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Spectral Properties, Reconstitution and Kinetics of *Paracoccus denitrificans* Isovaleryl-CoA Dehydrogenase

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Abstract

Isovaleryl CoA dehydrogenase (IVDH) is a flavoprotein that introduce a trans-double bond between C2 and C3 of the isovaleryl CoA substrate, an intermediate in leucine catabolism pathway. Interrogation of the *Paracoccus denitrificans* Pd1222 genome has identified Pden_3633 gene as a candidate to encode for IVDH. In previous study by Rafid *et al* (under the publishing), this putative IVDH was expressed in *E. coli* and purified as N-terminal *Strep*-tagged protein. In current study, spectral properties of the purified IVDH were conducted and the results showed that the enzyme was obtained as an apoprotein. For this reason, IVDH has been reconstituted by incubation with flavin adenine dinucleotide (FAD) and the experiment showed that the ratio 1: 20% (IVDH: FAD molar excess) was achieved fully reconstitution. Furthermore, kinetic parameters of the reconstituted IVDH were studied and the enzyme exhibited a specific activity for isovaleryl-CoA substrate while Vmax and K_M were estimated to be 600 mU mg^{-1} and 7.13 µM respectively. Finally, gel filtration by using HiLoad 16/60 Superdex 200 column was performed to investigate the quaternary structure of the recombinant IVDH. An apparent molecular weight of 171640 Da was determined.

Keywords: Acyl-CoA Dehydrogenase, *Paracoccus denitrificans*, Reconstitution

الصفات الطيفية وأعادة تشكيل وتقدير الثوابت الحركية لأنزيم Isovaleryl-CoA Dehydrogenase من بكتريا *Paracoccus denitrificans* Pd1222

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الخلاصة

ينتمي انزيم Isovalery–CoA Dehydrogenase (IVDH) الى مجموعة flavoproteins والذي يعمل على ادخال اصرة مزدوجة نوع ترانس بين ذرتي الكاربون 2 و3 لمادة التفاعل Isovaleryl–CoA، وهو مركب وسطي ينتج خلال مسار هدم الحامض الاميني الليوسين. تم خلال استجوات جينوم بكتريا *Paracoccus denitrificans* Pd1222 تشخيص الجين Pden_3633 على انه احد الجينات المرشحة للتشفير عن أنزيم IVDH. وفي دراسة سابقة استطاع رافد وجماعته (تحت النشر) من الحصول على ناتج تعبير هذا الانزيم المفترض في بكتريا *E. coli* ونقي كبروتين مرتبط N–terminal *strep*–tagged protein. تم التعرف في الدراسة الحالية على الصفات الطيفية للانزيم ودلت النتائج على انه بهيئة لب بروتين apoprotein لهذا السبب اعيد تشكيله من خلال الحضن مع FAD وأظهرت التجربة ان النسبة التي حققت أعادة تشكيل لكامل الأنزيم كانت (FAD: IVDH) 1: 20% مولار زيادة. كذلك قدرت الثوابت الحركية لأنزيم

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reconstituted IVDH، أذ أظهر الأنزيم فعالية نوعية تجاه مادة التفاعل isovaleryl–CoA وقد كانت قيم كل من ثابت ميكالس (Km) والسرعة القصوى (Vmax) 600 ملي وحدة/ ملغم و 7.13 مايكرومول على التوالي. وأخيرا تم التحري عن التركيب الرباعي للأنزيم المؤتلف IVDH بطريقة كروماتوكرافيا الترشيح بالهلام وبأستخدام عمود HiLoad 16/60 Superdex 200 أذ أظهر الأنزيم وزنا جزيئيا قدر ب 171640 دالتون.

