Cloning And Expression Of 3-Hydroxybutyrate Dehydrogenase Gene From Locally Isolated *Pseudomonas* sp.

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Abstract

The 3-HBDH gene from *Pseudomonas* sp. was amplified, cloned, and sequenced. The deduced amino acid sequence was highly matched with 3-HBDH sequences in DDBJ/GenBank/EMBL. But *Pseudomonas* sp. 3-HBDH was lack of the C-terminal region found in mammalian enzymes containing a lipid-binding domain that is important for the 3-HBDH's activity. The *E. coli* XL1 Blue cells transformed with the resultant plasmid, pBDH-1 showed 3-HBDH's activity. The nucleotide sequence of recombinant pBDH-1 indicate functional oligomers composed of subunits of 255 amino acid with a calculated M_r of 26,855 Da and proved on SDS-PAGE. Ammonium sulfate fractionation resulted in low yield of the enzyme but its activity was comparably high with other 3-HBDHs. The recombinant enzyme showed a broad range of stability with respect to pH and temperature.

Introduction

3-hydroxybutyrate dehydrogenase (3-HBDH) (EC 1.1.1.30) catalyzes the reversible oxidation of hydroxybutyrate to acetoacetate, utilizing nicotine adenine dinucleotide as a coenzyme [1] as shown in Figure 1a.

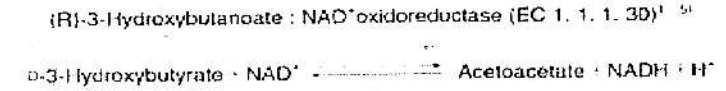

Figure 1a - Reversible reaction mechanism of 3-HBDH. The substrate, 3-hydroxybutyrate acid was oxidized to acetoacetate

This reaction provides a basis for determination of ketone bodies, acetoacetate and beta-hydroxybutyric acid. Deficient insulin availability in diabetes causes decrease of glucose intake and increase in ketone bodies formation and may cause diabetic ketoacidosis (DKA) [2]. To prevent this, the concentration of ketone bodies in blood or urine can be monitored with Williamson method [3], diazonium salt method [4] and the latest keto-film method [5] using 3-HBDH as part of the determination procedures. For a long time, sequence information was limited to mammalian 3-HBDHs such as human heart [6], bovine heart [7] and rat liver mitochondriaalthough 3-HBDHs from a number of bacterial sources had been partially purified such as *Rhodopseudomonas spheroids* [1] and *Paracoccus denitrificans*[(9]. Sequence analysis revealed that mammalian 3-HBDH is found belongs to the superfamily of short-chain dehydrogenases/reductases, which consists of coenzyme binding followed by the catalytic domain at N-terminai. In this study, we present the cloning and sequencing of 3-HBDH isolated from *Pseudomonas* sp. locally isolated from soil, as well as partial purification and characterization of the recombinant protein expressed in E.coli.

Materials And Methods

Pseudomonas sp. was isolated from soil and stored in a glycerin stock (-80C). *E. coli* XL1-blue was used as host , pGEMTeasy, and pKK223-2 were used as vectors. Large scale cultivation was carried out in Nutrient broth (1% meat extract, 1% polypeptone, 0.5% NaCl). *Pseudomonas* sp. Chromosomal DNA was extracted by a standard phenol extraction method. The degenerated oligoneucleotides were designed on the basis of conserved amino acid sequence among the reported bacterial 3-HBDHs. The primers were combined and by trial and error, changing the annealing temperature of the PCR condition, PCR was carried out in 50 µl reaction mixture containing GC buffer II (Takara). PCR products were cloned into pGEM-Teasy vector system (Promega). Positive clones were used for DNA sequencing by Sanger method using ALFExpress DNA sequencer.

