HUMAN-DNA LIG1 ENZYME AGAINST G-418 DISULFATE

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Abstract

In mammalian cells, the key of ligases for DNA replication was represented to DNA ligase enzyme type 1. It is considered as a vital enzyme and required in recombination and repair processes. Also, the Human DNA ligase enzyme type 1 is essential for encodes as a part of the family of ATP-dependent ligase. According to requirements for ligases function, they are classified into two groups; one group uses ATP such as Eukaryotes and the other needs NAD^+ such as bacteria. The main work in the current study is to clone, express and purify Human-DNA Ligase Enzyme type 1 gene (H-Lig1). The H-Lig1 gene consists of an open reading frame containing 2760 bp, which encoded for 919 amino acids residues. This gene was synthesized into an empty plasmid by GeneArt (Life Technologies, Fisher Scientific/UK) and sent as double-stranded DNA with individual plasmid. The key objective of cloning and purification of H-Lig1 is to demonstrate the effect of G-418 Disulfate on Human Lig1 protein activity *in vitro*, since DNA ligase of prokaryotic cells observe no similarity to the eukaryote ligases. Here in this study it is found that the activity of H-Lig1 protein (represented to Human-DNA Ligase 1) *in vitro* was not inhibited by using G-418 Disulfate and did **not** show any inhibition and the half-maximal inhibition concentration of G-418 [IC_{50}] was N/A, which is indicating the prospective DNA ligases as novel antibiotic targets against ligase of bacteria. The results have referred that in spite of DNA ligase in prokaryotes and eukaryotes are functionally similar. However, they are structurally different.

Keywords: DNA Ligase, Human-Lig1 Enzyme, G-418 Disulfate, Antibiotic, *in Silico*.

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same principle reaction mechanism, but are not interchangeable [18].

2. Materials and Methods

Gene Synthesis of H-Lig1.
The whole sequence coding collection of Human-DNA enzyme type 1 gene as received by the GeneBank database. The code of gene was 3978 (at Gene Bank). The H-Lig1 gene consists of an open reading frame containing 2760 bp, which encoded for 919 amino acids residues and with molecular weight of 101,736 Da. Because the percentage of C:G base pair was higher in the gene, the gene was synthesized into an empty plasmid by GeneArt (Life Technologies, Fisher Scientific/UK) and sent as double-stranded DNA with individual plasmid. The H-Lig1 was synthesized with SbfI and NotI site at the both end site of the plasmid.

Plasmids and Restriction Enzyme Digests.
The H-Lig1 gene (~1000 ng) was digested with SbfI and NotI (restriction enzymes) at 37°C for 1 hrs with 1× Restriction buffer (NEB) for each enzyme. The enzyme then destroyed by heating to 70 ºC for 15 min. Suitable vector (pMAL) for cloning and expression was ordered from NEB and received as a kit. The kit of pMAL (#E8200S) plasmid arrived with two types of plasmid: pMAL-p5x and pMAL-c5x. PMAL-c5x is similar to pMAL-p5x, but the Phusion protein stays in the cytoplasm not between membranes. Only fresh pMAL-c5x plasmids from the kit (1 µg) were digested with SbfI and NotI enzymes at 37°C for 1 hour plus 1× Restriction digest buffer (NEB), respectively.

Establishing DNA Ligase Assay.
The pre-engineered nucleotides [19] were thoroughly mixed in a 1:1:1 ratio at a concentration of 10 µl in 50 µl at a concentration of 1x of ligase buffer, which includes 30 mM Tris - HCl, pH 8.00, 2 mM $MgCl_2$, 1 mM DTT and 50 µg/ml BSA and connected by cool in the machine of PCR. The experiments on H-Lig1 protein were carried out in a 1 ×Ligase reaction buffer. The concentration of nicked oligoduplex was 1000 nM. Reaction of (50 µl) contained nicked oligoduplex (100 nM), 1 × buffer and 40 nM of pure protein. Experiment was routinely conducted at 16ºC. Time points (10 µl) were taken at regular intervals over 20 minutes (and added to 6 µl Stop buffer (80% (v/v) deionised formamide, 10 mM EDTA, 2 mM NaOH and then added 0.4% (v/v) bromophenol stain). The samples (10 µl) are heated at 90ºC for 2 minutes, loaded and then run at 50W for one hour and half. HEX-labelled DNA strands were visualised by excitation at 600 nm using fluorescence light. The varying concentration of G-418 Disulfate that used in this experiment to inhibit H-Lig1 protein in vitro was

