不对称还原制备光学纯 (*R*)-2-羟基-4-苯基丁酸乙酯的双酶共表达重组菌的构建

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摘要: 克隆了来自于枯草芽孢杆菌的羧基还原酶基因 *IoIS* 和葡萄糖脱氢酶基因 *GDH*, 采用 Ni-NTA 镍亲和层析柱对重组蛋白 *IoIS* 进行纯化, 并对纯酶进行了酶学性质研究. 结果表明, 该羧基还原酶的最适温度和 pH 值分别为 30 °C 和 6.0; 在 40 °C 以下具有较好的热稳定性; 在 pH 5.5–7.0 的偏酸性范围内能保持 75% 以上的酶活. 采用三种策略构建了 *IoIS* 和 *GDH* 的共表达重组质粒, 结果发现, 采用双启动子的重组质粒能够实现羧基还原酶 *IoIS* 的高效表达, 粗酶液中的 *IoIS* 和 *GDH* 的比酶活均达到 1.5 U/mg. 运用该重组菌对 10 g/L 的 OPBE 进行不对称还原, 反应 15 h 后, 底物转化率大于 99%, 产物 (*R*)-2-羟基-4-苯基丁酸乙酯的 ee 值达到 99.5%.

关键词: 羧基还原酶; 葡萄糖脱氢酶; 共表达; (*R*)-2-羟基-4-苯基丁酸乙酯; 不对称还原

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Two-Enzyme Coexpressed Recombinant Strain for Asymmetric Synthesis of Ethyl (*R*)-2-Hydroxy-4-phenylbutyrate

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Abstract: (*R*)-2-Hydroxy-4-phenylbutyrate (HPBE) is an important chiral intermediate for the synthesis of angiotensin-converting enzyme (ACE) inhibitors. Asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) to (*R*)-HPBE using a recombinant strain can provide high enantioselectivity. Cofactor regeneration is a critical issue in the application of a recombinant strain. A carbonyl reductase gene (*iolS*) and a glucose dehydrogenase (*GDH*) gene from *Bacillus subtilis* were cloned. Recombinant *IoIS* was purified using a Ni-NTA column and its enzyme activity properties were investigated. The purified *IoIS* exhibited maximum activity at pH 6.0 and 30 °C, and the enzyme showed good thermostability below 40 °C. It retained over 75% of its activity in the acidic pH range of 5.5–7.0. Three coexpression strategies were used for the recombinant vectors. The recombinant *E. coli* strain containing polycistronic plasmid pET-G-T7-I showed excellent carbonyl reductase activity, and the specific activity of both *IoIS* and *GDH* in the crude cell extract reached 1.5 U/mg. In the asymmetric reduction of OPBE by recombinant *E. coli* cells in aqueous system, the yield of (*R*)-HPBE reached over 99% with an enantiomeric excess of 99.5% at 10 g/L of OPBE within 15 h.

Key words: carbonyl reductase; glucose dehydrogenase; coexpression; (*R*)-2-hydroxy-4-phenylbutyrate; asymmetric reduction

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血管紧张素转化酶抑制剂 (ACEI) 是目前用于治疗高血压和充血性心力衰竭等心血管疾病的主要药物, 它通过抑制血管紧张素转化酶而减少血管紧张素 II (Ang II) 的生成, 从而控制血压, 保护靶器官^[1]. (R)-2-羟基-4-苯基丁酸乙酯 (HPBE) 是合成 ACEI 类药物的重要手性中间体, 可用于合成苯那普利 (Benazepril)、依那普利 (Enalapril) 和雷米普利 (Ramipril) 等重要普利类药物^[2].

近年来, (R)-HPBE 的合成一直是国内外研究的热点, 主要采用化学法和生物法制备, 每种方法又可分为拆分和合成两种途径. 其中化学不对称合成与拆分法需要昂贵的手性辅助试剂或手性起始原料, 且过程冗长, 又易伴随分解、异构、消旋和重排等反应^[3]. 生物拆分法制备 (R)-HPBE 的报道较多, 然而外消旋体拆分的理论转化率仅为 50%^[4~6]. 近些年, 利用生物合成法对 2-羧基-4-苯基丁酸乙酯 (OPBE) 的不对称还原成为热点. 与重组菌相比, 野生菌内部酶系复杂, 可能含有多种不同甚至相反的立体选择性酶, 因此对映体选择性很难达到 99% 以上^[7~9]. 本课题组曾筛选出一株克鲁斯假丝酵母 *Candida krusei* SW2026, 利用其对 OPBE 进行不对称还原, 当底物浓度为 20 g/L 时, ee 值达到 97.4%, 并从中分离得到了 NADPH 依赖型羧基还原酶, 研究了其酶学性质^[10,11].

目前, 有关改造的基因工程菌不对称还原 OPBE 的报道较少, Shen 等^[12]利用重组表达羧基还原酶和葡萄糖脱氢酶的大肠杆菌细胞催化还原 OPBE 得到 (R)-HPBE, 当底物浓度达到 206 g/L 时, ee 值大于 99%. 由于基因工程改造的菌株必须要通过外加辅酶才能达到转化的目的, 而这些辅酶价格昂贵, 稳定性低, 所以辅酶的再生问题对于重组菌还原制备 (R)-HPBE 至关重要.

最近共表达技术的广泛应用解决了辅酶再生的问题, 该技术可实现同一菌株内多个外源蛋白的活性表达. 文献大都利用葡萄糖脱氢酶 (GDH) 氧化葡萄糖生成 NAD(P)H, 完成辅酶的再生^[13~15]. 本文考察了来源于枯草芽孢杆菌的羧基还原酶基因 *iolS* 和葡萄糖脱氢酶基因 *gdh* 的三种共表达方式, 并在水相中进行不对称还原 OPBE 制备 (R)-HPBE.

