Cloning and Expression of Human Histone Deacetylase 2 in *Escherichia coli*

การโคลนและการแสดงออกของยีนฮิสโทนดีอะเซทิลเลส 2 ของมนุษย์ในเซลล์แบคทีเรีย *Escherichia coli*

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ABSTRACT

Histone deacetylase 2 (HDAC2), zinc dependent enzyme in histone deacetylase family class 1, is responsible for transcriptional repression. The deregulation of HDAC2 was correlated with several human diseases, mainly in cancer and neurological disease leading to the alleviation by HDAC inhibitor (HDACi). In this study we had attempted to clone and express of human *HDAC2 gene*; both *full-length-hHDAC2* and *truncated hHDAC2* in *E. coli* BL21 (DE3) pLysS. Accordingly, the GST-full-length hHDAC2 was overexpressed at both of 37 ˚C and 25 ˚C, but only 25 ˚C-overexpressed protein was soluble. The GST-truncated hHDAC2 was also overexpressed at 37 ˚C and 25 ˚C, however, neither 37 ˚C-overexpressed protein nor 25 ˚C-overexpressed protein was soluble. The 25 ˚C overexpressed GST-full length hHDAC2 will be test for deacetylase activity and will be used for further screening of HDACi from natural products.

บทคัดย่อ

เอนไซม์ HDAC2 จัดอยู่ในกลุ่ม histone deacetylase family class 1 หากเกิด deregulation ของเอนไซม์ชนิดนี้ พบว่ามีความสัมพันธ์กับการเกิดโรคหลายชนิด เช่น มะเร็ง และโรคที่เกี่ยวข้องกับความบกพร่องของสมอง ทำให้ HDAC inhibitor (HDACi) เข้ามามีบทบาทสำคัญและเป็นทางเลือกในการบรรเทาความไม่สมดุลนี้ ดังนั้นในงานวิจัยชิ้นนี้มีวัตถุประสงค์เพื่อต้องการโคลนและทำให้เกิดการแสดงออกของยีน *hHDAC2* จำนวน 2 ขนาด ได้แก่ *full-length hHDAC2* และ *truncated-hHDAC2* ในแบคทีเรีย *E. coli* และชักนำให้เกิดการแสดงออกของโปรตีนที่อุณหภูมิ 37 ˚C และ 25 ˚C 200 rpm พบว่าเฉพาะโปรตีน overexpressed GST-hHDAC2 ที่ 25 ˚C ส่วนใหญ่ละลายได้โดยปรากฏอยู่ในส่วนของ supernatant fraction ในขณะที่โปรตีน over expressed GST-truncated hHDAC2 ส่วนใหญ่ไม่ละลายและปรากฏอยู่ในส่วนของ pellet fraction ซึ่งโปรตีน GST-hHDAC2 ที่ overexpressed ที่ 25 ˚C จะถูกนำมาทดสอบกิจกรรมของเอนไซม์และนำไปทดสอบคัดเบื้องแรก (screening) หา HDACi จากสารสกัดธรรมชาติต่อไป

Keywords: Cloning, Histone deacetylase 2, *Escherichia coli*
คำสำคัญ: การโคลน เอนไซม์ฮิสโทนดีอะเซทิลเลส 2 แบคทีเรียอีโคไล

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Introduction

Histone acetylation and deacetylation, involving the addition and removal of acetyl groups from the histone protein, are regulated by two classes of enzymes with antagonism function; histone acetyltransferases (HATs) and histone deacetylases (HDACs) which play an important role in regulating gene expression by remodeling of chromatin structure (Choong, et al., 2016). HATs are responsible for transferring of acetyl group from acetyl CoA to amino of lysine residue in histone tail, acetyl group can neutralize the positively charged residues, leading to chromatin relaxation and transcriptional activation (Kramer, 2009). HDACs catalyze the removal of acetyl group from histone proteins, restoring the positive charge resulting in more condensed chromatin and transcriptional repression. However, the deregulation of histone acetylation/deacetylation levels play a significant role in many human disease such as cancer, diabetes, cardiovascular disease, memory impairment, and neurological disease (Volmar and Wahlestedt, 2015).

HDACs are divided into two major groups; the histone deacetylase family, zinc- dependent enzyme that share the similarity of nucleotide sequence as the deacetylase domain, and Sir2 regulator family, NAD^+-dependent enzyme that require NAD^+ to act as a cofactor. HDAC2 is grouped into histone deacetylase family class 1, a subunit of protein nuclear complex that plays crucial role for transcriptional repression (Seto and Yoshida, 2014). The upregulation of HDAC2 expression is associated with synaptic plasticity and memory in the mammalian nervous system (Yamakawa et al., 2017) leading to several neurological disease such as Alzheimer's disease (AD), Parkinson's disease (PD), chronic stress and etc (Konsoula and Barile, 2012). Interestingly, mouse model of AD treated with HDACi exhibited the recovery of better memories and impaired cognitive function; increased synapse member, increased dendritic spine density, reduced intranueronal beta amyloid (Ricobaraza et al., 2012). In MPTP-induced mice, PD model, the prevention of cell death by downregulation of p53-upregulation and of loss of dopaminergic neuron in substaintia nigra were observed after treated with HDACi, K560 (Choong et al., 2016). In depression mice model, upregulations of transcription factor induced by stress in brain; ERK, CREB and nAchours were observed after treated with HDACi, MS-275 (Covington et al., 2009).

