenter. Seed culture of the DMR532 strain, which had been activated by the LB medium, was mixed well and inoculated at 1% (v/v) into a sterilized fermentation medium (2 g glucose, 15 g yeast extract, 17.2 g Na2HPO4 12H2O, 3 g KH2PO4, 0.5 g NaCl, 3 g (NH4)2SO4, 1 g KCl, 0.5 g MgSO4, 0.011 g CaCl2, glycercol 20 g dissolvd in 1 L of deionized water) in a volume of 200 L. The incubation temperature was 37°C. During incubation, dissolved oxygen was controlled at 30 to 50% and pH was maintained at 6.9 to 7.0. The feeding medium was 15% glycercol, 2.25% yeast extract, and 1.5% anhydrous magnesium sulfate (m/v). When the glycerol was exhausted, the fermentation medium was added at a flow rate of 50 mL/h. When the OD600 reached 80 to 90, the culture temperature was gradually reduced to 25°C, then, IPTG at a final concentration of 0.1 mmol/L was also added for induction. After 12 hours, the fermentation broth was processed by disc centrifugation and the solid content was controlled at 50 to 70%. The fermentation broth was processed by disc centrifugation and the solid content was controlled at 50 to 70%. The methods were used to obtain a homogenate of DMR532.

Exploration and Optimization of Clarification Processes

To each 100 mL of DMR532 homogenate was added 0.05 to 0.2% chitosan solution, 0.5 to 1.5 mol/L of (NH4)2SO4, as well as 13% diatomite plus 0.08% polyethyleneimine (PEI) solution, separately. The mixture was threaded for 60 minutes at room temperature and the supernatant was obtained by centrifugation at 12,000 × g for 20 minutes. In the final group of homogenate, 0.6 mol/L of ammonium formate was added. The mixture was heated at 40°C for 60 minutes and centrifuged to the supernatant was added PEI (Sigma-Aldrich, Shanghai, China) at a final concentration of 0.06%, stirred at room temperature for 30 minutes, and centrifuged at 12,000 × g for 10 minutes. Then, a clarified enzyme solution was obtained. The activity of TIPDHm in the solution was detected and the supernatant was graded for turbidity according to the first method of clarity check in Pharmacopoeia of the People's Republic of China (ChP), which was a visual method and divided into five grades (0.5, 1, 2, 3, and 4). Samples with a judged grade ≤2 were considered as clarified solutions, where insoluble particles are invisible and will not affect the microfiltration or ultrafiltration.

Effects of different salt solutions ((NH4)2SO4, NH4H2PO4, (NH4)2CO3, and ammonium acetate), different experimental temperatures (5–60°C), and different concentrations of ammonium formate (0.1–1.2 mol/L) on clarification of the homogenate were further explored according to the steps described above. After an optimized clarification process, the homogenate was concentrated to one-fifth of the volume using an ultrafiltration membrane with a molecular weight cutoff of 100 kDa.

Reaction Condition Optimization

Reaction mixture 1 contained keto acid A (0.1 mol/L), ammonium formate (0.6 mol/L), NAD (1 mmol/L), and DTT (0.1 mmol/L). pH was adjusted with 10 mol/L NaOH. The reaction volume was 2 mL per group (1 mL reaction mixture and 1 mL clarified solution). The reaction was performed for 12 hours. HPLC was used for analysis. Furthermore, reaction conditions including pH (6–8), temperature (20–60°C), as well as A concentration were also explored, using a concentrated clarified solution. Reaction mixture 2 contained: keto acid A (0.1–0.5 mol/L), ammonium formate (0.9 mol/L), NAD (1 mmol/L), and DTT (0.1 mmol/L). pH was adjusted with 10 mol/L NaOH. The reaction volume was 2 mL per group (1 mL reaction mixture and 1 mL concentrated clarified solution), and samples were taken for analysis at 8 and 12 hours, respectively.