1 实验部分

1.1 菌株、质粒及试剂

本文所用的菌株及质粒如表 1 所示. 限制性内切酶 *NdeI*, *HindIII*, *NotI* 和 *XhoI*, T4 DNA 连接酶购自宝生物 (大连) 生物工程有限公司; PCR 相关产品购自广州东盛生物科技有限公司; OPBE 和 (R)-HPBE 购自 Sigma 公司; NADP⁺和 NADPH 购自生工生物 (上海) 有限公司; 其余试剂和药品均为进口或国产分析纯.

表 1 本文所用菌株及质粒
Table 1 Strains and plasmids in this study

Strain and plasmid	Application	Source
<i>Bacillus subtilis</i>	source of CR and GDH	our laboratory
<i>E. coli</i> K12	source of CR	our laboratory
<i>E. coli</i> JM109	cloning host	our laboratory
<i>E. coli</i> BL21 (DE3)	expression host	our laboratory
pMD18-T	cloning vector	Takara
pET-24a	expression vector	Novagen
pET-20b	expression vector	Novagen

1.2 葡萄糖脱氢酶基因的克隆和表达

使用细菌基因组提取试剂盒提取枯草芽孢杆菌基因组. 根据 GenBank 中登记号为 M12276 的葡萄糖脱氢酶基因设计引物进行 PCR 扩增, 本文所用的引物如表 2 所示. 将扩增获得的 GDH 基因片段经纯化后连接到 pMD18-T 载体上, 构建好的克隆载体转化至大肠杆菌 JM109 中并送生工生物 (上海) 有限公司进行测序. 用 *NdeI* 和 *HindIII* 分别酶切 pET-24a 及克隆载体 pMD18-T-*gdh*, 将酶切产物胶回收后, 利用 T4 连接酶连接过夜, 次日将连接产物 pET24a-*gdh* 转化至大肠杆菌 BL21 (DE3), 以载体所带卡那霉素抗性筛选阳性转化子. 阳性重组子采用菌落 PCR 和提取质粒双酶切进行鉴定. 将验证成功的重组子接种于含卡那霉素的 LB 培养基中, 在 37 °C 培养过夜, 次日转接于新鲜培养基中, 培养至 OD₆₀₀ 约为 0.6 时, 加入终浓度为 0.4 mmol/L 的 IPTG, 于 25 °C 诱导 4 h 后, 离心收集菌体, 超声破碎后根据标准步骤进行 SDS-PAGE 分析^[16].

1.3 羧基还原酶的筛选、克隆及表达

使用细菌基因组提取试剂盒提取枯草芽孢杆菌

表 2 本文所用引物
Table 2 Primers used in this study

Name	Primer sequences (5'→3')	Restriction site
GDHf	GGAATTCCATATGTATCCGGATTTAAAAGC	<i>Nde</i> I
GDHr	CCCAAGCTTTTAACCGCGCCTGC	<i>Hind</i> III
YcsNf	GGAATTCCATATGCAGCGTATTCAAT	<i>Nde</i> I
YcsNr	CCGCTCGAGTGGAATATCGTAACCCTGTAC	<i>Xho</i> I
IolSf	GGAATTCCATATGAAAAAAGCGAAGCTCGG	<i>Nde</i> I
IolSr	CCGCTCGAGTGCGAACAGCTTATCAAT	<i>Xho</i> I
YccKf	GGAATTCCATATGGATCAAACACGTACAC	<i>Nde</i> I
YccKr	CCGCTCGAGTTTGAATAATGTCGCTGATG	<i>Xho</i> I
YdhFf	GGGAATTCCATATGGTTCAGCGTATTACT	<i>Nde</i> I
YdhFr	TTCCGCTCGAGCGGTACGTCGTACCCAGT	<i>Xho</i> I
YajOf	GGAATTCCATATGCAATACAACCCCTTAG	<i>Nde</i> I
YajOr	CCGCTCGAGTTTAAATCCTACGACAGG	<i>Xho</i> I
SDf	AAGGAAAAAAGCGGCCGCTAAGGAGGATAT ACATATGAAAAAAGCGAAGCTCGG	<i>Not</i> I
T7f	AAGGAAAAAAGCGGCCGCTAATACGACTCA CTATAG	<i>Not</i> I

和大肠杆菌 K12 的基因组, 分别进行克隆表达。如表 2 所示, 根据 GenBank 中的序列信息设计引物, 分别扩增来源于枯草芽孢杆菌的基因 *ycsN* (NP_388296.1), *iolS* (AL009126.3) 和 *yccK* (NC_000964), 以及来源于大肠杆菌的基因 *ydHf* (P76187.2) 和 *yajO* (P77735.2)。其它步骤同 1.2 节。

1.4 葡萄糖脱氢酶和羧基还原酶的酶活测定

葡萄糖脱氢酶的酶活测定条件: 总反应体系为 0.25 ml, 包括 75 mmol/L Tris-HCl (pH 8.0), 2.0 mmol/L NADP⁺, 0.1 mmol/L 葡萄糖以及适量酶, 测定 340 nm 处吸光度的增加。羧基还原酶的酶活测定条件: 总反应体系为 0.25 ml, 包括 100 mmol/L 磷酸钾缓冲液 (pH 6.0), 1 mmol/L OPBE, 5 mmol/L NAD(P)H 以及适量酶, 测定 340 nm 处吸光度的下降。酶活力单位 (U) 定义为: 在上述条件下, 每分钟催化 1 μmol NAD(P)H 氧化所需的酶量、或每分钟催化 1 μmol NADP⁺ 还原所需的酶量。蛋白质含量测定采用 Bradford 法, 以小牛血清白蛋白 BSA 为标准品^[17]。

1.5 不同来源的羧基还原酶对 OPBE 的不对称还原反应

采用来源于枯草芽孢杆菌和大肠杆菌的不同羧基还原酶不对称还原 OPBE 的反应体系: 总反应体积为 2.5 ml, 在 100 mmol/L pH 6.0 磷酸钾缓冲液加入 10 mmol/L NAD(P)H, 20 mmol/L 底物 OPBE, 适