1. Introduction

Mammalian cells have 3 ATP-dependent DNA ligases, which are required for DNA replication and repair [1]. Ligases are a part of the nucleotidyl transferase superfamily. In general, all kind of DNA ligases have the some mechanism for repairing the damage inside the DNA [2,3]. NAD^+-dependent ligase are found particularly in micro-organisms and eukaryotic viruses [4,5,6] . In mammalian cells the key ligase for DNA replication is DNA ligase I (represent Human-Lig1 enzyme) and has been stated to become required in recombination and repair processes [7]. Unsurprisingly, DNA ligase I had been stated to be a potent factor for mammalian cells boosting *in vitro* and *in vivo* [8]. Strongly evidence shows that DNA ligase I is to form of base excision repair (BER) and it shared as well in nucleotide excision repair (NER) with the aid of in vitro restoration [9,10].
In Mammals (eukaryotic cell) the DNA ligases that seal these damages in DNA have three genes that are different from each other in their sequencing, function and physical properties, which all of them have a good conserved catalytic domain responsible for the ligation. ATP-dependent DNA ligase contains of three mainly groups in eukaryotes cells called LigI, LigIII and LigIV. The first and third groups or family found just in all eukaryotes, while the second one is only detected in vertebrates**.** All of them have essential catalytic domains called the NTase [11]. Eukaryotic DNA ligase I (refers to H-Lig1) is the potent protein for ligation of single-strand breaks within the nucleus, such as the ligation of Okazaki fragments (lagging strand) at some stage in discontinuous DNA replication [12]. Sun and Rheanna in 2004 reported that Eukaryotic DNA ligase I is required in different sides of DNA metabolism and responsible for the activity with proliferating cells. Also they stated that the levels of DNA ligase I are up-regulated in human tumor versus normal tissues and is important for the survival of tumor. The loss of DNA ligase1 activity leads to random joining of Okazaki fragments during the replication process [13]. The smallest ATP-dependent DNA ligase was encoded via Chlorella virus PBCV-1, which has about 298 amino acids, and consisting of only two domains and resembling bacteriophage DNA ligases [14]. The NTase domain is considered as the larger domain that binds ATP and contains many of the active-site residues [11]. DNA ligase III gene (LIG3) is essential for mitochondrial DNA replication and repair [1,15]. DNA ligase IV gene (LIG4) is required for repair of nuclear double-strand breaks and for nonhomologous end joining and recombination [16,17]. All of the three DNA Ligases (I, III, IV) that explained above use the

3. Results

Ligate H-Lig1 gene into the plasmid.

The SbfI/NotI-cut gene of H-Lig1 was ligated into pMAL-c5x. **Figure 1.** shows un-cut and SbfI / NotI - cut about 100 ng for original H-Lig1 gene and pMAL-c5x plasmids that are loaded on 2% agarose gel. The ratio between ligated gene and the plasmid was 1 to 20 and was used (10 µL), 1µl of T4 enzyme with 1x T4 ligase buffer. The ligation mixture of SbfI/NotI-cut gene of H-Lig1 gene in pMAL-c5x was incubated on ice to the competent cells named *E. coli* 10-Beta cells (NEB) for 15 min. And then they have been plated on agar plates that include ampicillin (50ug/ml) and then incubated for one night at 37°C till the colonies of bacteria have been grown.

500,1000, 1500, 2000, 2500, 3000, 3500 and 4000 uM, which was much more high than the concentration of G-418 Disulfate that used to inhibit the bacterial LigA protein in my previous study [19]. The time-points that used in this experiments for each concentrations of ligase enzyme was 0.5, 1, 1.5, 3 and 20 minutes. The intensity of band have been analysed via using ImageJ and put them in the programme of Excel to see the V_0 for each time-course of Geneticin. The initial data from the experiments above [V_0] were put in Grafit software with the Geneticin to find the [IC_{50}] for this compound. The IC_{50}, which is refer to the half-maximal inhibition concentration of G-418, indicate to the inhibition concentration that is fit to inhibit the protein.

