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硕 士 学 位 论 文

一种海洋来源苯丙氨酸脱氢酶的挖掘及酶
学性质的研究

Mining and study on enzymatic properties of a marine
source of phenylalanine dehydrogenase

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厦门大学博硕士论文摘要库

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摘要

苯丙氨酸脱氢酶（PheDH）是一种氧化还原酶，其正反应可催化苯丙氨酸和辅酶 NAD⁺氧化脱氨基形成苯丙酮酸，逆反应可催化苯丙酮酸和氨以及辅酶 NADH 合成苯丙氨酸。此外，PheDH 可用于苯丙酮尿症（PKU）的检测，目前国内测 PKU 的酶试剂盒所需的 PheDH 大多需要进口。独特海洋环境造就具有耐盐、嗜热、嗜冷、耐压和耐酸、耐碱等特性的极端酶。海洋微生物在新基因、新蛋白开发等方面的应用潜力极大。有机溶剂中酶促反应可增加底物溶解度、调整或提高酶的立体选择性甚至可提高酶的热稳定性等众多优点。为了从海洋菌株中挖掘一种耐有机溶剂、耐盐、耐金属离子的 PheDH，本文所研究的主要内容和取得的主要结果如下：

一、目标菌株的筛选。从实验室保存的 90 株海洋菌株出发，通过菌株培养，粗酶液酶活的测定，筛选出 15 株具有 PheDH 酶活的野生型菌株。其中野生菌 *Bacillus nanhaiensis* 粗酶液酶活最高，酶活达到 0.940 ± 0.04 U/mg，故以 *Bacillus nanhaiensis* 作为目标菌株。

二、构建表达 PheDH 的重组大肠杆菌。通过 NCBI 数据库，在 *Bacillus* 属中搜索出 12 条 PheDH 基因，由同源性比对，设计 6 对引物，以 *Bacillus nanhaiensis* 基因组为模板，克隆出 PheDH 基因，经 *Nde* I 和 *Xho* I 双酶切，并连接至载体 pET28a，转化至 *E.coli* BL21（DE3），得到了能够表达 PheDH 的重组大肠杆菌。

三、考察 PheDH 酶促反应最适温度、温度稳定性及最适 pH、pH 的稳定性。经镍柱纯化得到 PheDH，其酶促反应最适温度为 40 °C，45 °C 以下孵育 1 h，酶活都能保持在 80 % 以上；最适酶促反应 pH 为 10.0，不同 pH 的缓冲溶液，50 °C 孵育 1 h，PheDH 在 pH=7.5 时最为稳定。

四、测定 PheDH 的耐有机溶剂性及催化反应动力学参数。分别测定体积浓度为 10 %、20 %、30 % 的甲醇、乙醇、乙二醇、异丙醇、乙二醇单乙醚、乙腈、

丙酮的有机溶剂中 PheDH 的酶活及酶活稳定性, 结果显示在体积浓度为 10 %、20 %、30 % 的甲醇、乙醇、乙二醇、乙二醇单乙醚、丙酮的有机溶剂中酶活保留 80 % 以上, 而在体积浓度为 10 %、20 %、30 % 的甲醇、乙醇、乙二醇、乙二醇单乙醚、丙酮的有机溶剂中酶活稳定性增加。计算出在不含有机溶剂和含 30 % 的甲醇或 30 % 的乙二醇单乙醚中的 PheDH 的催化反应动力学参数。

五、PheDH 的耐盐性质的考察。加入 NaCl, 使反应体系中 NaCl 的终浓度为 0、1、2、3、4 M, 测定 PheDH 的酶活及酶活稳定性, 当盐离子终浓度为 2 M 时, 酶活达到最大, 是不加盐离子时的 1.2 倍。25 °C, 200 rpm 的摇床内孵育 24 h, 加入 2 M NaCl 体系的剩余酶活约为不加 NaCl 体系剩余酶活的 2 倍, 酶活稳定性也得到提高。

六、PheDH 的耐金属离子性质的考察。分别测定 K^+ 、 Ni^{2+} 、 Cd^{2+} 、 Sr^{2+} 、 Cu^{2+} 、 Mg^{2+} 、 Co^{2+} 、 Ba^{2+} 、 Ca^{2+} 、 Mn^{2+} 、 Zn^{2+} 和 Al^{3+} 等 12 种金属离子对 PheDH 酶活的影响, 其中当 Mn^{2+} 终浓度为 20 mM 时, 酶活达到最大, 为原始酶活的 2.2 倍。

