Recombinant Production and Purification of Inosine 5-Mono Phosphate Dehydrogenase 1 Retinal Isoforms for Functional Studies

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Abstract- Inosine 5-monophosphate dehydrogenase 1 (IMPDH1) is the rate-limiting enzyme in the *de novo* purine nucleotide biosynthesis. IMPDH1 catalyzes IMP-oxidation to XMP, which in continue is converted to guanine nucleotides. Like mammals, the mouse IMPDH1 (mH1) has retinal-specific isoforms named H1 (546) and H1 (603). Mutations in the *IMPDH1* gene are believed to cause retinal degenerative disease, retinitis pigmentosa, mediated by retinal-specific gene variants. After RNA extraction from the mouse retina, RT-PCR was done using *Nde*I and *Xho*I harboring primers. Tree mH1 isoform genes were amplified and cloned into a pET26b+ vector separately. The recombinant expression vectors were then transformed in *E. coli* BL21 (DE3) strain, expressed under IPTG-induced conditions and purified with Ni-NTA agarose resin. Activity assay of recombinant proteins was done by using spectrophotometric methods. Here, we cloned and optimized the expression and the purification of recombinant mH1 canonical and retinal isoforms in *E. coli* to gain soluble and highly active protein for further functional assays. Recombinant protein production in prokaryotic hosts, especially *E. coli*, is the most common method in large-scale of protein production for functional and structural studies. However, maximal yield and activity of recombinant proteins require optimal conditions for expression and purification, which is what we showed in the present study for the mouse IMPDH1 recombinant isoforms.

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Keywords: Expression vector; Inosine 5-monophosphate dehydrogenase 1 (IMPDH1); Recombinant protein; Retinal isoform

Introduction

Retinitis pigmentosa (RP) is one of the most common forms of inherited retinal degenerative diseases. It is characterized by photoreceptor cells (rods and cones) degeneration, leading to progressive loss of vision (1). Over 50 genes have been identified involving in non-syndromic RP (http://www.sph.uth.tmc.edu/RetNet). The worldwide prevalence of RP is about 1 in 3700. RP is genetically heterogeneous and has several modes of inheritance as autosomal dominant (adRP), autosomal recessive, and X-linked (2). Mutations in the *IMPDH1* gene cause RP10 disease which involves 2.5% of all cases of autosomal dominant RP (3-5).

IMPDH catalyzes IMP-oxidation to XMP using NAD^+ as a cofactor, which is the first committed step in purine nucleotide biosynthesis (6). In mammals, the *IMPDH* gene encodes two isoforms, H1 and H2, with 84% similarity to each other (7). The *IMPDH1* gene is highly expressed in the retina. The level of IMPDH1 is more about ten times in the retina than the other tissues. (8). Furthermore, the majority of GTP production in the mouse retina is advanced by the IMPDH1 isoform and not the IMPDH2 isoform or the salvage pathway (9). In addition to the canonical IMPDH1 isoform or H1 (514), the IMPDH1 has some retinal-specific transcripts generated by alternative splicing or translation start site

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(10). In the mouse, H1 (514) results from the transcription and translation of exons 1-14, excluding exon 13b and consisting of 514 residue amino acids and the other retinal isoforms, named H1 (546) and H1 (603) are more frequent (Figure 1) (10). Retinal isoforms of human IMPDH1 have similar catalytic activity but different DNA binding activity to the canonical isoform (11). The real function of IMPDH1 in RP10 disease pathogenicity has not been discovered; because RP10-caused mutations don't affect the catalytic activity of the IMPDH1 (11).