Synthesis of Saxagliptin Intermediate

Considering the reaction efficiency and cost control, 0.4 mol/L of substrate was chosen for the following study. To 20 or 40 mL of double-distilled water was added A (17.92 g) and ammonium formate (11.35 g), adjusting pH = 9.2 with 10 mol/L NaOH. Then, NAD 0.133 g, DTT 0.03 g, and 100 mL of the concentrated clarified solution (containing 11435.34 U/L of TIPDH) were added. The reaction volume was fixed to 200 mL with double-distilled water. The pH was adjusted to 9.0 at room temperature and then controlled at 49 to 51°C. During the reaction process, the pH was regularly monitored and maintained between 8.9 and 9.1. At the end of the reaction, the mixture was heated to 70 to 90°C for 30 minutes,7 cooled to room temperature, and centrifuged at 12,000 × g for 15 minutes to obtain the supernatant containing product B, which had a purity over 90%.
Results

Identification of Co-expressed Enzymes and High-Density Fermentation

SDS-PAGE analysis showed that the relative molecular weight of the mixed homogenate of TIPDHm and PpFDH was 40 kDa, which was consistent with the theoretical value (►Fig. 2). The protein band of PpFDH was located above the TIPDHm. Furthermore, activity of PpFDH and TIPDHm was 63.65 and 214.16 U/L respectively in the fermentation broth from shake flasks (►Table 1).

Zhao et al reported that the maximal cell density (OD600) reached 103.87 with the dry cell weight (DCW) of 52.32 g/L. However, in this study, the OD600 reached 186.77, which may be associated with a much higher DCW than the reported result. High-density fermentations at the 5 L and 300 L scales yielded 301.93 and 296.60 g/L of wet cells, respectively (►Table 1), both close to 30% of total weight, indicating that this medium is suitable for DMR532 growth. Moreover, 300 L scale fermentations reached a final enzyme activity of 2,042.52 U/L (PpFDH) and 7,674.24 U/L (TIPDHm), respectively, which are approximately 32- and 35-fold increase compared with the shake flasks (LB). The fermentation time was only 24 hours, much shorter than the reported study (36.5–40 hours). Given above, the fermentation process is stable and reproducible and meets the requirements for scale-up production.

Exploration of the Clarification Process for Homogenate

Clarification Methods for Screening

Our preliminary research showed that FDH easily catalyzes the coenzyme NADH cycle, and TIPDH is a key enzyme that restricts the conversion rate of reductive amination. Thus, a higher concentration of the enzyme may be associated with higher conversion efficiency. Considering the low concentration of the enzyme in the homogenate, the enzyme needs to be concentrated. Ultrafiltration technology is widely used for the separation and concentration of biomacromolecules due to its mildness and efficient characteristics. But the premise is that the feed liquid should have a certain degree of clarity to avoid the rapid decline of the processing speed caused by the clogging of the membrane pores. In our process, the crude enzyme was used to catalyze the reaction directly, which avoids the fine purification of the crude enzyme solution and makes a simple preparation of the enzymes. Therefore, it is necessary to explore clarification methods of the turbid homogenate to facilitate the ultrafiltration, while removing impurities as much as possible and retaining the catalytic activity of the TIPDH to the greatest extent.

As shown in ►Table 2, the combination of ammonium formate and PEI gave the best effect, which not only achieved the clarification effect of the homogenate, but also retained 95.30% activity of TIPDHm. The centrifuged precipitate, treated with ammonium formate, was viscous, indicating that the salt had a better removal effect on viscous impurities such as cell debris in a warm bath. Further use of PEI removed negatively charged impurities, and finally yielded a clarified enzyme solution. In the reductive amination experiments, ammonium formate was also a reaction substrate, which avoids additional introduction outside the reaction system that may affect the conversion rate.