Consequently, HDAC inhibitors have been used as an alternative way to alleviate symptom from imbalance of HDAC2 level. In this study, we attempted to synthesize the rhHDAC2 protein in bacterial expression system to obtain a specific enzyme to be used as the important component in screening for natural HDAC inhibitors. We aimed to clone and express *full-length hHDAC2* and *Truncated hHDAC2* in *E. coli* BL21 (DE3) pLysS to obtain active GST-hHDAC2 protein for screening of HDACi from natural product.

Objective of the study

We aimed to clone and express of *hHDAC2 gene* in *E. coli* to obtain rhHDAC2 protein for screening of HDACi from natural product.

Materials and methods

Cloning of *full-length hHDAC2 gene*

mRNA was extracted from HeLa cells by RNeasy Mini kit (QIAGEN, Valencia, CA, USA). The mRNA was used as a template for cDNA synthesis by reverse transcription polymerase chain reaction (RT-PCR). *Full-length-hHDAC2 gene* was cloned into pGEX-6P-2 vector by using the *Bam*HI and *Xho*I restriction sites. The *full-length-hHDAC2 gene* was amplified by PCR using specific primers which were designed based on coding sequence data of *hHDAC2 gene*, obtained from GenBank (NM_001527.4); forward primer, 5′-TACAGGATCCATGGCGTACAGTCAAGGAGG-3′with *Bam*HI site (underline) and reverse primer, 5′-CGATCTCGAGTCAGGGGTTGCTGAGCTGTTC-3′ with *Xho*I site (underline). The PCR cycling condition consists of an initial denaturation at 98 °C for 30 sec; 30 cycles of 98 °C for 10 sec, 60 °C for 20 sec, 72 °C for 45 sec and final extension at 72 °C for 7 min. After digestion with *Bam*HI and *Xho*I, PCR products were ligated into pGEX-6P-2 vector. The ligated products were transformed into *E. coli* DH5α by heat shock method and transformed *E. coli* colonies were selected by ampicillin resistance on LB agar plate. Recombinant clone were screened by restriction enzyme analysis. pGEX-6P-2-*full-length-hHDAC2* plasmids were extracted by Presto™ Mini Plasmid kit (Geneaid, Taiwan). The nucleotide sequence was confirmed by DNA sequencing and analyzed using BioEdit.

Expression of rhHDAC2 in *E. coli* strain BL21(DE3) pLysS containing pGEX-6P-2-*full-length-hHDAC2* plasmid

The pGEX-6P-2-*full-length-hHDAC2* plasmid was transformed into *E. coli* BL21 (DE3) pLysS. For preparation of pre-cultures, transformed colony was inoculated and grown at 37 ˚C under constant shaking 230 rpm for 16-18 hours in fresh LB medium (10, 5, 10 g/L of tryptone, yeast extract and NaCl) containing 50 µg/ml of ampicillin and 37 µg/ml of chloramphenicol. Pre-cultures were diluted (1:100) and cultured in 100 ml of fresh LB medium containing antibiotic to an OD_{600} value reached between 0.4-0.6. The expression of rhHDAC2 protein was performed as 3 hours induction with IPTG under constant shaking 200 rpm at 25 ˚C and 37 ˚C and of varying concentration of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM. The collected samples were resuspended in lysis buffer (500 mM NaCl, 20 mM NaH_2PO_4, 1 mg/ml lysozyme and 1 mM PMSF) with 2x loading dye and were further boiled for 5 min. The samples with OD_{600} = 0.1 were loaded on 12.5 % polyacrylamide gel.

Solubility assay of recombinant GST-hHDAC2 protein

After preparation of pre-cultures, the pre-cultures were further diluted and grown around 3 hours before IPTG induction step. rhHDAC2 protein was expressed at 37 ˚C with 0.05 mM IPTG and at 25 ˚C with 0.05, 0.1, 0.2 mM IPTG for 3 hours under constant shaking 200 rpm. The cultured cells were collected and resuspended in lysis buffer (1:20 w/v). The collected cells were disturbed by an ultrasonic cell disruptor using a pulse setting of 25% duty (10 cycle, 3 s: 15 s of pulse on: off). The sonicated cells were centrifuged at 4 ˚C 10,000 rpm for 30 min and the supernatant was collected whereas the pellet was washed twice time with lysis buffer and resuspended with lysis buffer containing 8 M urea. The samples (20 µl each) were further analyzed by SDS-PAGE in 12.5% polyacrylamide gel.