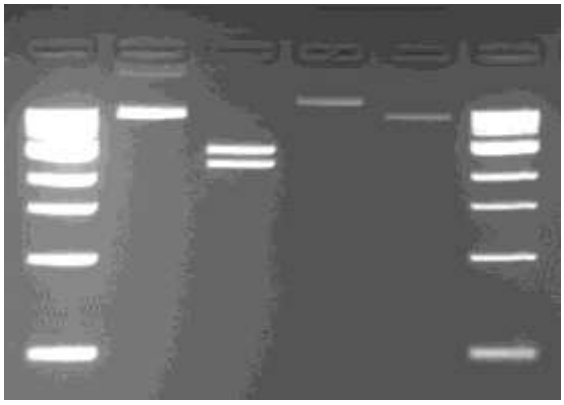


Figure 1. This figure shows the digestion of H-Lig1 gene and pMAL-p5x plasmid (MBP) on 2% agarose gel. The figure shows agarose gel (2%) with different lanes: the lane M shows a marker. Lane 1 was H-Lig1 gene without cut. Lane 2 was the gene with SbfI and NotI cut, it was appear two bands after cut: the first band was pure gene of h-Lig1 (2760 bp), the second band was empty plasmid used by GeneArt to clone the gene because the high percentage of G:C. Lane 3 was uncut fresh pMAL-c5x plasmid. Lane 4 was cut pMAL-c5x plasmid with SbfI and NotI restriction enzymes.

Forward primer 5'-AGCTCGAACAACAACAACAATAACAATAAC

Reverse primer 5'-AAAACGAAAGGCCCAGTCTTTCGACTGAGC

Successful cloning of the H-Lig1 gene in pMAL-c5x was inserted and shown with an arrow next to each gel. For Human ligase in pMAL-c5x, the PCR product was 2933 bp. **Figure 2** shows the successful cloning of H-Lig1 in pMAL-c5x plasmids in 2% agarose gel. Finally, these colonies PCR were sent for sequencing.

Colony PCR for H-Lig1.

For investigation of bacterial recombination H-Lig1 gene (pMal-c5x plasmid with Lig1 gene), 40 samples of ampicillin-resistant colonies were forwarded to Colony-PCR assay. Bifidly, 50 µl of PCR reaction, 25 µl of Taq Mastermix and a pair of specific primers have been used. Amplification the part of pMAL-c5x at both side of the gene was obtained. The forward and reverse PCR primers of pMal-c5x that used are shown below of 30 base:

Figure 2. This figure shows the agarose gel 2% shows the colony PCR for H-Lig1 in pMAL-c5x plasmid. The figure shows the colony of PCR, the first lane showed a PCR result with 2760 bp size (red box) plus initial sequencing of the plasmid (meant up to 3000 bp), showing the H-LIG1 gene in pMAL-c5x.

strands of the plasmids. Sequencing data was opened in SnapGene software (4.0.6) and the sequence of DNA was checked by base pair physically. **Figure 3** shows the image of the correct sequencing of cloned H-Lig1 in pMAL-c5x by Beckman Coulter/ Genewiz.

Gene Sequencing of H-LIG1.

The recombinant H-Lig1 pMAL-c5x Plasmids which about 100 ng were sent for reading the sequencing of DNA by the company of Beckman Coulter. The primers of sequencing that used separately sequenced to the Forward and Reverse

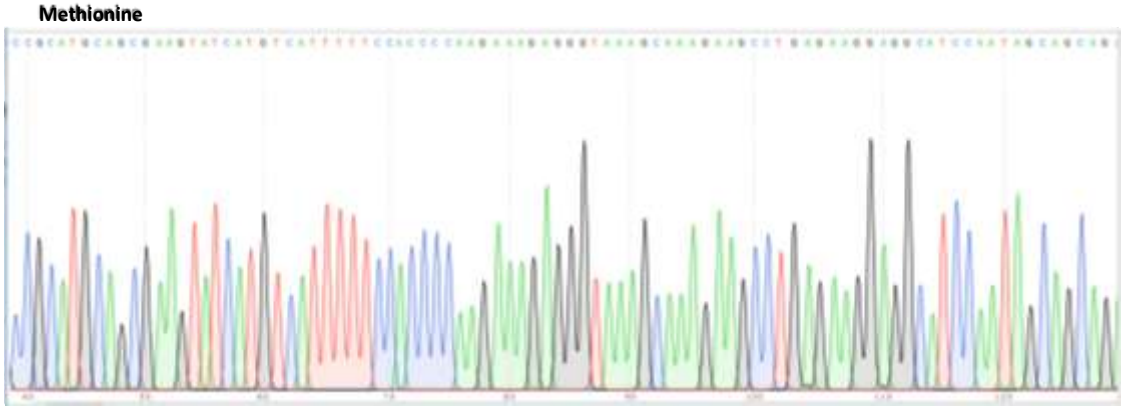


Figure .3 Shows the correct sequencing of cloned H-Lig1 in pMAL-c5x. This figure is showing the example of the up sequencing of cloned H-Lig1 gene in pMAL-c5x.