Optimization of the Clarification Process

As shown in ►Fig. 3, the better clarifying categories were ammonium formate and ammonium acetate. The optimum concentration of ammonium formate was 0.6 to 0.9 mol/L and the temperature was within 40 to 50°C. With an

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Table 1 Results of fermentation expression and activity assays of DMR532

<table>
<thead>
<tr>
<th>Fermentation methods</th>
<th>OD600</th>
<th>Wet cells* (g/L)</th>
<th>Activity of PpFDHb (U/L)</th>
<th>Activity of TIPDHmc (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake Flask</td>
<td>9.33</td>
<td>10.61</td>
<td>63.65</td>
<td>214.16</td>
</tr>
<tr>
<td>5 L Fermenter</td>
<td>197.65</td>
<td>301.93</td>
<td>2,181.30</td>
<td>7,753.56</td>
</tr>
<tr>
<td>300 L Fermenter</td>
<td>186.77</td>
<td>296.60</td>
<td>2,042.52</td>
<td>7,674.24</td>
</tr>
</tbody>
</table>

*The content of wet cells per liter of fermentation broth.

bPpFDH activity per liter of fermentation broth.

cTIPDHm activity per liter of fermentation broth.

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In economical point in mind, DMR532 homogenate was treated with 0.6 mol/L ammonium formate at 40°C, and concentrated to one-fifth volume by ultrafiltration. Our data showed that both TiPDHm and PpFDH maintained high yields during the processes of clarification and ultrafiltration (Table 3). However, the molecular weight of the dimeric form of PpFDH is similar to that retained by the ultrafiltration membrane, resulting in a small amount of permeation during ultrafiltration, with a loss of activity of PpFDH approximately 10% after ultrafiltration. As shown in Fig. 2, the clarified solution (lane 2) has a lighter TiPDHm band by comparison to the homogenate (lane 1), and bands with molecular weight >150 kDa appeared. It is presumed that a high concentration of ammonium formate promoted the formation of the TiPDHm molecule in a polymeric form. We directly used the enzyme solution containing high concentrations of TiPDHm and PpFDH, obtained from the above clarification and ultrafiltration treatment, to catalyze the formation of amino acid B from keto acid A.

### Table 2 Effect of different treatments on clarification of DMR532 crude enzyme solution

<table>
<thead>
<tr>
<th>Processing</th>
<th>Turbidity</th>
<th>Activity recoverya</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% chitosan</td>
<td>&gt;4</td>
<td>80.21%</td>
</tr>
<tr>
<td>0.1% chitosan</td>
<td>&gt;4</td>
<td>75.22%</td>
</tr>
<tr>
<td>0.2% chitosan</td>
<td>&gt;4</td>
<td>20.38%</td>
</tr>
<tr>
<td>0.5 mol/L (NH₄)₂SO₄</td>
<td>&gt;4</td>
<td>96.33%</td>
</tr>
<tr>
<td>1 mol/L (NH₄)₂SO₄</td>
<td>&gt;4</td>
<td>69.59%</td>
</tr>
<tr>
<td>1.5 mol/L (NH₄)₂SO₄</td>
<td>&gt;4</td>
<td>1.47%</td>
</tr>
<tr>
<td>13% diatomaceous earth + 0.08% PEI</td>
<td>&gt;4</td>
<td>57.85%</td>
</tr>
<tr>
<td>0.6 mol/L ammonium formate + 0.06% PEI</td>
<td>= 2</td>
<td>95.30%</td>
</tr>
</tbody>
</table>

Abbreviation: PEI, polyethyleneimine.

aBased on the first method of clarity check in ChP.
bActivity ratio of TiPDHm in treatment solution to homogenate.