Cloning of *truncated-hHDAC2 gene*

The *truncated hHDAC2 gene* was amplified by PCR using pGEX-6P-2-*full-length-hHDAC2* plasmid as a template with specific primers designed cover coding sequence at nucleotide position 9-1,125 bp; forward primer, 5′-TACAGGATCCGGCGGCAAAAAAAAAGTCTGC-3′with *Bam*HI (underline) and reverse primer, 5′-

CGATCTCGAGTCAAGGTAACATGCGCAAATTTT-3′with *Xho*I (underline). The PCR cycling condition consists of initial denaturation at 98 °C for 30 sec; 30 cycles of 98 °C for 10 sec, 57 °C for 20 sec, 72 °C for 45 sec and final extension at 72 °C for 7 min. After digestion with *Bam*HI and *Xho*I, PCR products were ligated into pGEX-6P-2 vector. The ligated product were transformed into *E. coli* DH5α by heat shock method and the transformed *E. coli* colony was selected by ampicillin resistant in LB agar plate. Recombinant clones were screened by restriction enzyme analysis. pGEX-6P-2-*truncated-hHDAC2* plasmid was extracted by Presto™ Mini Plasmid kit. And the nucleotide sequence was confirmed by DNA sequencing and analyzed using BioEdit.

Expression of recombinant truncated hHDAC2 in *E. coli* strain BL21 (DE3) pLysS containing pGEX-6P-2-*truncated-hHDAC2* plasmid

The pGEX-6P-2-*truncated-hHDAC2* plasmids were transformed into *E. coli* BL21(DE3) pLysS. For preparation of pre-cultures, transformed colony was inoculated and grown at 37 ˚C under constant shaking 230 rpm for 16-18 hours in fresh LB medium containing 50 µg/ml of ampicillin and 37 µg/ml of chloramphenicol. Pre-cultures were diluted (1:100) and cultured in 100 ml of fresh LB medium containing antibiotic to an OD_{600} value reached between 0.4-0.6. The expression of recombinant truncated-hHDAC2 protein was performed as 3 hours-induction under constant shaking 200 rpm at 25 ˚C and 37 ˚C and of varying concentration of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM. The cultured samples were collected to analyze the expression of rhHDAC2 protein by centrifugation. The collected samples were resuspended in lysis buffer with 2x loading dye and were further boiled for 5 min. The samples (OD_{600} = 0.1) were loaded on 12.5 % polyacrylamide gel.

Solubility assay of recombinant GST-truncated-hHDAC2 protein

After preparation of pre-cultures, the pre-cultures were further diluted and grown around 3 hours before IPTG induction step. Truncated-rhHDAC2 protein was expressed at 37 ˚C in 0.4 mM IPTG and at 25 ˚C in 0.4 and 0.8 mM IPTG for 3 hours under constant shaking 200 rpm. The cultured cells were collected and resuspended in lysis buffer (1:20 w/v). The collected cells were disturbed by an ultrasonic cell disruptor using a pulse setting of 25% duty (10 cycle, 3 s: 15 s of pulse on: off). The sonicated cells were centrifuged at 4 ˚C 10,000 rpm for 30 min and the supernatant was collected whereas the pellets was washed twice timewith lysis buffer and resuspended with lysis buffer containing 8 M urea. The samples (20 µl each) were further analyzed by SDS-PAGE in 12.5% polyacrylamide gel.

Results

Cloning* of *full-length-hHDAC2 gene

A *full-length hHDAC2* cDNA gene was amplified by RT-PCR using the specific primers. The PCR Products were digested and ligated into pGEX-6P-2 vector by using the *Bam*HI and *Xho*I restriction sites. The recombinant clones were screened by enzymatic digestion analysis. The expected DNA band around 1.5 Kb correlated with actual coding sequence of *hHDAC2 gene* size; 1,467 bp was observed (data not shown). DNA sequencing result showed that pGEX-6P-2-*full-length-hHDAC2* plasmid from desired colony had 1 point of silent mutation; CAT > CAC (underlined) position 504, remaining Histidine residue as shown in figure 1.