Introduction

Paracoccus denitrificans is a beta-proteobacteria and denitrifying organism that is useful in understanding the biochemical basis of the environmental emissions of the powerful greenhouse gas nitrous oxide (N_2O) from agricultural soils. In addition, the bacterium has respiratory transport chain which comparable to that in eukaryotic mitochondrion making it the favorite model for the study of oxidative phosphorylation [1-2]. Isovaleryl-CoA dehydrogenase (IVDH) is a FAD dependent enzyme and has a commission number (EC 1.3.99.10). The enzyme catalyzes the conversion of the isovaleryl-CoA to the 3-methylcrotonyl-CoA in the leucine catabolism pathway, or in other words in the first step of the β-oxidation involving isovaleryl-CoA, transferring electrons from isovaleryl-CoA substrate to the electron transferring protein (ETF) and then the electrons transfer to the ETF:QO which in turn transfers the electrons to the electron transport chain for synthesis of ATPs [3-5]. IVDH belongs to the Acyl-CoA dehydrogenases family. Members of this family can be classified based on their specificity for chain length fatty acid Acyl-CoA to a distinct groups; short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD) and very long-chain (VLCAD) acyl-CoA dehydrogenases. Four members of ACADs family involved in the amino acids catabolism; IVDH for leucine, short branched-chain acyl-CoA dehydrogenase (SBCAD) for isoleucine, isobutyryl-CoA dehydrogenase (IBD) for valine and glutaryl-CoA dehydrogenase (GCD) for lysine and tryptophan catabolism [4, 6, 7]. All members of ACADs have glutamate, which acts as the catalytic base, in their active site. E376 is the catalytic base residue in MCAD and SCAD and it conserved in all other members of ACADs family but not in IVDH and LCAD which they have E254 [8] and E261 [9] at the corresponding position respectively. ACADs have been identified in bacteria, human, plants, rat and many other organisms [10-15]. Characterization of ACADs homology in bacteria is of interest, it would give an insight to comparison of fatty acid and amino acid metabolism with those of corresponding animals providing an overview of the evolutionary value of ACADs enzymes.

Materials and Methods

Reconstitution of purified IVDH

The recombinant IVDH was reconstituted by incubation with FAD [16]. Three molar excess ratios of FAD were used to optimize the ratio with a totally reconstituted holoenzyme. Table-1 shown the molar excess ratios of FAD used for reconstitution of IVDH.

Table 1-Reconstitution of the recombinant IVDH with different molar ratios of FAD

Components	Tubes		
	Tube 1	Tube 2	Tube 3
IVDH (233 µM)	1000 µl	1000 µl	1000 µl
FAD (12mM)	23.3 µl	29.1 µl	38.3 µl
Final molar excess ratio	1: 20%	1: 50%	1: 100%

The tubes were then incubated overnight on ice. After that, all samples were re-purified, by using Strep-Tactin Superflow Plus cartridge (a kit from QIAGEN for purification of *Strep*-tagged proteins), to eliminate unbound FAD. Spectral properties were then recorded. For the ratio with a total reconstitution, 280: 430 nm should be 5-6.

Enzyme assay and determination of Kinetic parameter values

IVD activity was assayed as described by Engel [17]. The principle of the assay depends on the reduction of 2,6-dichlorophenolindophenol (DCPIP) by phenazine methosulfate (PMS) which acts as an intermediate electron carrier. The assay was carried out at 30°C. The reaction mixture contained in 2500 µl sodium phosphate buffer (100 mM, pH 7.6), 37.5 µl DCPIP, 25 µl PMS, 12.5 - 25 µl of 15 – 60 µg recombinant IVDH and 25 µl isovaleryl-CoA (IV-CoA). The assay was started by adding 10 µl of 5 mM (20 µM final concentration) of the IV-CoA, or by adding different concentrations, and the

absorbance at 600nm was then monitored with an extinction coefficient 22 mM^{-1} cm^{-1} for the DCPIP. One unit activity is represented in conversion of 1 µmol IV-CoA per minute. Vmax and Km values were calculated by using Lineweaver–Burk plot.

Spectral analysis

Spectral properties of protein samples were scanned by using Beckman DU640 UV/ Vis spectrophotometer. The sample was dissolved in 50 mM sodium phosphate buffer, pH 8.0, at 30 °C under aerobic conditions.