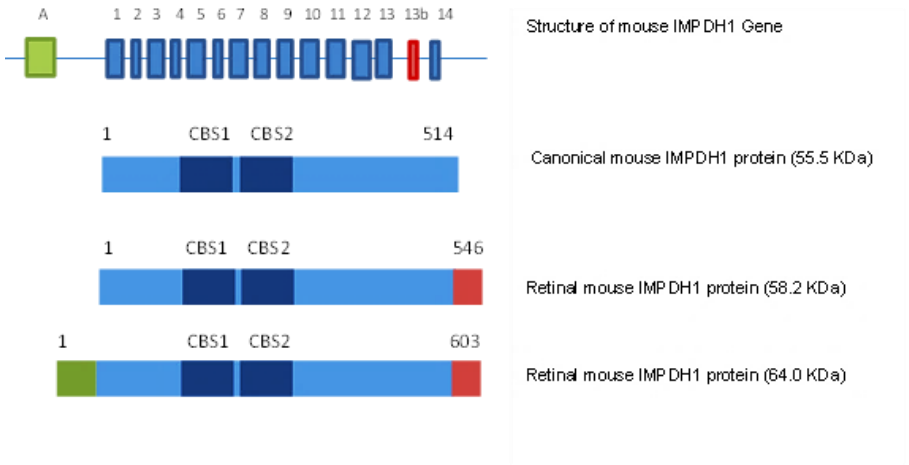

Figure 1. Mouse IMPDH1 transcripts and proteins. All IMPDH proteins form active homotetramers, and each IMPDH monomer is composed of an (α/β)8 barrel structure, which performs the enzymatic function, and a flanking subdomain, which is composed of two CBS regions similar to the cystathionine β-synthase gene (22). Canonical isoform results from the translation of exons 1-14 except for exon 13b. Retinal isoforms H1 (546) results from the translation of exons 1-14 pulse exon 13b and H1 (603) results from the translation of exons 1-14 pulse exon 13b and exon A (11)

It has been provided that the IMPDH1 may have a retinal-specific function, independent of its enzymatic activity, mediated by retinal isoforms; so many different functional assays require identifying the pathological mechanism of IMPDH1 mutations.

Nowadays, the high-level production of desired proteins is achieved by DNA recombination in the prokaryotic or eukaryotic host. Moreover, high-level expression and purification are critical for functional and structural assays of recombinant proteins. *E. coli* is the most commonly used prokaryotic host in recombinant protein production; because it grows fast in a simple environment and is easy to genetically manipulate (12). The recent signs of progress in the genetics of *E. coli* at the molecular level show a great opportunity for the rapid and economical production of recombinant proteins (13).

According to the Center for Eukaryotic Structure Genomics (CESG) statistical reports, only 30% of all proteins which be cloned in *E. coli* are expressed in soluble and active forms (14). Low-concentration or insoluble proteins are not suitable for structural and functional studies. So the solubility by detergents and refolding process must be done after expression. Thus, improvement of the expression and the purification conditions are very important in recombinant protein production.

Today, many genetics tools, such as modified expression vectors, suitable bacterial strains, and commercial cloning kits, are available for researchers in recombinant protein production fields. For example, expression vectors, which have fusion tags for solubility and purification of overexpression proteins, are accessible. It has previously been shown that genetically fused purification tags enhance the level of purity and protein recovery (15).

In this study, we cloned the retinal isoforms of mouse *IMPDH1* besides canonical isoform in the pET26b+ expression vector and transformed them in the *E.coli* BL21 (DE3) strain. The expression was optimized under conditions to reach highly active proteins for later functional assays.

Materials and Methods

Cloning of *IMPDHI* gene isoforms in the expression vector

Total RNA was extracted from the C/57 mouse retina using the Trizol protocol (Ambion). Full-length

cDNA was synthesized from the *IMPDHI1* gene (*Mus musculus*, ID 23917) with reverse transcriptase, oligo dT, and random hexamer (Thermo scientific). The H1 (514), H1 (546), and the H1 (603) cDNA were amplified separately by pfu DNA polymerase (Thermo Scientific) and gene-specific primers (Table 1).

Table 1. Primers designed for the amplification of three isoforms of the *IMPDH1* gene

Primer	Length (bp)	Sequences
IF1	27	5′-GAATTCCATATGGCGGACTACCTGATC-3′
IR1	22	5′-CGCTCGAGGTACAGCCGCTTCT-3′
IF2	28	5′-GAATTCCATATGGAGGAACCGCTCTCAC-3′
IR2	24	5′-CGCTCGAGGTTCTGGAGGGAGGCT-3′

Amplified products were gel purified and double digested with *Nde*I and *Xho*I restriction endonucleases (Thermo scientific). Inserts containing restriction sites at the N- terminal and C- terminal ends were ligated into the pET 26b+ vector (Novagene) separately. Recombinant vectors were then transformed into *E. coli* BL21 (DE3) strain. All clones were completely sequenced using an ABI genetic analyzer to confirm the sequence and verify the absence of any mutation during PCR.