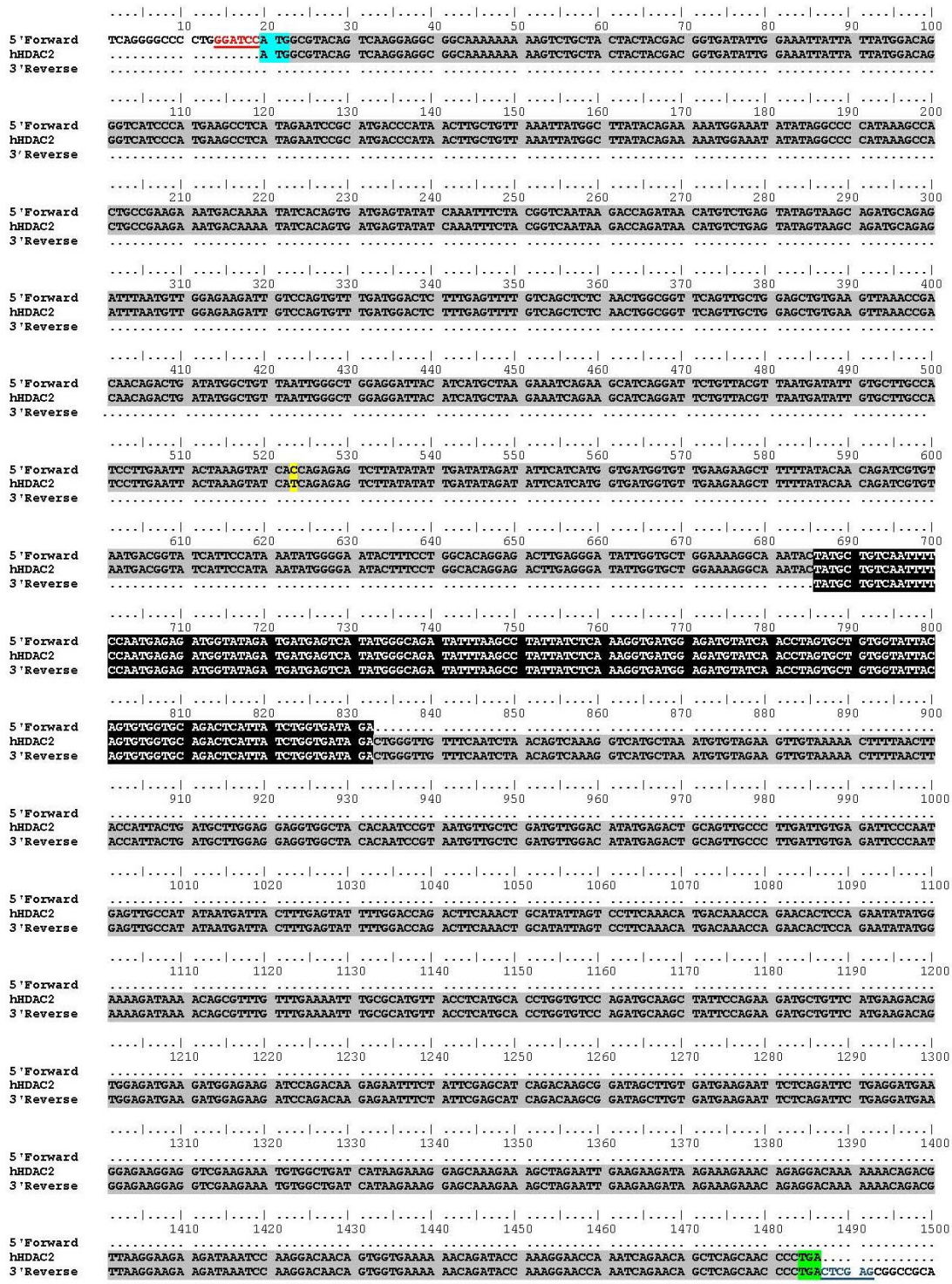

Figure 1 Alignment of full length-hHDAC2 gene in pGEX-6P-2-full-length-hHDAC2 plasmid with sequence data of hHDAC2 obtained from GenBank (NM_001527.4)

Expression of *E. coli* strain BL21 (DE3) pLysS containing pGEX-6P-2-*full-length-hHDAC2* plasmid

The pGEX-6P-2-*full-length-hHDAC2* plasmids were transformed into *E. coli* BL21 (DE3) pLysS. The expression of GST-rhHDAC2 protein was done for 3 hours of induction time under constant shaking 200 rpm at 25 ˚C and 37 ˚C and of varying concentration of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM. As shown in figure 2, the

overexpressed band protein around 75 kDa in all IPTG induced condition both 25 ˚C and 37 ˚C was observed. The theoretical molecular mass of GST-rhHDAC2 protein is around 81 kDa. At 37 ˚C condition, the level of GST-rhHDAC2 expression seemed to have equal protein level although using different IPTG concentrations as shown in Figures 2. Whereas, at 25 ˚C, the level of GST-full length rhHDAC2 expression significantly increased in a dose dependent manner as shown in Figures 3.