Gel filtration

For determination of protein size, the ÄKTAexplore 10 system supported to the unicorn software and HiLoad 16/60 Superdex 200 pg (GE Healthcare) gel filtration column were used. The column has 120 ml bed volume, and fractionation range between 10 000 and 600 000. Two column volumes of eluent buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 8.0) was used to equilibrate of the column with a flow rate 1 ml/min. Amersham filtration calibration kit (GE Healthcare) was used for column calibration, the kit contains catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) as standard proteins. The molecular size of the IVDH was determined by comparing its retention time with those of the standard proteins at 280 nm.

Results and discussion

Reconstitution of *Strep*-Tag IVDH

Binding of cofactors are required by many proteins to conduct their biological function. Also, cofactors have an important role in protein folding. They serve as a nucleation or an attractive site that facilitates the overall folding [18]. It was found that isoalloxazine ring of the FAD exerts a nucleation effect folding MCAD into a functional holoprotein. Furthermore, a low concentration of FAD has an enhancement effect on thermal stability and activity of short and midum chain acyl-CoA dehydrogenases [19-21].

The present study showed that the recombinant IVDH, which was already purified in a previous study by Rafid *et al* (under the publishing), was colorless or pale yellow suggesting that the protein was purified as an apoprotein, no FAD bound. And to confirm that, spectral properties of the purified *Strep*-Tag *Paracoccus denitrificans* IVDH (*Strep*-Tag P. d IVDH) was studied and it was found a very high value for the ratio of the enzyme absorbance at 280 nm to that of a visible region at 450 nm. Also, no absorption maxima at ~450 and ~375 nm compared to the FAD spectra indicating that the enzyme is an apoprotein Figure-1. As a consequence of binding FAD to the protein, the protein bound FAD will exerts a similar absorption of the FAD at ~450 and ~375 nm but with lower peaks [22]. To get on a holoprotein, recombinant IVDH has been reconstituted with the FAD. And according to the experiment, the protein was re-purified after incubation duration (overnight) with the FAD to exclude the excess unbound FAD with wash.

Thus, only the IVDH bound to the FAD (holoprotein) will eluted as a purified protein which then acquired an obvious yellow color resulted from FAD binding Figure-2. Also, the results showed that the molar excess ratio 1: 20% (IVD: FAD) was the ratio in which the protein was fully reconstituted Figure-3, one FAD per protein molecule calculated by absorbance ratio 280⁄450 nm of approximately 5 [23].

This study agreed with other studies that dealt with heteroexpression of IVDH in *E. coli*, apo-IVDH was obtained by these studies and authors suggested that loss in FAD was occurred during enzyme purification [24-26].