Expression of canonical and retinal IMPDH1 in *E. coli*

BL21 (DE3) cells containing plasmid-encoded each of three IMPDH1 isoforms were grown separately in 10 mL of Luria Broth (LB) medium containing 30 $\mu g.mL^{-1}$ kanamycin and incubated at 37° C overnight. Next day 0.5 mL was sub cultured into 25 ml of fresh LB-kanamycin at 37° C with a shaking rate of 250 rpm to reach an OD_{600} of 0.6-0.8 and induced with 0.1-1 mM IPTG (isopropyl-b-D-thiogalactopyranoside). After induction cells were incubated at 25° C with the same shaking rate to reach high solubility proteins. 1 mL of bacteria was taken out at 0, 3, 6, and 16 hours post-induction and centrifuged. Pelet was resuspended in SDS-PAGE loading dye and electrophoresed on 12% SDS-polyacrylamide gel after heating at 95° C for 5 minutes.

Purification of IMPDH1 isoforms under native conditions

After induction for 6 hours at 25° C, cells were harvested from 100 mL LB-Kanamycin medium, centrifuged at 4000× g for 15 minutes at 4°C, and frozen at -70° C for 2 hours or overnight. After thawing, the bacterial pellets were resuspended in binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole), treated with 1 $mg.ml^{-1}$ lysozyme for 30 minutes on ice, and sonicated. After centrifuging at 12000× g for 20 minutes, the supernatant was enriched for His-tagged protein using equilibrated Ni-NTA agarose resin (ABT) and mixed gently for 1 hour at room temperature. Then resin was washed three times with washing buffer (50mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH: 8.0). After centrifuging at 500× g for 5 minutes, elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH: 8.0) was added to the resin and mixed for 10 minutes at room temperature. Finally, the resin was centrifuged, and the supernatant was carefully decanted in a new tube and stored on ice or at -20° C.

Purified proteins were run on 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue.

Dialysis of proteins

Purified protein samples were dialyzed at a 1:100 ratio dialysis buffer containing (50 mM Tric-HCl pH: 8.0, 100 mM KCl and 1 mM DTT) at 4° C for overnight, with one buffer exchange after 2 hours. After dialysis, Protein samples were mixed by 20% glycerol and stored at -70° C.

Protein quantification

All purified protein samples were quantified by the lowry method using Bovine serum albumin as the standard (16).

Enzyme activity

Enzyme activity was assayed for canonical and retinal isoforms of recombinant IMPDH1. The assay solution contained 100 mM Tris-HCl (pH 8.0), 1 mM DTT, and 100 mM KCl, and the substrates 2 mM IMP (Inosine 5-mono Phosphate) and 4 mM NAD^+ (Nicotinamide Adenine Dinucleotide). The reaction was started by the addition of 5 $\mu g.ml^{-1}$ purified recombinant enzyme to buffer in 1 cm cuvettes. The initial rate of activity was measured at 25° C in a Cary win UV–vis spectrophotometer by monitoring the absorbance

increase at 340 nm for 10 minutes.

Results

Cloning of canonical and retinal isoforms of *IMPDH1*gene

cDNA fragments encoding three isoforms, H1(514), H1(546), and H1(603), were PCR amplified and cloned in pET26b+ expression plasmid, which insert 6His-tag at the C-terminal of recombinant proteins (Figure 2). Sequence analysis of the clones displayed 100% identity with the sequence published in GenBank.

Optimization of IMPDH1 isoforms expression in *E. coli*

In order to optimize the expression of canonical and retinal isoforms of IMPDH1 in *E. coli*, we investigated the effect of two important parameters, including inducer concentration and post-induction incubation time. After the growth of transformed BL21 (DE3) bacteria in 25 mL liquid LB-Kanamycine medium and achieving cell density (OD_{600}: 0.6-0.8), the culture medium was divided into 4×5 mL, and IPTG was added to them with the final concentration of 0.1, 0.25, 0.5, and 1 mM respectively and incubated at 25^{O} C for 16 hours with shaking rate of 250 rpm. 1 mL of induced bacteria was taken out at 0, 3, 6, and 16 hours after induction for SDS-PAGE analysis (Figure 3).

The optimum expression was achieved at an IPTG concentration of 0.1 mM for the H1 (514) isoform and 1 mM for the H1 (546) and H1 (603) isoforms. Also, the best post-induction period was 6 hours at temperatures of 25^{O} C. SDS-PAGE analysis of the induced bacteria revealed an expected protein band that was consistent with the size of each H1 isoform (Figure 4).