量粗酶液。该体系于 30 °C 和 220 r/min 条件下反应 12 h, 通过气相色谱测定产率和 ee 值。

1.6 羧基还原酶 IolS 的纯化

将诱导表达后离心获得的大肠杆菌 BL21(DE3)/pET20b-iolS 湿菌体用缓冲液 A (20 mmol/L 磷酸钠, 500 mmol/L NaCl, 5 mmol/L 咪唑, pH 7.4) 悬浮, 超声破碎 (功率 285 W, 超声 1 s, 间歇 3 s, 共 10 min), 离心取上清液待上样。将镍柱用缓冲液 A 平衡 10 min 后上样, 先用缓冲液 A 将未结合的蛋白洗脱下来, 再用 0~1000 mmol/L 的磷酸盐缓冲液进行梯度洗脱, 收集洗脱液测定酶活, 并用 SDS-PAGE 检验蛋白纯度。

1.7 羧基还原酶 IolS 酶学性质的研究

1.7.1 最适反应温度和热稳定性

总体积为 250 μl 的体系包括 1 mmol/L 底物 OPBE, 5 mmol/L 辅酶 NADPH 和 0.1 mol/L 磷酸钾 (pH = 6.0) 缓冲液, 在不同温度 (10~70 °C) 的水浴中恒温 2 min, 分别加入等量的电泳纯 IolS 酶液测定其酶活, 考察该酶的最适温度。以所测的不同反应温度下最高酶活为 100% 酶活 (对照), 其它温度条件下所测酶活以对照酶活的百分比表示。

将电泳纯 IolS 酶液在不同温度 (10~60 °C) 下分别保温 1 h, 然后取等量的酶液测定其残余酶活, 考察该酶的热稳定性。以 pH 6.0 条件下新鲜电泳纯酶液的酶活作为 100% 酶活对照, 其它温度下酶活以对照酶活的百分比表示。

1.7.2 最适反应 pH 和 pH 稳定性

总体积为 250 μl 的体系包括 1 mmol/L 底物 OPBE, 5mol/L 辅酶 NADPH 和 0.1 mol/L 不同 pH 值缓冲液, 在 30 °C 恒温 2 min, 分别加入等量的电泳纯 IolS 酶液测定其酶活, 考察该酶的最适 pH。以所测定不同 pH 缓冲液中的最高酶活作为 100% 酶活对照, 其它 pH 条件下所测酶活以对照酶活的百分比表示。

将电泳纯 IolS 酶液在不同 pH (4~10) 缓冲溶液中, 于 4 °C 分别放置 24 h 后, 取等量的酶液测定残余酶活, 考察该酶的 pH 稳定性。以 4 °C, pH 6.0 条件下新鲜电泳纯酶液的酶活作为 100% 酶活对照, 其它 pH 条件下酶活以对照酶活的百分比表示。

1.8 GDH 和 IolS 的共表达

为同时实现 GDH 和 IolS 在重组大肠杆菌中的

图1 大肠杆菌重组质粒 pET-G-SD-I 结构图

Fig. 1. Structure of coexpression plasmid pET-G-SD-I.

高水平表达, 本文探索了三种共表达方式.

(1) 构建重组质粒 pET-G-SD-I (见图 1). 首先以 pET20b-iolS 为模板进行 PCR 扩增, 上游引物为 SDf, 下游引物为 IolSr, PCR 产物为 5'端带有 SD 序列的 IolS 基因. 然后将扩增产物按 1.2 节中的方法克隆到 pET24a-gdh 中. 最后将重组质粒 pET-G-SD-I 转入大肠杆菌 BL21(DE3) 中.

(2) 构建重组质粒 pET-G-T7-I (见图 2). 首先以 pET20b-iolS 为模板进行 PCR 扩增, 上游引物为 T7f, 下游引物为 IolSr, PCR 产物为 5'端带有 T7 启动子和 rbs 序列的 IolS 基因. 然后将扩增产物按照 1.2 节中的方法克隆到 pET24a-gdh 中. 最后将重组质粒 pET-G-T7-I 转入大肠杆菌 BL21(DE3) 中.

图2 大肠杆菌重组质粒 pET-G-T7-I 结构图

Fig. 2. Structure of coexpression plasmid pET-G-T7-I.

(3) 将 pET20b-iolS 和 pET24a-gdh 同时转化入大肠杆菌 BL21(DE3) 中, 将此重组菌株培养于含有卡那霉素和氨苄青霉素的 LB 液体培养基中.

1.9 诱导表达条件的优化

诱导方法同 1.2 节对重组大肠杆菌 BL21(DE3)/pET24a-gdh 以及大肠杆菌 BL21(DE3)/pET20b-iolS 考察诱导温度 (15, 20, 25, 30, 35 和 40 °C) 和诱导剂

量 (IPTG 终浓度为 0.2, 0.4, 0.6, 0.8, 1.0 和 1.2 mmol/L) 对 GDH 和 IolS 酶活的影响.

1.10 共表达重组菌催化 OPBE 的不对称还原

利用大肠杆菌 BL21(DE3)/pET-G-T7-I 作为转化反应的重组菌. 取 2 g 洗涤后的湿菌体悬浮于 20 ml, 100 mmol/L 磷酸钾缓冲液 (pH 6.0) 中, 加入 200 mmol/L 的 D-葡萄糖, 50 mmol/L 的 OPBE 和 0.05 mmol/L 的 NADP^+ . 于 30 °C, 220 r/min 摇床震荡反应 15 h. 反应结束后, 离心分离菌体, 上清液用乙酸乙酯萃取 3 次, 加入少量无水 MgSO_4 干燥过夜, 供气相色谱分析^[2].