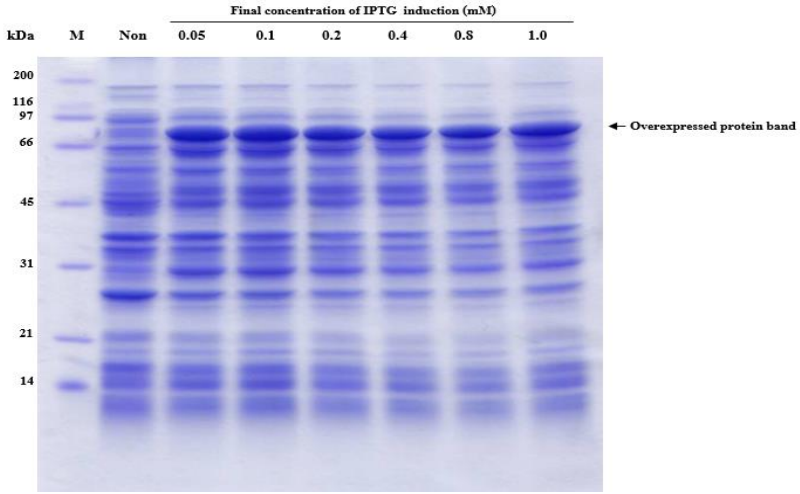

Figure 2 SDS-PAGE analysis of recombinant GST-hHDAC2 protein of 3 hours of induction time under constant shaking 200 rpm, and varying concentrations of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM, at 37 ˚C

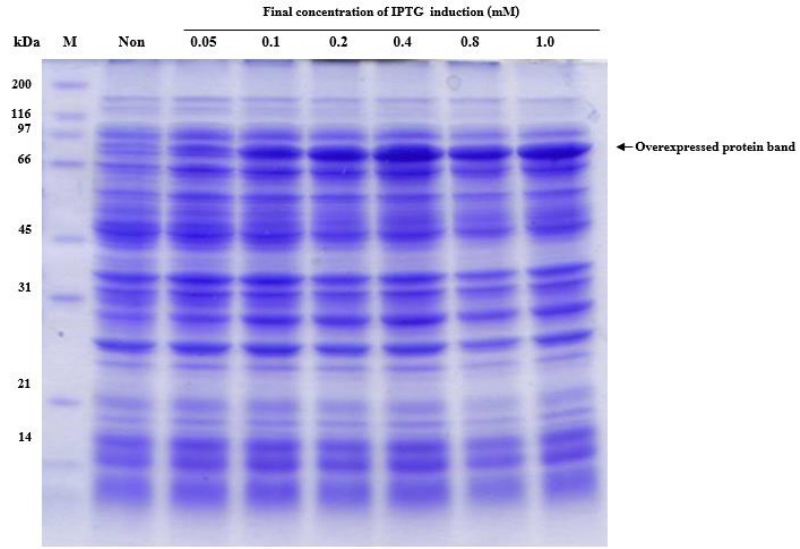

Figure 3 SDS-PAGE analysis of recombinant GST-hHDAC2 protein of 3 hours of induction time under constant shaking 200 rpm, and varying concentration of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM, at 25 ˚C

Solubility assay of recombinant GST-hHDAC2 protein

The pre-culture of *E. coli* BL21 (DE3) pLysS containing pGEX-6P-2-*full length-hHDAC2*, was cultured in LB medium with 50 µg/ml ampicillin and 37 µg/ml chloramphenicol at 37 °C to an absorbance of 0.4-0.6 at 600 nm. The cultures were induced by 0.05 mM IPTG at 37 ˚C and 0.05, 0.1 and 0.2 mM IPTG at 25 ˚C under constant

shaking 200 rpm for 3 hours. The cultures were harvested and fractionated as described in material and method section. The results from 37 ˚C condition demonstrated that most of expressed GST-full length-hHDAC2 protein band was found in the pellet fraction as shown in Figure 4. Whereas, at 25 ˚C condition, most of expressed GST-hHDAC2 protein band was found in the supernatant fraction in all IPTG-induced cultures as shown in Figure 5.

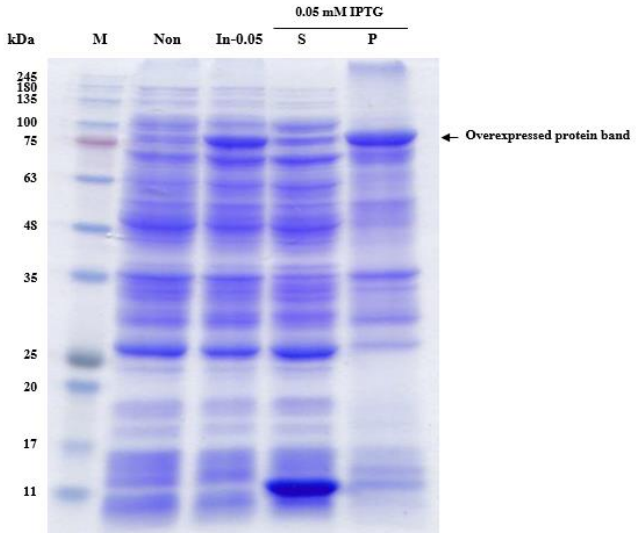

Figure 4 Solubility assay of recombinant GST-hHDAC2 protein at 37 ˚C, 3 hours , 200 rpm and 0.05 mM IPTG, Lane M: protein marker, Lane In: IPTG induction, Lane non: non induction, Lane S: supernatant, Lane P: pellet