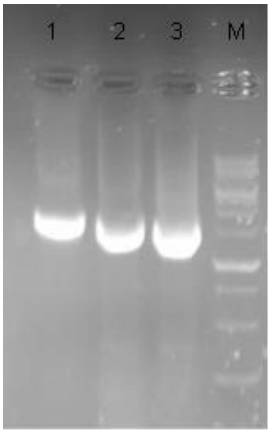

Figure 2. PCR amplified of three mIMPDH1 isoforms. Lane 1: H1 (603)1812 bp, lane 2: H1 (546)1641 bp, lane 3: H1 (514)1545 bp, M: 1 Kb ladder

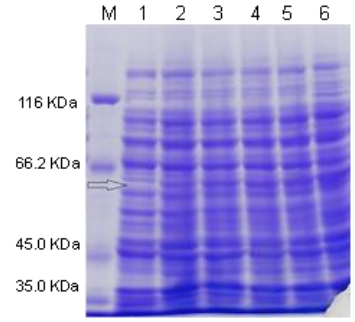

Figure 3. SDS-PAGE analysis for setting up inducer concentration. (Transformed bacteria were induced with 0, 0.1, 0.25, 0.5, and 1 mM IPTG (lane 1-5) and harvested after 6 hours at 25^{O} C. lane6: induced with 1 mM IPTG and harvested after 16 hours. This experiment was done for canonical and two retinal isoforms of mIMPDH1 separately). M: Protein size marker

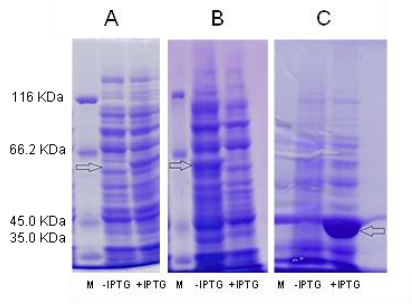

Figure 4. SDS-PAGE from the optimized expression of three mIMPDH1 isoforms. M: Protein size marker
A) H1(546), B) H1(603) and C) H1(514)

Purification of IMPDH1 isoforms under native conditions

The crude extract was isolated by treating 0.5-0.8 g cell pellets from 100 ml culture medium with lysis buffer followed by sonication and treatment with a strong nuclease (Benzonase-sigma) to remove the viscosity of the solution and the slurry was cleared by high-speed centrifugation. Lysis buffer contains 10 mM imidazole to suppress non-specific binding. The lysate was then enriched for His-tag protein by equilibrated Ni-NTA agarose resin and finally, we purified about 0.3-1 mg protein in 500 mM imidazole elution buffer. The mouse-purified proteins have a predicted mass of 55.5, 58.2, and 64.0 kDa corresponding to the H1 (514), H1 (546) and H1 (603) isoforms respectively (17). The purity of the proteins was assayed by 12% SDS–PAGE (Figure 5).

Dialysis and quantification

Purified proteins were dialyzed to remove imidazole, and measured their concentration with the lowry

method; data are shown in table 2.

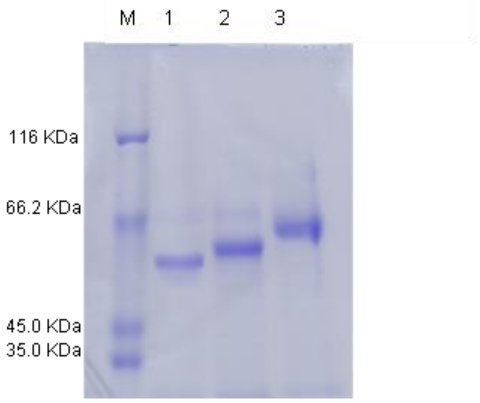

Figure 5. SDS-PAGE analysis of purified mIMPDH1 isoforms. Protein bands was consistent with the size of each H1 isoform. M: Protein size marker, 1: H1 (514), H1 (546), H1 (603)

Table 2. Data from purification and activity assay of canonical and retinal isoforms of IMPDH1 enzyme

protein	Vol. of medium (mL)	Weight of cell pellet (g)	Total concentration ($\mu g.mL^{-1}$)	Activity ($\mu mol.min^{-1}$)
H1(514)	100	0.5	245	1.1
H1(546)	100	0.8	315	1.07
H1(603)	100	0.8	317	1.7

Enzymatic activity

Activity assay was determined by measuring the absorbance increase caused by the reduction of NAD^+ to NADH. As it has been shown in table 2, enzyme activity for canonical mouse IMPDH1 isoform was achieved at 1.1 $\mu mol.min^{-1,}$ and for retinal isoforms was about 1.07 for H1 (546) and 1.7 for H1 (603) (Table 2). The Time course activity of enzymes was plotted in the first 2 minutes, in which the formation of NADH from NAD^+ was linear (Figure 6).