2 结果与讨论

2.1 gdh 的克隆表达及诱导条件优化

按照方法 1.2 节扩增获得 GDH 基因. 序列分析显示 gdh 的大小为 783 bp, 编码 260 个氨基酸, 酶分子量大小约为 28 kDa. 核酸序列与 GenBank 中登记号为 M12276 的葡萄糖脱氢酶基因序列相似性为 98%, 蛋白序列与 GenBank 中登记号为 AAA22463.1 的葡萄糖脱氢酶序列相似性为 100%. SDS-PAGE 电泳结果见图 3, 大肠杆菌 BL21(DE3)/pET24a-gdh 经诱导表达后在 28 kDa 处有明显的蛋白条带, 表明 GDH 在重组菌中得到了高效表达.

图3 重组表达 GDH 和 IolS 的 SDS-PAGE 电泳图谱

Fig. 3. SDS-PAGE analysis of recombinant expression of GDH and IolS. Lane 1: protein molecular weight marker; Lane 2: cell-free extract of *E. coli* BL21(DE3); Lane 3: cell-free extract of *E. coli* BL21(DE3)/pET24a-gdh; Lane 4: cell-free extract of *E. coli* BL21(DE3)/pET20b-iolS.

按照 1.9 节方法所述培养大肠杆菌 BL21(DE3)/pET24a-gdh, 加入 IPTG (终浓度为 0.4 mmol/L) 后分别在 15, 20, 25, 30, 35 和 40 °C 诱导 4 h 后测定酶活. 结果如图 4 所示, 30 °C 诱导 GDH 时酶活最高.

表 3 不同来源羧基还原酶酶活、产率及对映体选择性的比较

Table 3 Comparison of carbonyl reductases of different origins

Strain	Gene	Activity (U/mg)	Cofactor-dependency	Yield (%)	ee (%)
<i>Bacillus Subtilis</i>	<i>ycsN</i>	0.41	NADH	25.2%	92.2%
	<i>iolS</i>	1.45	NADPH	89.6%	99.5%
	<i>yccK</i>	0	—	ND	ND
<i>E. coli</i> K12	<i>ydhF</i>	0.012	NADH	ND	ND
	<i>yajO</i>	0.027	NADPH	ND	ND

ND means not detected.

因此在最适温度 30 °C 下, 分别加入终浓度为 0.2, 0.4, 0.6, 0.8, 1.0 和 1.2 mmol/L 的 IPTG, 诱导 4 h 后测定酶活. 结果如图 4 所示, IPTG 终浓度 0.8 mmol/L 时 GDH 酶活最高. 最终确定诱导条件为诱导温度是 30 °C, IPTG 终浓度为 0.8 mmol/L, 粗酶液中比酶活 9.5 U/mg.

图 4 诱导温度 (a) 和 IPTG 浓度 (b) 对大肠杆菌重组表达 GDH 酶活的影响

Fig. 4. Effects of inducing temperature (a) and IPTG concentration (b) on recombinant GDH activity.

2.2 羧基还原酶的筛选、克隆表达及诱导条件优化

为获得高效不对称还原 OPBE 生成 (R)-HPBE 的羧基还原酶基因, 本文按 1.3 节所述方法对 5 种不同来源的羧基还原酶进行了克隆表达, 并按 1.4 节和 1.5 节所述方法考察了它们的酶活、产率及对映体选择性, 结果见表 3. 可以看出其中来源于枯草芽孢杆菌的羧基还原酶 *IolS* 表现出了较高的催化

能力和对映选择性. 序列分析显示 *iolS* 基因的长度为 933 bp, 编码 310 个氨基酸, 酶分子量大小约为 36 kDa, 属于醛酮还原酶超家族. 此序列已提交至 GenBank, 登记号为 JQ782389. SDS-PAGE 电泳结果见图 3, 大肠杆菌 BL21(DE3)/pET20b-*iolS* 经诱导表达后在 36 kDa 处有明显的蛋白条带, 表明 *IolS* 在重组菌中得到了高效表达.

按照 1.9 节方法接种大肠杆菌 BL21(DE3)/pET20b-*iolS*, 加入终浓度为 0.4 mmol/L 的 IPTG 后分别在 15, 20, 25, 30, 35 和 40 °C 诱导 4 h 后测定酶活. 结果如图 5(a) 所示, 在 25 °C 诱导 *IolS* 酶活最高. 因此在 25 °C 分别加入终浓度为 0.2, 0.4, 0.6,

图 5 诱导温度 (a) 和 IPTG 浓度 (b) 对大肠杆菌重组表达 *IolS* 酶活的影响

Fig. 5. Effects of inducing temperature (a) and IPTG concentration (b) on recombinant *IolS* activity.

0.8, 1.0 和 1.2 mmol/L 的 IPTG, 诱导 4 h 后测定酶活. 结果如图 5(b) 所示, IPTG 终浓度为 0.4 mmol/L 时 IolS 酶活最高. 最终确定诱导条件为 25 °C, IPTG 终浓度 0.4 mmol/L, 粗酶液中比酶活 1.5 U/mg.

2.3 羧基还原酶 IolS 的纯化

利用镍柱对 IolS 进行纯化, 获得单一条带 (见图 6). 纯化后 IolS 的比酶活为 5.1 U/mg, 是粗酶液比酶活的 3.4 倍.

图 6 IolS 纯化的 SDS-PAGE 电泳图

Fig. 6. SDS-PAGE analysis of purified IolS. Lane 1: protein molecular weight marker; Lane 2: cell-free extract of *E. coli* BL21(DE3)/pET20b-*iolS*; Lane 3: purified enzyme.

2.4 羧基还原酶 IolS 的酶学性质

该羧基还原酶 IolS 的最适温度和热稳定性如图 7 所示. 可以看出, 随温度的升高, 酶活也逐渐升

图 7 IolS 纯酶的最适温度和热稳定性

Fig. 7. Optimum temperature and thermostability of purified IolS. Enzyme assay was performed using standard assay procedure: 1 mmol/L OPBE and 5 mmol/L NADPH at various temperature conditions. Thermostability of IolS was measured after pre-incubation at various temperatures for 1 h. Activity at pH 6.0 and 4 °C without pre-incubation was taken as 100%.