关键词: 苯丙氨酸脱氢酶; 蛋白表达; 有机溶剂; 耐盐

Abstract

Phenylalanine dehydrogenase (PheDH) is an oxidoreductase, which can catalyze the oxidation deamination of phenylalanine into phenylpyruvic acid and coenzyme NAD⁺ reduction for the forward reaction, the reductive amination of phenylpyruvic acid into phenylalanine and the oxidation of NADH reduction for the backward reaction. It had been applied for the diagnosis of phenylketonuria (PKU). However, the PheDH for producing the kit was mostly imported from foreign companies. The unique marine environment has rendered the enzyme to evolve special characteristics like salt-resistant, thermophilic, cold-adapted, high pressure, acid, alkali resistance, etc. Marine microorganisms have great potential for applications in new genes, proteins discovery and other aspects of development. Enzymatic reactions in organic solvent have many advantages such as improving stereoselective, increasing substrate solubility and improving the thermal stability of enzymes. To develop a novel PheDH with organic solvents resistances, salt tolerance and metal ion resistances from ocean strains, the main content of this paper and the main results obtained are as follows.

Screening of target strains. Ninety ocean strains from our laboratory were cultured, collected, ultrasonicated, and measured for crude PheDH activity. We found that 15 wild-type strains exhibited PheDH activity. Among them, *Bacillus nanhaiensis* had the highest activity which reached 0.940 ± 0.04 U / mg. Therefore, *Bacillus nanhaiensis* was chosen to be our target strains for the next experiments.

Construction of recombinant *E. coli* for the expression of PheDH. 12 PheDH genes from *Bacillus* genus was obtained using NCBI database. Six pairs of primers were designed after homology alignment. PheDH gene was cloned using *Bacillus nanhaiensis* genome as template. PheDH gene was digested by *Nde* I and *Xho* I, ligated with vector pET28a and transformed to BL21(DE3),

Investigation of the optimal temperature, thermal stability, optimal pH and pH stability. PheDH was purified by nickel column and investigated for the optimum

temperature, thermostability, optimum pH and pH stability. We found that the optimum temperature was 40 °C. PheDH retained over 80 % of initial activity after incubation for 1 h below 45 °C. The optimum pH for the enzymatic reaction was 10.0. PheDH exhibited best pH stability at pH=7.5 when incubated at 50 °C.

Determination of organic solvent resistance and kinetic parameters. PheDH activity and stability were measured in 10 % (V/V), 20 % (V/V) and 30 % (V/V) organic solvents including methanol, ethanol, ethylene glycol, isopropanol, ethylene glycol monomethyl ether, acetonitrile and acetone. The results showed that PheDH can retain above 80 % activity with 10 %, 20 % and 30 % methanol, ethanol, ethylene glycol, ethylene glycol monomethyl ether and acetone. The enzyme stability was greatly increased with 10 %, 20 % and 30 % methanol, ethanol, ethylene glycol, ethylene glycol monomethyl ether and acetone. K_m , V_{max} , K_{cat} , K_{cat} / K_m of PheDH without organic solvents, and 30 % methanol and 30 % ethylene glycol monomethyl ether were determined and compared.

Determination of the salt tolerance of PheDH. The salt tolerance of PheDH was evaluated by adding NaCl in the PheDH reaction mixture with a final concentration of 0 M, 1 M, 2 M, 3 M, and 4 M. When 2 M NaCl was added, PheDH exhibited highest activity which was 1.2-folds of that of PheDH without NaCl. When 2 M NaCl was added, the stability of PheDH was greatly enhanced.

The effect of metal ions on PheDH activity. Metal ions including K^+ , Ni^{2+} , Cd^{2+} , Sr^{2+} , Cu^{2+} , Mg^{2+} , Co^{2+} , Ba^{2+} , Ca^{2+} , Mn^{2+} , Zn^{2+} and Al^{3+} were added in the PheDH reaction mixture to investigate the effect of metal ions on PheDH activity. The results showed that Mn^{2+} could increase the activity of PheDH. When 20 mM Mn^{2+} was added, PheDH activity was 2.2-folds of that of the original activity.

Key words: Phenylalanine dehydrogenase; protein expression; organic solvent; salt-resistant

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