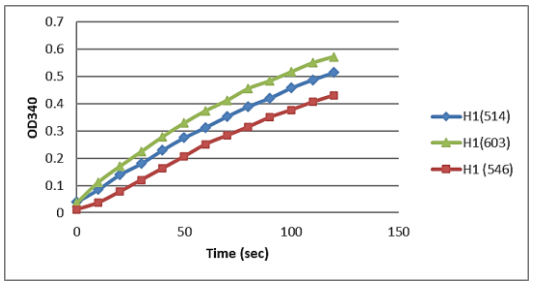

Figure 6. Time course activity plots of retinal and canonical isoforms in the first 2 minutes which the rate of reaction was linear

Discussion

Recombinant protein production on a large scale is critical for protein functional and structural studies. The selection of a strong expression system for the high-level production of recombinant proteins depends on many parameters, including cell culture medium, expression vector, appropriate host cell, and biological activity of target protein. The choice of effective expression vector and competent host cell can significantly increase the activity and amount of target protein present in the soluble fraction. The pET System is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*, and BL21 (DE3) strain is the most widely used host for overexpression of target gene.

In the present study, the target gene was successfully cloned in the pET26b+ expression vector and transformed into the BL21 (DE3) strain. The pET26b+ carries the His-tag sequence for introducing it to the C-terminal of the target protein and facilitating the later protein purification.

We tried to find the optimum inducer concentration, post-induction time, and post-induction temperature in order to produce highly the canonical and retinal isoforms of the mouse IMPDH1 enzyme, soluble and biologically active form, for later functional studies. Canonical isoform or H1 (514) has been expressed frequently in past studies, and finding out the optimum expression conditions of this isoform was a relatively

straightforward task (12,9,10,18-20). We showed that canonical isoform was successfully produced at a high level after the induction with 0.1 mM of IPTG. However, the recombinant production of two retinal isoforms of the mouse IMPDH1, H1 (546) and H1 (603), was more difficult than to canonical isoform. Nevertheless, we could express two retinal isoforms with 1 mM IPTG concentration, which was 10-fold greater than for canonical isoform. The effect of post-induction incubation period affects the overall folding, accumulation, and productivity of recombinant proteins in *E. coli* (21). The post-induction incubation period of time in *E. coli* culture is affected by several factors, such as the power of the promoter, inducer concentration, solubility, and intrinsic properties of recombinant protein. Finally, retinal isoforms were expressed after 6 hours at 25º C post-induced incubation. The lower temperature in post-induction enhances the production of soluble recombinant proteins (9).

Immobilized metal affinity chromatography (IMAC) is a general purification method that is widely used to purify recombinant proteins containing a short polyhistidine affinity tag (22). In our study, the His-tag sequence was inserted in the C-terminal of three recombinant proteins, which facilitates their purification via the IMAC method by using Ni-NTA agarose resin. We didn't observe any significant difference in total protein extraction from canonical and two retinal isoforms of the mouse IMPDH1 (Table 2).

Activity assay was the final approach to confirm the successful expression of functionally active recombinant enzymes. In this context, we showed that the recombinant IMPDH1 catalyzes the oxidation of IMP to XMP by using NAD^+ as a cofactor. Conversion of NAD^+ to NADH was measured by spectrophotometer at 340 nM wavelength. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μM of NADH per minute.

We finally concluded that two isoforms of the mouse H1 have an insignificant difference in total activity. However the retinal isoform H1 (603) shows an increased activity compared to the other isoforms. This result is consistent with previous studies performed on the mouse IMPDH1 isoforms (23). The higher retinal isoform H1 (603) activity was achieved in the saturated concentration of substrates. It may be due to more expression of this isoform compared to the canonical isoform in the retina; the H1 (603) is knowingly one of the major transcripts in the mouse retina (10). However, more functional and structural investigations are necessary for final confirmation.

We sum up that the canonical and two retinal isoforms of the mouse IMPDH1 were successfully expressed in the *E. coli* BL21 (DE3), yielding high-level recombinant protein. The purified enzymes were confirmed, showing functionally active that can be used for later functional assays.

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