图 8 IolS 纯酶的最适 pH 和 pH 稳定性

Fig. 8. Optimum pH (a) and pH stability (b) of purified IolS.

高; 当温度高于 30 °C 后, 酶活开始下降, 可见, 最适温度为 30 °C. 另外, 该酶在 40 °C 以下保温 1 h, 酶活损失不明显, 可保持原有活力的 80% 以上, 说明该酶具有一定的热稳定性. 但当温度高于 40 °C 时, 酶的失活较为严重, 70 °C 时的酶活为 0.

羧基还原酶 IolS 的最适 pH 和 pH 稳定性如图 8 所示. 可以看出, 该酶催化还原反应的最适 pH 为 6.0; 在 pH = 5.5–7.0 的缓冲液中较稳定, 24 h 后可以保留原有酶活的 75% 以上.

2.5 GDH 和 IolS 的共表达

大肠杆菌 BL21(DE3) 中三种不同共表达方式的 GDH 和 IolS 酶活见表 4. 可以看出, 携带有共表达质粒 pET-G-SD-I 的大肠杆菌具有最高的 GDH 酶活, 粗酶液中的比酶活为 4.8 U/mg, 但同时 IolS 的比酶活是三种共表达方式中最低的, 仅有 0.5 U/mg.

表 4 大肠杆菌中不同共表达方式对 GDH 和 IolS 酶活的影响

Table 4 Effect of different coexpression strategies on GDH and IolS activity

Coexpression	GDH activity (U/mg)	IolS activity (U/mg)
pET-G-T7-I	1.5	1.5
pET-G-SD-I	4.8	0.5
Two plasmids	3.4	0.9

Induction conditions: recombinant *E. coli* cells were induced in LB medium with 0.8 mmol/L IPTG for 4 h at 25 °C and 220 r/min.

两种质粒同时转入大肠杆菌中的重组菌粗酶液中 GDH 和 IolS 比酶活分别为 3.4 和 0.9 U/mg. 携带有共表达质粒 pET-G-T7-I 的重组菌表现出最高的 IolS 酶活, 粗酶液中 GDH 和 IolS 两者的比酶活均为 1.5 U/mg. 对携带 pET-G-T7-I 重组菌的诱导条件优化结果显示: IPTG 浓度为 0.8 mmol/L, 诱导温度为 25 °C 时, 有利于 GDH 和 IolS 两者的比酶活同时达到较高的水平 (数据未给出). 由于羧基还原酶 IolS 是转化反应中最关键的酶, 其酶活与 GDH 相当有利于反应的进行和辅酶的再生, 因此选择了共表达质粒 pET-G-T7-I 作为进一步研究的对象. 从转录翻译的角度分析, 由于 IolS 基因上游具有独立的 T7 启动子和 rbs 序列, 重组质粒 pET-G-T7-I 可以转录两条 mRNA, 其中一条 mRNA 含有 GDH 和 IolS 两种酶的编码信息, 另一条 mRNA 只含有 IolS 的编码信息, 两条 mRNA 都能够正常翻译, 也就是说 IolS 可以被转录和翻译两次, 所以蛋白含量增加, 酶活也相应升高, 也与本文结果相符. SDS-PAGE 电泳结果见图 9 所示, 大肠杆菌 BL21(DE3)/pET-G-T7-I 经诱导表达后在 28 和 36 kDa 处有明显的蛋白条带, 表明 IolS 和 GDH 在重组菌中均得到高效表达.

图 9 *E. coli* BL21(DE3)/pET-G-T7-I 重组蛋白表达的 SDS-PAGE 电泳图

Fig. 9. SDS-PAGE analysis of recombinant protein expression of *E. coli* BL21(DE3)/pET-G-T7-I. Lane 1: protein molecular weight marker; Lane 2: soluble proteins of *E. coli* BL21(DE3); Lane 3: whole cell extract of *E. coli* BL21(DE3)/pET-G-T7-I; Lane 4: soluble proteins of *E. coli* BL21(DE3)/pET-G-T7-I.

2.6 重组菌催化 OPBE 的不对称还原

将重组大肠杆菌 BL21(DE3)/pET-G-T7-I 诱导表达后, 收集菌体, 利用菌悬液转化 OPBE. 在单水相中, 底物浓度为 10 g/L 时, 反应进程曲线见图 10. 可以看出, 反应约 13 h 基本达平衡, ee 值始终保持

图 10 *E. coli* BL21(DE3)/pET-G-T7-I 不对称还原 OPBE 的反应时间曲线

Fig. 10. Time course of the asymmetric reduction of OPBE by recombinant *E. coli* BL21(DE3)/pET-G-T7-I.

在 99.5% 左右, 转化率大于 99%.

2.7 讨论

本文筛选到一种来源于枯草芽孢杆菌的羧基还原酶 IolS, 它具有以很高的立体选择性 (ee 值为 99.5%) 不对称还原 OPBE 生成 (*R*)-HPBE 的催化活性, 是目前基因工程菌报道中较高的, 并对该酶进行了纯化以及酶学性质的研究. 为解决重组菌株还原反应中的辅酶循环问题, 采用了羧基还原酶基因 *iolS* 与葡萄糖脱氢酶基因 *gdh* 共表达的方法. 目前有三种常用的共表达方法, 比较常见的是在一个质粒上串联表达 2 个基因, 在这 2 个基因之间可以添加 SD 序列, T7 启动子等特殊序列^[18,19]. 第二种方法是将分别含有不同抗性, 不同目的基因的质粒同时转入一个宿主细胞中, 以实现两种蛋白的同时表达^[20,21]. 第三种方法是通过引物的设计在两个基因间加入能翻译出连接蛋白的核酸序列, 翻译出一个新的融合蛋白, 这个双功能蛋白既可以完成底物的转化又可以催化辅酶循环再生^[22]. 本文对前两种方法进行了比较分析, 并在串联基因时采用了不同的策略. 结果表明, 串联基因时在两基因间加入 T7 启动子和 rbs 序列有利于下游 IolS 基因的表达. 利用构建的重组菌株 *E. coli* BL21(DE3)/pET-G-T7-I 在水相中催化 OPBE 的不对称还原反应只需在反应起始时添加极少量的辅酶 (<0.05 mmol/L NADP⁺), 降低了生产成本. 为进一步提高反应效率和底物浓度, 目前正在进行反应体系的研究, 包括水/有机溶剂两相反应体系的选择、反应中 pH 的调控以及底物的流加方式等.