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Recombinant sequence was compared with other 3-HBDHs using Blast program (NCBI) via internet coupled with Genetyx-mac software. *E. coli* XL1-blue harboring pKK/BDH was aerobically cultured in 20 litres of N-broth containing ampicillin at 37°C for 17 hours, using a jar fermentor. Ammonium sulfate fractionation and DEAE-TOYOpearl column were used in partial purification of 3-HBDH recombinant as indicate in Table 1. After each purification steps, fractions identification of 3-HBDH presents, formation of diformazan method consists of reaction mixture (1.0 ml) of 100 mM Tris-HCl (pH 8.5), 0.2% Triton X-100, 25 mM DL-3-hydroxybutyric Na salt, 2 mM NAD+, 0.001% Phenazine Methosulfate (PMS)l, 0.01% nitroblue tetrazolium salt (NBT) and 0.1 ml of enzyme. Thermal and pH stability were tested on partially purified enzyme with temperature incubation range between 0-60 C and pH range of 6 to 9.5.

Table 1 - Partial purification steps of recombinant pBDH-1.

Steps	Total Protein (mg)	Total Activity (Units)	Specific activity (Units/mg)	Yield (%)
Cell extract	97500	1600000	1.6	100
Protamine sulfate treatment	30780	149040	4.8	93
Ammonium sulfate fractionation	2080	19600	9.4	9.6
DEAE-Toyopearl fractionation	48	2660	55	1.6

Results And Discussion

PCR product with expected size of 0.77 kbp which has a EcoRI and HindIII site at each forward and reverse primer was produced. The PCR condition consisted of 1 cycle of denaturation for 5 min at 94°C, 27 cycles of denaturation at 95°C for 45 s, annealing at 62°C for 45 s and extension at 72°C for 1 min. The reaction for primer extension continued for 7 min. The PCR product were clone with pGEMTeasy vector and positive clones were sequenced. The open reading frame is found to be 765 nucleotides and encoding 255 amino acid (Figure 1b) residues with a calculated monomer size of 26,855 Da. Fasta search of the recombinant enzyme against the GenBank database yielded almost exclusively members of the short-chain dehydrogenase superfamily (SCAD). *Pseudomonas aeruginosa* 3-HBDH sequence has a very high similarities of 77% in nucleotide and comparable similarities of 72% in amino acid. One of the conserved domain $G_{11}XXXG_{15}XG_{17}$ at the N-terminal is know to be a NADH-binding site for most SCAD. The catalytic center featuring the putative active site residues Ser, Tyr, and Lys are also present. However, the recombinant enzyme is not preceded by a leader peptide nor followed by sequence containing lipid-binding site LPGAISDMIYIR which occurs in most mammalian type 3-HBDH [15]. This suggests that the recombinant enzyme is independent on lipid for its activity. Nevertheless, mammal type 3-HBDH and the recombinant enzyme have quite similar features. For expression in E.coli, the gene was cloned into an expression vector, pKK223-3 featuring a tac promoter. Following a large scale cultivation at 37 C, overnight, the cells were first cell disrupt with Dynomill before applied into purification steps (Table 2). The specific activity is 55 U/mg which is high comparable to all types of 3-HBDHs to date. The SDS-PAGE analysis of the enzyme showed a band of approximately 26 kDa (Figure 2) but the band became unclear after further purification with ammonium sulfate. The partially purified recombinant enzyme was stable up to 40°C and 50% activity was maintained even after the incubation at 55°C for 15 mins (Figure 3). This is a contrast to one wild-type 3-HBDH from *Rhodopseudomonas spheroids*, which lost 70% of activity rapidly at 37°C. The enzyme was stable between pH 7 and pH 9 and maximum activity was shown at pH 8-8.5 (Figure 3).

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PBDH-1          1 ----------------------------------------------------M-LKGKVAL   8
P.Aeruginosa    1 ----------------------------------------------------MTLKGKTAL   9
Human heart     1 MGLPPPPGRFSRLPGKTLSACDRENGARRPLLLGSTSFIPIGRRTYASAAEP-VGSKAVL  59
                    GxxxxGxG
PBDH-1          9 VTG-STGIGLGIATALAAQGADIVLNGFGDAAEIEKAAGLAAQHGVKVLYDGADLSKG-EA  67
P.Aeruginosa   10 VTGSTSGIGLGIALSLAEAGADLLLNGFGEVDAAL-AQV-RA-RGVRAEHHPADLSDV-AQ  68
Human heart    60 VTGCDSGFGFSLAKHLHSKGFL-VFAGCLMKDKGHDGVKELDSLNSDRLRTVQLNVFRSEE 120