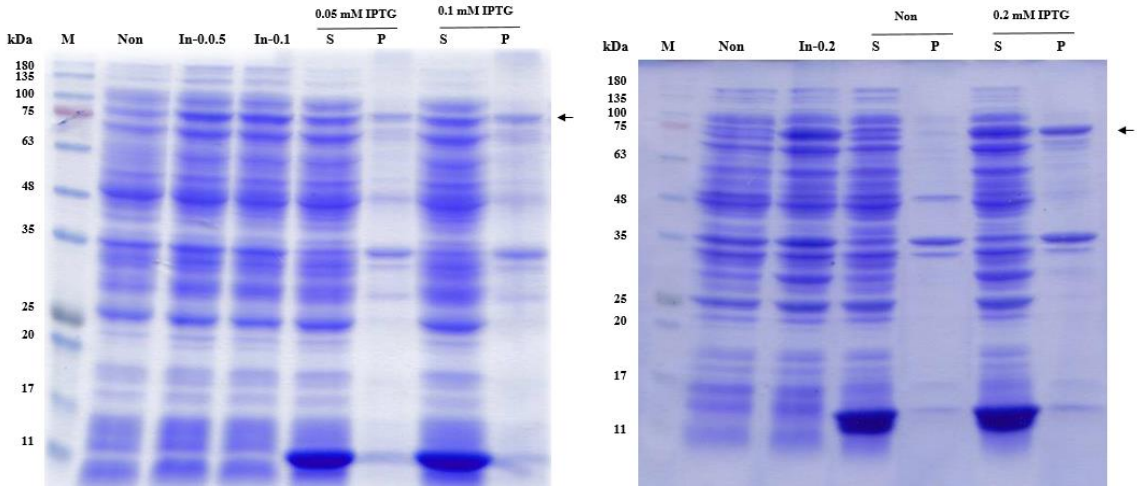

Figure 5 Solubility assay of recombinant GST-hHDAC2 protein at 25 ˚C, 3 hours of induction time, 200 rpm constant shaking and (A) 0.05 and 0.1 mM IPTG, (B) 0.2 mM IPTG, Lane M: protein marker, Lane In: IPTG induction, Lane non: non induction, Lane S: supernatant, Lane P: pellet

Cloning of *truncated hHDAC2 gene*

A *truncated hHDAC2 gene* covering range of coding sequence of hHDAC2 gene at position 19-1,125 bp was amplified by PCR using pGEX-6P-2-*full-length-hHDAC2* plasmid as a template. The PCR products were

digested and ligated into pGEX-6P-2 vector by using the *Bam*HI and *Xho*I restriction sites. The recombinant clones were screened by enzymatic digestion analysis. The expected DNA band upper 1.0 Kb in 0.1% agarose gel electrophoresis correlated with actual coding sequence of *hHDAC2 gene* size; 1,107 bp was observed (data not shown). DNA sequencing result found that pGEX-6P-2-*truncated-hHDAC2* plasmid from desired colony had 1 point of silent mutation (underlined); CAT > CAC position 504 remaining Histidine residue.

Expression of recombinant truncated hHDAC2 in *E. coli* strain BL21 (DE3) pLysS containing pGEX-6P-2-*truncated-hHDAC2* plasmid

pGEX-6P-2-*truncated-hHDAC2* plasmids were transformed into *E. coli* BL21 (DE3) pLysS. The expression of recombinant truncated-hHDAC2 protein was performed for 3 hours of induction time under constant shaking 200 rpm at 37 ˚C and 25 ˚C, and of varying concentrations of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM. The overexpressed protein band around 63 kDa (the theoretical molecular mass of GST-rhHDAC2 protein is around 68 kDa) was observed in all IPTG induced cultures both at 37 ˚C and 25 ˚C. At 37 ˚C condition, the level of recombinant GST-truncated hHDAC2 expression seemed to have equal protein level at IPTG concentrations of 0.1-1.0 as shown in Figures 6. Whereas, at 25 ˚C condition, the level of recombinant GST-truncated-hHDAC2 significantly increased in dose dependent manner at concentrations of 0.05-0.8 as shown in Figures 7.