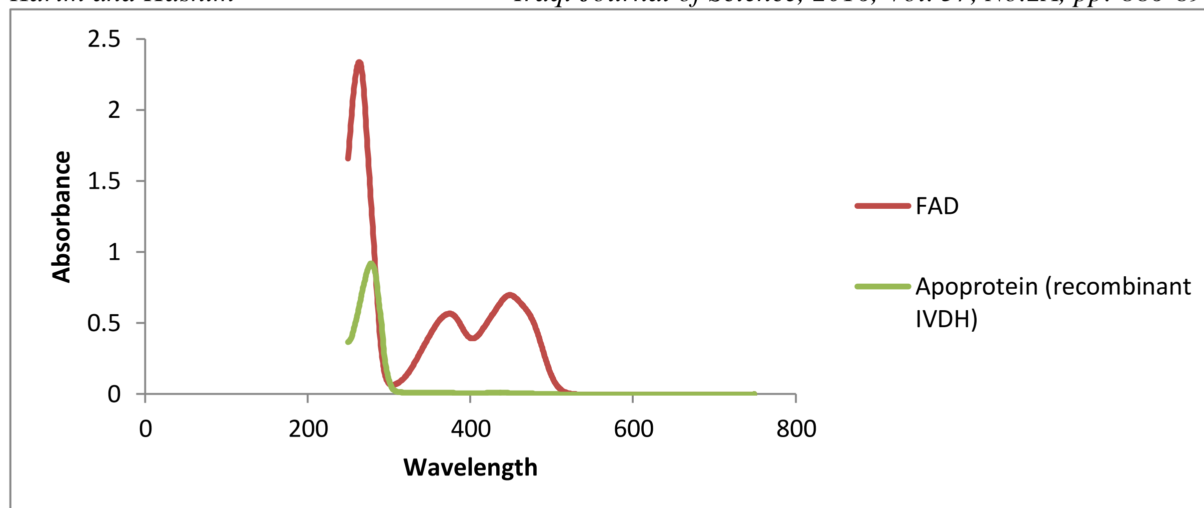

Figure 1- Spectral forms of purified *Strep*-Tag P. d IVDH (100 µM) under aerobic conditions, apoprotein, and FAD (12 mM). The spectrum was recorded in 50 mM sodium phosphate buffer, pH 8.0, at 30 °C.

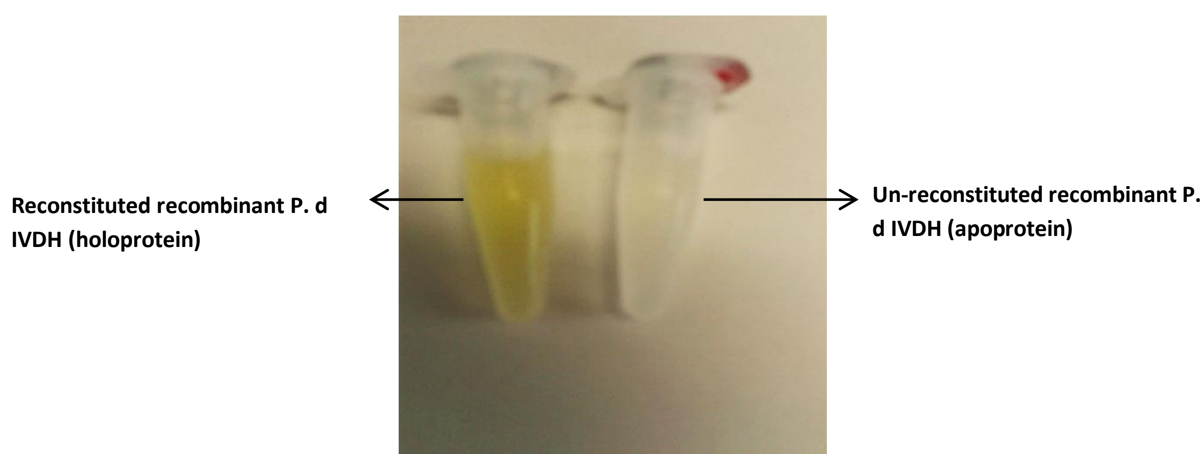

Figure 2-Reconstitution of the recombinant P. d IVDH.