PBDH-1        239 DQVRGAAWNMDGGWVAQ                                             255
P.Aeruginosa  240 DQVRGAAWNMDGGWVAQ                                             256
Human heart   301 DAVTHALTATTPYTRYHPMDYYWWLRMQIMTHLPGAISDMIYIR                   344
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Figure 1b - Alignment of pBDH-1 with Pseudomonas aeruginosa 3-HBDH and Human heart 3-HBDH. *The GXXXGXG motif and also lipid binding site in mammalian 3-HBDH are shown in red and green respectively.*

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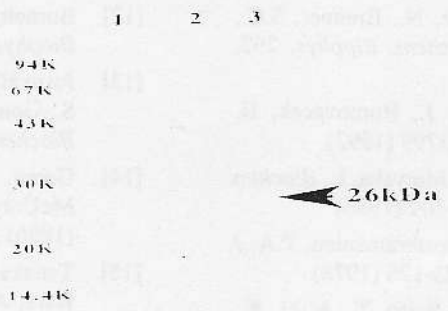
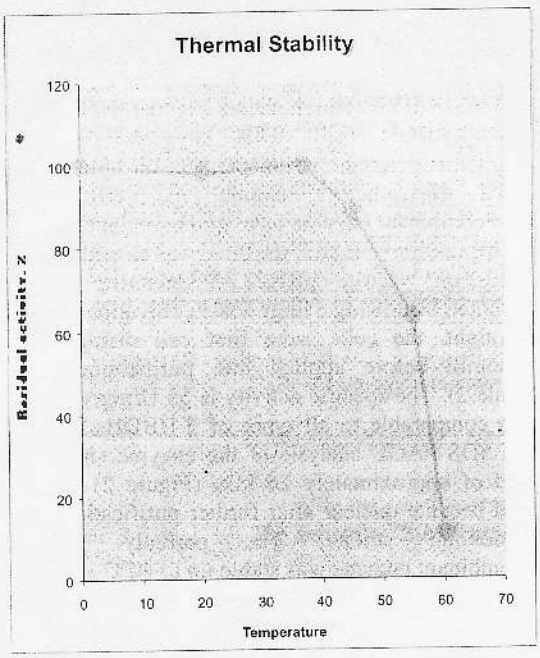


Figure 2 - SDS-PAGE analysis of the partially purified recombinant 3-HBDH. Lane 1, recombinant (crude extract); Lane 2, marker; Lane 3, wild-type 3-HBDH (67 kDa band is BSA that stabilizes the enzyme for storage)



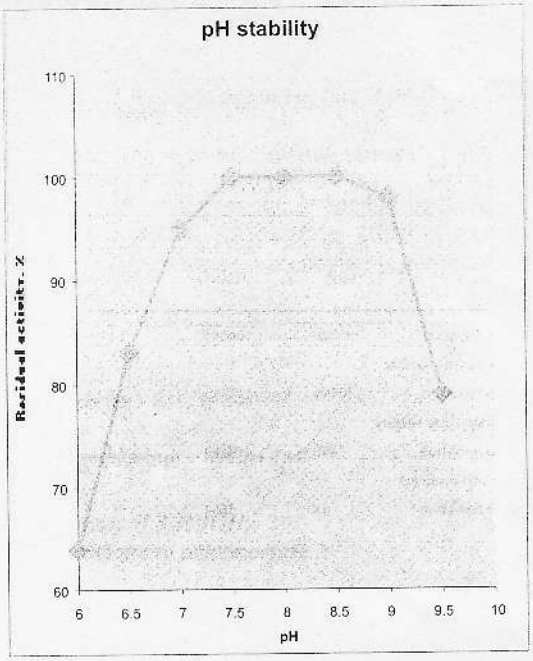


Figure 3 - Thermal and pH stability patterns.

Conclusion

The 3-HBDH gene was successfully amplified and expressed by using pKK 223-3 vector. The high stability of the recombinant enzyme is an advantage for clinical diagnosis. The phenomenon of enzyme denaturation after ammonium sulfate precipitation can be investigated in the near future. With an efficient purification method, a reasonable amount of pure enzyme can be obtained. The structure and functions are to be clarified in the future.

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