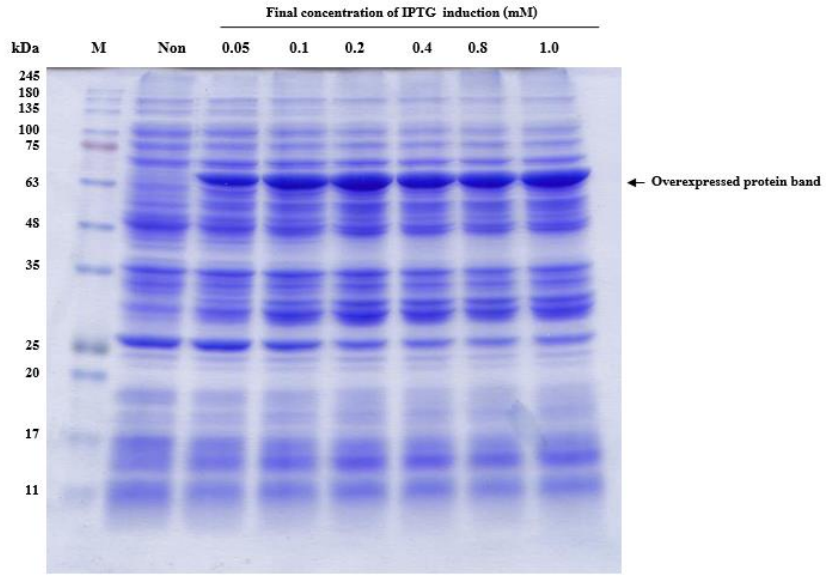

Figure 6 SDS-PAGE analysis of recombinant GST-truncated-hHHDAC2 protein of 3 hours of induction time under constant shaking 200 rpm, varying concentrations of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM, at 37 ˚C

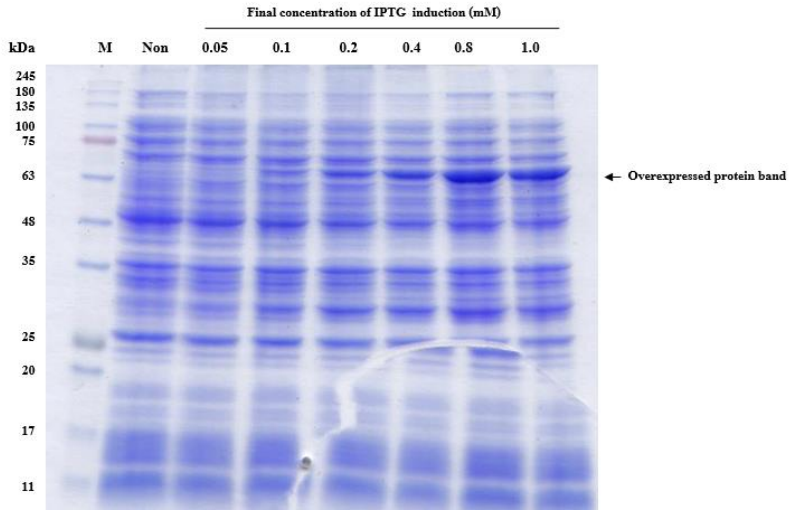

Figure 7 SDS-PAGE analysis of recombinant GST-truncated-hHHDAC2 protein of 3 hours of induction time under constant shaking 200 rpm, varying concentrations of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM, at 25 ˚C

Solubility assay of recombinant GST-truncated hHDAC2 protein

The pre-culture of *E. coli* BL21 (DE3) pLysS containing pGEX-6P-2-*truncated-hHDAC2*, was cultured in LB medium with 50 µg/ml ampicillin 37 µg/ml at 37 °C to an absorbance of 0.4-0.6 at 600 nm. The cultures were induced by 0.4 mM IPTG in 37 ˚C and 0.4 and 0.8 mM IPTG in 25 ˚C under constant shaking 200 rpm for 3 hours. Bacterial cells were harvested and fractionated as described above. Most of expressed GST-truncated-hHDAC2 protein band was found in the pellet fraction in all IPTG induced cultures both at 37 ˚C and 25 ˚C as shown in Figure 8.