3 结论

综上, 本研究克隆了来自于枯草芽孢杆菌的羧基还原酶基因 *iolS* 和葡萄糖脱氢酶基因 *gdh*, 并对其共表达方法进行了探索, 构建了可应用于高立体选择性还原 OPBE 制备 (*R*)-HPBE 的重组大肠杆菌, 实现了辅酶的内部循环, 为实现生物催化法制备 (*R*)-HPBE 的工业化生产奠定了基础。

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英 译 文

English Text

Angiotensin-converting enzyme (ACE) inhibitors are widely used for the treatment of hypertension, congestive heart failure, and other cardiovascular diseases because they can reduce the Angiotensin II receptor (AngII) to control blood pressure and protect target organs [1]. (*R*)-2-hydroxy-4-phenylbutyrate (HPBE) is an important chiral intermediate for the synthesis of ACE inhibitors such as benazepril, enalapril, and lisinopril [2]. Various approaches for the preparation of (*R*)-HPBE have been reported in recent years, including chemical and biological methods. Both of these can be carried out in two ways: resolution and synthesis. The chemical method usually involves multiple steps and a large amount of organic reagents [3]. In the case of resolution, the theoretical maximum yield is only 50% [4–6]. The microbial reduction of ethyl 2-oxo-4-phenyl -butyrate (OPBE) to (*R*)-HPBE has been intensively studied in the last decade. As compared with a recombinant whole-cell catalyst, wild strains produce (*R*)-HPBE in relatively lower enantiomeric excess (*ee*) due to their multiple intracellular carbonyl reductases that have variable or opposite stereospecificity [7–9]. Previously, a highly potent carbonyl reductase-producing strain *Candida krusei* SW 2026 was isolated in our laboratory, which catalyzed the enantioselective reduction of OPBE to (*R*)-HPBE. At 20 g/L of OPBE, the *ee* and yield were 97.4% and 82.0%, respectively. The carbonyl reductase was purified to homogeneity using three chromatograph columns and its enzyme activity properties were investigated [10, 11]. There have been few reports on the asymmetric reduction of OPBE to (*R*)-HPBE catalyzed by recombinant strains so far. Shen et al. [12] were successful in getting (*R*)-HPBE with good *ee* and yield (99% and 100%, respectively) at 1 mol/L (approximately 206 g/L) of OPBE using recombinant *E. coli* strain with both CgKR2 and GDH encoding genes. Since the expensive cofactor NAD(P)H was required for the oxidoreduction process, in situ cofactor regeneration is critical for the economics of the industrial scale biotransformation using the recombinant strain.

The use of a coexpression system solves the problem of cofactor regeneration. The technology enables the expression of a number of heterologous proteins within the same strain. Glucose dehydrogenase (GDH) has been widely used for co-enzyme regeneration to recycle the nicotinamide coen-

zyme NAD(P)H from NAD(P)⁺ [13–15]. In this study, three methods were used for the construction of a two-enzyme coexpression system comprising the carbonyl reductase gene (*iolS*) and glucose dehydrogenase gene (*gdh*) from *Bacillus subtilis*. The bioconversion catalyzed by the recombinant whole cells were achieved in aqueous system

1 Experimental

1.1 Strains, plasmids, and chemicals

The strains and plasmids used in this study are listed in Table 1. Restriction enzymes and T4 ligase were purchased from TaKaRa Biotechnology Co. PCR related products were purchased from Dongsheng Biotech Co., China. (*R*)-HPBE and OPBE were purchased from Sigma-Aldrich Chemical Co. NADP⁺ and NADPH were purchased from Sangon Biotech Co., China. All other reagents and solvents were from local commercial suppliers and were of analytical grade or biochemical reagents.

1.2 Cloning and expression of glucose dehydrogenase

Genomic DNA was isolated from *Bacillus subtilis* using a bacterial genomic DNA extraction kit. Two oligonucleotide primers for the amplification of the full length *gdh* were designed based on its nucleotide sequence (GenBank No. M12276). These are listed in Table 2. The amplified DNA fragment was ligated into the pMD18-T to create the recombinant plasmid pMD18-T-*gdh*. DNA sequencing was performed by Sangon Biotech Co. The pMD18-T-*gdh* was double digested with *Nde* I and *Hind* III and then inserted into the expression vector pET-24a. The resulting plasmid, pET24a-*gdh*, was transformed into *E. coli* BL21(DE3), and positive clones were screened and confirmed by colony PCR and restriction enzyme digestion. The expression of the GDH was induced with 0.4 mmol/L IPTG when the OD₆₀₀ reached 0.6, and the incubation temperature was changed to 25 °C for the induction of protein expression. Then the cells were harvested after 4 h. Protein concentration was determined by SDS-PAGE analysis performed according to standard procedures [16].

1.3 Screening, cloning, and expression of carbonyl reductases

The genomic DNA of *Bacillus subtilis* and *E. coli* K12 was isolated as described above. The primers designed based on the nucleotide sequences of the various carbonyl reductases (CRs) are listed in Table 2. The CR encoding genes *ycsN* (NP_388296.1), *iolS* (AL009126.3), and *yccK* (NC_000964) were from *Bacillus subtilis*, while *ydhF* (P76187.2) and *yajO*

(P77735.2) were from *E. coli* K12. The cloning, expression, and analysis methods were the same as described for the *gdh* gene in section 1.2.