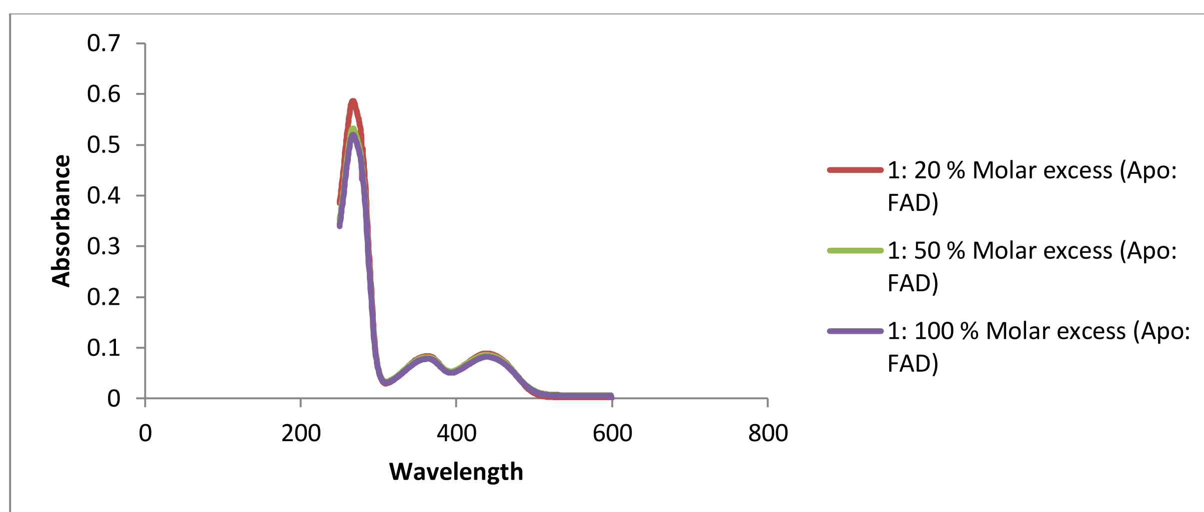

Figure 3- Reconstitute of recombinant P. d IVDH at different molar excess ratios of FAD. The concentration of the protein after re-purification was (58 µM). The spectrum was recorded under aerobic conditions in 50 mM sodium phosphate buffer, pH 8.0, at 30 °C.

Activity and kinetic parameters calculation

The specific activity and kinetic parameters of the reconstituted P. d IVDH were carried out by addition different concentration of the Isovaleryl-CoA to the reaction mixture. Values of Vmax and K_M were calculated by using Lineweaver–Burk plot. The results showed that the reconstituted IVDH has a specific activity towards Isovaleryl-CoA with a 600 mU mg^{-1} and 7.13 µM for Vmax and Km respectively Figure-4 while the Kcat and catalytic efficiency (Kcat/ K_M) were 1.9 S^{-1} and 0.26 $\mu M^{-1} S^{-}$

[1] respectively. The little K_M value confirmed that the IVDH from *P. denitrificans* Pd1222 has a highly specificity for isovaleryl-CoA substrate. However, it seemed close to those K_M values of IVDH (3.1 µM and 2.3 µM) calculated for Human [27] and *P. aeruginosa* PAO 1 [28] repectively.

Determination quaternary structure of recombinant P. d IVDH

A manifest molecular weight of 171640 Da Figure-5 was calculated which can be interpreted to mean that the recombinant P. d IVDH existing as a homotetramer. This molecular weight was harmonious with that estimated for *P. aeruginosa* PAO1 (176000 Da), Human (172400 Da), Rat (175000 Da) and *Solanum tuberosum* (188000 and 146000 Da for St-IVDH1 and St-IVDH2 respectively) [29, 24, 30, 28].