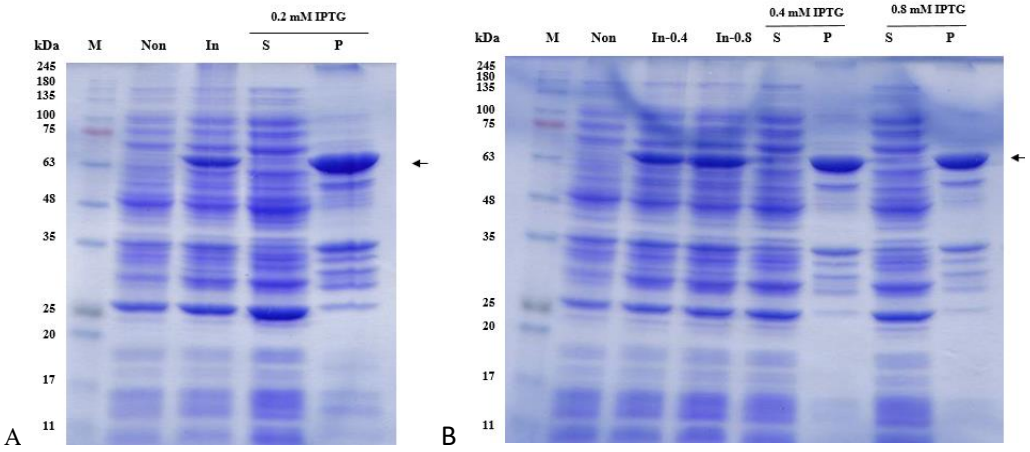

Figure 8 Solubility assay of GST-truncated hHDAC2 protein (A) 37 ˚C 0.05 mM IPTG (B) 25 ˚C, 0.4 and 0.8 mM IPTG, 3 hours of induction time, 200 rpm constant shaking and, Lane M: protein marker, Lane In: IPTG induction, Lane non: non induction, Lane S: supernatant, Lane P: pellet

Discussion

In this study, we had attempted to clone and express recombinant human HDAC2 protein in bacterial expression system, more advantage; easier to manipulation, inexpensive, short time of life cycle (Sofia et al., 2014). For avoid insoluble inclusion protein from overexpressed product, limiting factor in *E. coli* expression system (Sevastsyanovich et al., 2010), we used *E. coli* BL21 (DE3) pLysS as a host. *E. coli* BL21 (DE3) containing pLysS plasmids which constitutively expresses low levels of T7 lysozyme, should improve the digestion of bacterial cell wall to obtain more produced bacterial protein. Moreover, the *full-length hHDAC2 gene* were prepared by PCR using cDNA as a template. A *full length hHDAC2 gene* was cloned into pGEX-6P-2 vector, comprised of GST-fusion protein which encourage overexpressed protein to more solubility in *E. coli* (Malhotra, 2009). DNA sequencing result showed that pGEX-6P-2-*full length-hHDAC2* plasmid from desired colony had 1 point of silent mutation (underlined); CAT > CAC position 504 remaining Histidine residue. The expression of GST-full length rhHDAC2, was performed for 3 hours of induction time under constant shaking 200 rpm, at 25 ˚C and 37 ˚C, and of varying concentrations of IPTG; 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mM. The overexpressed protein size was around 75 kDa which was smaller than that of the theoretical molecular mass of GST-full length rhHDAC2 protein; around 81 kDa, in all concentration of IPTG induction. We had obtained more soluble overexpressed protein by reducing of protein synthesis rate, decreasing of hydrophobic interaction associated protein accumulation, by lowering growth temperature and IPTG inducer concentration (Schumann and Ferreira, 2004). Therefore, the conditions of 0.05, 0.1 and 0.2 mM IPTG induction at 25 ˚C and of 0.05 mM IPTG induction at 37 ˚C were further chosen and used to elucidate the solubility of recombinant GST-hHDAC protein. At 37 ˚C condition, most of expressed GST-hHDAC2 protein band was found in the pellet fraction. Whereas, at 25 ˚C, most of GST-full length hHDAC2 protein was found in the supernatant fraction in all IPTG induced cultures.

Additionally, we had attempted to obtain more level of GST-hHDAC2 protein in the supernatant fraction by cloning and expression of *truncated hHDAC2 gene* in *E. coli*. The data from RCSB PDB data bank (https://www.rcsb.org/structure/5IX0) demonstrated that *truncated hHDAC2* gene was successfully expressed in *E. coli* and the soluble protein was obtained. In this regard, we hypothesized that the smaller protein should be easier to undergoes folding. From nucleotide sequence data of *hHDAC2 gene*, we chose range of nucleotide around 19-1,125 of coding *hHDAC2 gene* which covered deacetylase domain following the data from PDB data bank (RCSB PDB - 5IX0: HDAC2 WITH LIGAND BRD7232) to further cloned and expressed in *E. coli* BL21 (DE3) pLysS. The *truncated hHDAC2 gene* was prepared by PCR using pGEX-6P-2-*full length-hHDAC2* plasmid as a template. Unfortunately, the recombinant GST-hHDAC2 protein was found in the pellet fraction both at 25 ˚C and 37 ˚C IPTG induction.

In summary, we successfully expressed GST-hHDAC2 protein by using 0.05 or 0.1 or 0.2 mM IPTG induction under constant shaking 200 rpm, at 25 ˚C in BL21 (DE3) pLysS. The activity of recombinant GST-hHDAC2 protein will be elucidated. If it has the deacetylase activity, it will be used for screening of HDAC inhibitors from natural source and synthetic compound libraries.

Conclusion

We could obtain soluble recombinant GST-hHDAC2 protein. The expression of GST-hHDAC2 will be tested for the effect of time induction and enzymatic activity. The active GST-hHDAC2 protein will be used as the important component for further screening of HDACi from natural products.

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