1.4 Enzyme activity assays

Dehydrogenase and carbonyl reductase activities were determined spectrophotometrically by monitoring the increase or decrease in the absorption of NADPH at 340 nm and 30 °C. The reaction mixture for GDH comprised 75 mmol/L Tris-HCl (pH 8.0), 2.0 mmol/L NADP⁺, 0.1 mmol/L glucose, and an appropriate amount of enzyme in a final volume of 0.25 ml. The reaction mixture for CR was composed of 100 mmol/L phosphate buffer (pH 6.0), 1.0 mmol/L OPBE, 5.0 mmol/L NADPH, and an appropriate amount of enzyme in a final volume of 0.25 ml. One unit of activity was defined as the amount of enzyme required for catalyzing the oxidation of 1 μmol of NADPH per minute (CR) or reduction of 1 μmol NADP⁺ per minute (GDH). The protein concentration was measured by the Bradford method using bovine serum albumin as the standard.

1.5 Bioreduction of OPBE to (*R*)-HPBE

A 2.5 ml reaction mixture containing 100 mmol/L potassium phosphate buffer (pH 6.0), OPBE (20 mmol/L), NAD(P)H (10 mmol/L), and an appropriate amount of enzyme was incubated at 30 °C and 220 r/min for 12 h. Then the mixture was centrifuged and the supernatant was extracted three times with ethyl acetate, which was followed by drying over anhydrous MgSO₄ for further GC analysis.

1.6 Protein purification

The induced recombinant cells were harvested and suspended in buffer A (20 mmol/L sodium phosphate, pH 7.4, containing 500 mmol/L NaCl, and 5 mmol/L imidazole). The cells were disrupted by ultrasonication (285 W, pulse 1 s, pause 3 s) for 10 min. Cell debris was removed by centrifugation at 8000 g for 15 min at 4 °C, and the supernatant was sent to a HisTrap-FF crude chelating affinity column equilibrated with buffer A. The bound enzyme was eluted by a stepwise gradient of imidazole concentration, from buffer A containing 5 mmol/L imidazole to the same buffer containing 1000 mmol/L imidazole. The protein samples were analyzed by SDS-PAGE.

1.7 Effects of pH and temperature on purified enzyme

The optimum temperature was determined by incubation in the range of 10 to 70 °C. To determine the optimum pH, activities were assayed in 100 mmol/L buffers with various

pH (4.0–10): sodium citrate buffer (pH 4.0–6.0), sodium phosphate buffer (pH 6.0–8.5), and glycine-NaOH buffer (pH 8.5–10). For thermal stability, aliquots of the purified enzyme were incubated at temperatures in the range of 10–60 °C in 100 mmol/L phosphate buffer (pH 6.0) for 1 h followed by cooling on ice before activity assay. The pH stability was determined after incubating the purified enzyme in the same buffers with different pH values (4.0–9.0) for 24 h at 4 °C. All activities were measured under the standard conditions described in section 1.4.

1.8 Coexpression of IolS and GDH

To achieve a high level expression of both IolS and GDH, three coexpression strategies were attempted in this study. The first method was to construct recombinant plasmid pET-G-SD-I (Fig. 1). The forward primer (SDf) and reverse primer (IolSr) were designed for PCR using pET20b-*iolS* as template. The resulting fragment, *iolS* gene with SD sequence at 5' end, was cloned into pET24a-*gdh* to give a two-enzyme coexpression plasmid pET-G-SD-I. A second method was to construct recombinant plasmid pET-G-T7-I (Fig. 2). The forward primer (T7f) and the reverse primer (IolSr) were designed for PCR using pET20b-*iolS* as the template. The resulting fragment, *iolS* gene with T7 promoter and rbs sequence at 5' end, was cloned into the pET24a-*gdh* to give a two-enzyme coexpression plasmid pET-G-T7-I. In the third method, both pET-20b-*iolS* and pET-24a-*gdh* were transformed into *E. coli* BL21(DE3) to result in a two-plasmid system. The strain was grown on a LB plate containing ampicillin and kanamycin.

1.9 Optimization of expression conditions

E. coli BL21(DE3)/pET24a-*gdh* and *E. coli* BL21(DE3)/pET20b-*iolS* were induced at different temperatures (15, 20, 25, 30, 35, and 40 °C) and different concentrations of IPTG (final concentration of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/L).

1.10 Bioreduction of OPBE to (*R*)-HPBE catalyzed by recombinant *E. coli*

The reaction mixture (20 ml) containing 100 mmol/L potassium phosphate buffer (pH 6.0), NAD⁺ (0.05 mmol/L), glucose (200 mmol/L), OPBE (50 mmol/L), and 2 g of wet cells was incubated at 30 °C and 220 r/min for 15 h. After the reaction, the supernatant was extracted three times with ethyl acetate and dried over anhydrous MgSO₄ for further GC analysis.

2 Results and discussion

2.1 Cloning and expression of GDH and optimization of inducing conditions

The sequence analysis indicated that the *gdh* (783 bp) encoded 260 amino acids. The calculated molecular mass of GDH was 28 kDa. The cloned *gdh* showed 98% similarity with the nucleotide sequence of *B. subtilis* *gdh* (GenBank No. M12276), and 100% similarity with its amino acid sequence. Figure 3 shows the SDS-PAGE of the cell-free extract of *E. coli* BL21(DE3)/pET24a-*gdh*. The recombinant GDH product was observed at 28 kDa, which was consistent with our prediction based on the gene sequence.

The effects of IPTG concentration and temperature on enzyme activity of GDH are shown in Fig. 4. The optimal activity was reached at the IPTG concentration of 0.8 mmol/L and 30 °C, and the enzyme activity of GDH was 9.5 U/mg.