### TiPDHm- and PpFDH-Mediated Reductive Amination Reaction

#### Reaction Condition Optimization

In this study, a lower concentration of A (0.1 mol/L) was used to avoid substrate inhibition according to a reported study. Results from pH screening (Fig. 4A) showed that when pH = 8 to 10, a high conversion efficiency was achieved with the maximum being seen at pH = 9.0. Results from temperature screening (Fig. 4B) showed that the catalytic activity of the enzymes is significantly at 40 to 50°C with the highest conversion being at 50°C. However, the enzyme was gradually deactivated above 50°C, leading to a low conversion of the reaction. Thus, pH = 9.0 and a reaction temperature of 50°C were chosen for the following study. Results from substrate concentration screening (Fig. 4C) suggested that the conversion efficiency was gradually inhibited with an increase in substrate concentration. When substrate concentration was within 0.3 mol/L, more than 95% of the conversion could be achieved. However, at higher substrate concentrations, the conversion efficiency decreased significantly.
completed in 8 hours. When the concentration was increased to 0.4 mol/L, it needs to take 12 hours to complete 95% of the conversion. In Basch et al reports, the reaction time of catalyzing A (0.45 mol/L) into B was approximately 42 hours; however, in the present study, under the optimized reaction conditions, the reaction time is greatly reduced.

**Efficient Preparation of Saxagliptin Intermediate Materials**

As shown in Fig. 5, when a concentrated enzyme solution was used to catalyze the generation of B from A (0.4 mol/L), the conversion rate could reach 95% at 12 hours. At 20 hours, a complete conversion was achieved. In fact, during industrial preparation, the remaining 3 to 5% may be discarded to control reaction time and save production costs. Therefore, it is feasible to achieve efficient conversion of chiral amino acids by concentrating enzymes.

The prepared B was subjected to HPLC (Fig. 6) and chiral analysis with the standard control. Our data showed that peak positions of the sample and the control were consistent. Chiral analysis showed an enantiomeric excess value of the sample >99.9%. Liquid chromatography-tandem mass spectrometry showed that the molecular weight of B was in agreement with the theory, indicating that the amino acid B prepared by biocatalysis was correct in structure.

**Discussion**

Biocatalytic synthesis of chiral compounds is identified of mild reaction conditions, high stereoselectivity, and high...
Table 3  Enzyme activity changes after clarification and concentration

<table>
<thead>
<tr>
<th></th>
<th>Turbidity</th>
<th>Activity (P)&lt;sup&gt;a&lt;/sup&gt; (U/L)</th>
<th>Activity (P)&lt;sup&gt;c&lt;/sup&gt; (U/L)</th>
<th>Activity (F)&lt;sup&gt;d&lt;/sup&gt; recovery</th>
<th>Activity (P)&lt;sup&gt;d&lt;/sup&gt; recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated solution</td>
<td>2</td>
<td>701.22</td>
<td>2,446.74</td>
<td>96.37%</td>
<td>95.30%</td>
</tr>
<tr>
<td>Concentrated solution</td>
<td>3</td>
<td>3,146.37</td>
<td>11,435.34</td>
<td>89.60%</td>
<td>93.27%</td>
</tr>
</tbody>
</table>

*Based on the first method of clarity check in ChP.

<sup>a</sup>PFDH concentration in solution.

<sup>b</sup>TIPDHm concentration in solution.

<sup>c</sup>Activity ratio of PFDH in clarified solution or concentrated solution to homogenate.

<sup>d</sup>Activity ratio of TIPDHm in clarified solution or concentrated solution to homogenate.

strategy was used to introduce the protease genes, TIPDHm and PpFDH, into E. coli for expression. Then, high-density fermentation culture was investigated. TIPDHm was used to catalyze the synthesis of key intermediate of saxagliptin (compound B). PpFDH was used for regeneration of NAD(P)H. The co-expression of the recombinant organisms solved the complicated preparation problem of multi-enzymes, and to some extent had the effect of cooperative expression, which is verified on the 300 L fermentation scale.

TIPDHm is a hexameric enzyme. Compared with monomers, oligomerization confers structural stability and kinetic changes to the protein, leading to improved catalytic efficiency of the enzyme. Protein–salt interaction regulates protein solubility and stability, and ion concentration influences protein hydrophobicity. In the present study, ammonium formate was found to have the ability to clarify the solution, and this may be due to the effect of cooperative expression, which is verified on the 300 L fermentation scale.

Conflicts of Interest
None declared.

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