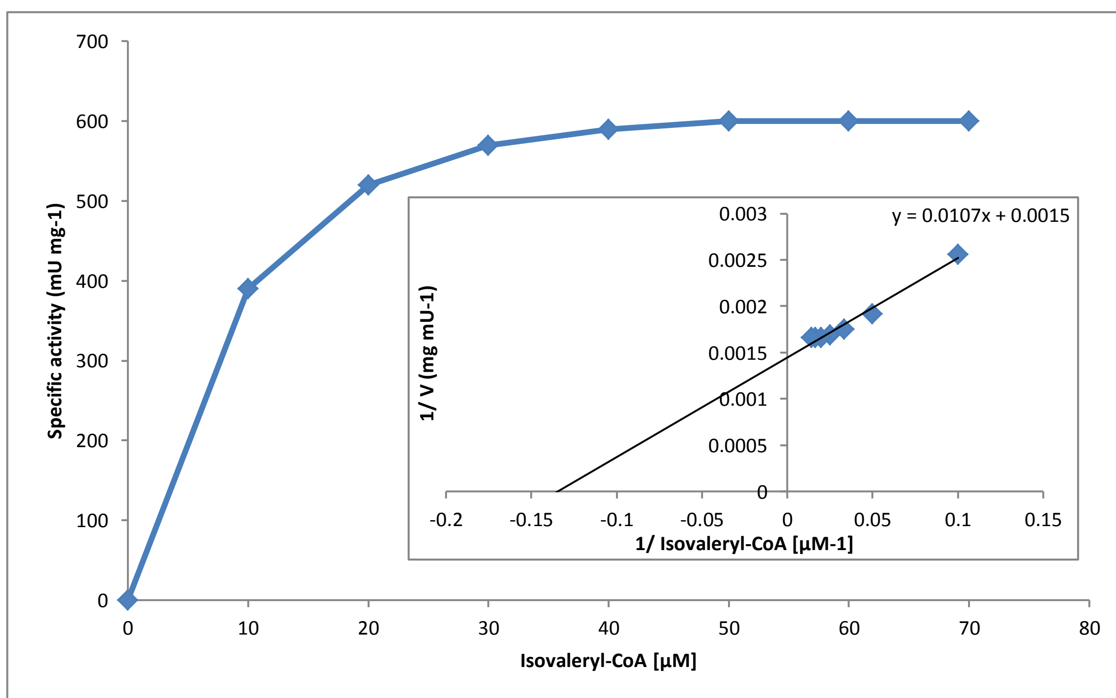

Figure 4- Calculating specific activity of reconstituted P. d IVDH at different concentrations of Isovaleryl-CoA. Inset shows Lineweaver–Burk plot to evaluate Vmax (600 mU mg^{-1}) and K_M (7.13 µM).

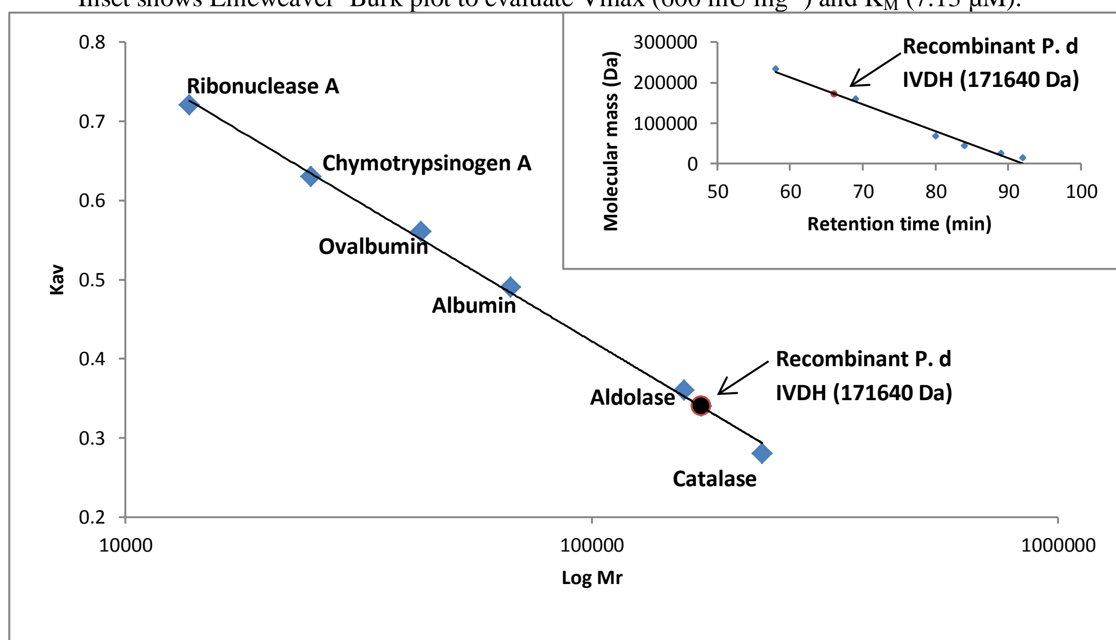

Figure 5-Calibration curve of Kav for the standard proteins on HiLoad 16/60 Superdex 200 pg column. Inset shows standard curve of the molecular mass based on retention time of the standard proteins.

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