2.2 Screening, cloning, and expression of CR genes and optimization of induction conditions

To obtain new reductases capable of reducing OPBE to (*R*)-HPBE with a high product *ee* and yield, five kinds of carbonyl reductases of different origins were cloned and their enzyme activities were investigated (Table 3). The IolS from *Bacillus subtilis* exhibited the highest enzyme activity and enantioselectivity. The sequence analysis indicated that the *iolS* (933 bp) encoded 310 amino acids, and the calculated molecular mass of IolS was 36 kDa. IolS belongs to the Aldo-keto reductases (AKRs) superfamily. The nucleotide sequences of *iolS* have been deposited in GenBank with the accession No. JQ782389. Figure 3 shows the SDS-PAGE of the cell-free extract of *E. coli* BL21(DE3)/pET20b-*iolS*. The expressed recombinant IolS product was observed at about 36 kDa, which agreed with the prediction from the gene sequence.

The effects of IPTG concentration and temperature on the enzyme activity of IolS are shown in Fig. 5. The optimal activity was achieved at the IPTG concentration of 0.4 mmol/L and 25 °C, and the enzyme activities of GDH were 1.5 U/mg.

2.3 Protein purification

The *N*-terminal His-tagged recombinant enzyme IolS was purified to electrophoretic homogeneity with a HisTrap-FF crude chelating affinity column. The specific activity of purified IolS was 5.1 U/mg, which corresponded to a 3.4 fold increase in purity as compared with the crude extract. Figure 6 shows the SDS-PAGE of the crude extract and target fraction after purification.

2.4 Enzyme activity characterization of carbonyl reductase

The carbonyl reductase showed maximum activity at 30 °C. The activity dropped rapidly when the temperature was increased. The enzyme was extremely weak at temperatures higher than 40 °C, which is demonstrated in Fig. 7. The effect of pH on the enzyme activity was also studied. The results are shown in Fig. 8. The maximum enzyme activity was observed at pH 6.0 (sodium phosphate buffer) and > 75% of the activity was retained between pH 5.5 and 7.0 after 24 h.

2.5 Coexpression of *IolS* and GDH

The *IolS* and GDH activities of *E. coli* BL21(DE3) harboring different plasmids are shown in Table 4. The highest GDH activity was observed with *E. coli* BL21(DE3) harboring pET-G-SD-I, where the GDH activity was 4.8 U/mg. However, a much lower *IolS* activity (0.5 U/mg) was observed. *E. coli* BL21(DE3) with two separate plasmids (pET20b-*iolS* and pET24a-gdh) exhibited *IolS* and GDH activities of 0.9 and 3.4 U/mg, respectively. Of the three strategies used, the highest *IolS* activity (1.5 U/mg) was attained with *E. coli* BL21(DE3) carrying pET-G-T7-I, where GDH showed a comparable activity (1.5 U/mg) to *IolS*.

The induction condition for *E. coli* BL21(DE3)/pET-G-T7-I was optimized, and the results suggested that the IPTG concentration of 0.8 mmol/L and induction temperature of 25 °C gave a high level expression of both GDH and *IolS* (data not shown). Because *IolS* is the key enzyme for the reduction of OPBE, and a similar GDH activity was achieved, *E. coli* BL21(DE3)/pET-G-T7-I was selected as the biocatalyst for the subsequent experiments. From the perspective of transcription and translation, with the use of an independent T7 promoter and a rbs sequence located upstream of *iolS*, pET-G-T7-I can effectively transcript two mRNA: one of which contained the coding information of GDH and *IolS* and the other contained the coding information of *IolS*. Therefore, *iolS* can be transcribed and translated twice, to lead to an enhanced enzyme expression level. This was also consistent with our experimental results. The expressions of GDH and *IolS* are shown in Fig. 9, which indicated that *IolS* and GDH were highly expressed in the recombinant *E. coli* strain carrying pET-G-T7-I.

2.6 Bioreduction of OPBE to (*R*)-HPBE

The time course of the bioreduction of OPBE to (*R*)-HPBE in an aqueous system catalyzed by *E. coli* BL21(DE3)/pET-G-T7-I is shown in Fig. 10. After 13 h of reaction, over 99% yield and 99.5% *ee* were achieved at 10 g/L of OPBE.

2.7 Discussion

A carbonyl reductase (*IolS*) was identified in *Bacillus subtilis*, which exhibited high enantioselectivity (> 99.5%) in the reduction of OPBE to (*R*)-HPBE. The recombinant *IolS* was purified to homogeneity and characterized. The coexpression of *iolS* and *gdh* was adopted to address the cofactor regeneration issue in the oxidoreductase catalyzed reaction. Two coexpression methods were pursued: the tandem expression of the *gdh/cr* in one expression plasmid, in which the SD sequence, T7 promoter, and other special sequences were inserted between two genes [17,18] and a second method where two separate recombinant plasmids were transformed into *E. coli* cells to construct a two-plasmid system [19,20]. The results suggested the insertion of the T7 promoter and rbs sequence was beneficial for the expression of *iolS*. Only 0.05 mmol/L NADP⁺ was necessary for the bioreduction of OPBE to (*R*)-HPBE catalyzed by *E. coli* BL21(DE3)/pET-G-T7-I in aqueous system, and this resulted in 99% yield and 99.5% *ee* at 10 g/L OPBE. To further enhance the reaction efficiency and substrate concentration, studies on the optimization of the reaction process including substrate feed batch, aqueous/organic biphasic system, and pH regulation were carried out.

3 Conclusions

In summary, a carbonyl reductase gene (*iolS*) and a glucose dehydrogenase (GDH) gene from *Bacillus subtilis* were cloned. A recombinant *E. coli* capable of the highly stereoselective reduction of OPBE to (*R*)-HPBE have been constructed and internal coenzyme regeneration to give a feasible approach for the industrial production of (*R*)-HPBE by biocatalysis.

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