degrees for three hours till an OD as much as 1.50. Bacterial cultures were collected using a centrifuge at 6000 rpm for a period of 20 minutes at a temperature of 4 °C, and then the filtrate was poured and kept on the pellets. The bacteria precipitate was collected again and broken down using a buffer containing 10 mM sodium phosphate, pH: 7.0 and in the presence of protein inhibitors called PMSF and cocktail protease. Bacterial cells were broken using sonicating device for three minutes, and then using the same centrifugation conditions above, and the cells were separated into a supernatants and pellets. **Figure 4 and Figure 5** show the 10% (w/v) of the Main induction of SDS PAGE gel for Human Lig1 in pMAL-c5x in *E. coli* BL21 (DE3), and showing the pre and post-induction, soluble and insoluble protein. And then the supernatant of lane 4 was applied to a 5 ml of maltose column to purify the hLig1 protein. It was washed with 5 ml of maltose buffer (it came with the kit) to remove non-

Large-scale expression of H-Lig1 protein

The cultures of *E. coli* bacteria were grown in a nutrient media containing milligram ampicillin, and the H-Lig1 pMAL-c5x plasmids were transformed into new reproducible bacterial cells called BL21 E. coli (DE3) in a cool place for thirty minutes, and then suddenly heated up to 100°C for ten seconds, transferred and incubated in a bacteria incubator for a whole day. At 37 degrees and using a vibrator. These bacterial cells were separated into nutrient media containing ampicillin 50 mg per ml and left for a whole day at 37°C. Then only one colony was taken from these colonies and transferred to a liquid nutrient medium containing ampicillin at 37°C for 24 hours using a vibrator. 10 mL of sample of bacterial was subculture of both recombinant Human Lig1 plus 1% glucose brought to the LB broth containing kanamycin. The expression of protein was stimulated by adding one ml of the chemical substance called IPTG, and then leaving the culture mixture at a temperature of 37

using amylose cleave protease to remove the specifically bound proteins. The hLig1 was eluted bound between the hLig1 protein and maltose with ratio 1:50. Finally, the sample of hLig1 protein was collected through the column and stored in storage buffer (20).

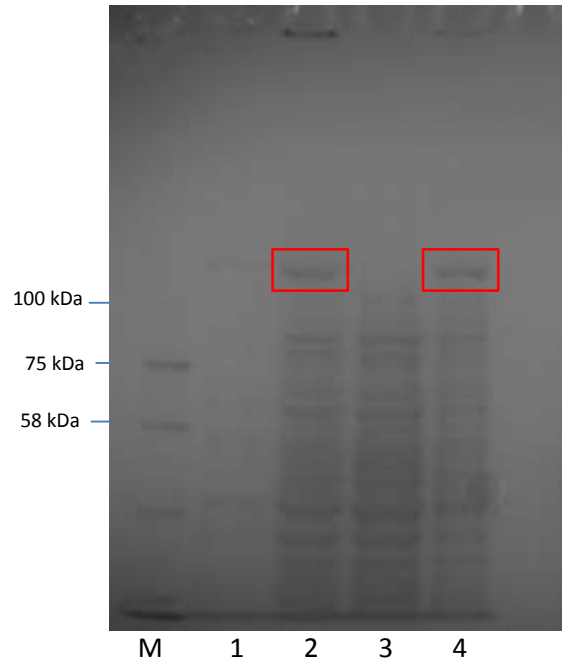


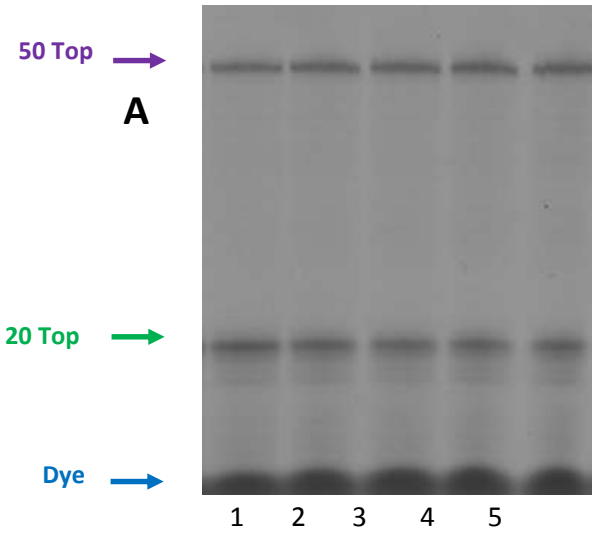
Figure 5 shows the Main Inductions of H-Lig1 pMAL-c5x in BL21 E. coli (DE3).

Lane M indicate to the marker of protein by KDa. The first Lane showed pre-induction of hlig1. The second Lanes is indicated to post-induction of expressed hLig1 (red box). The third Lanes is showing a sample of debris (pellets) of the expressed. Forth Lane is supernatant of expressed of hLig1, as well see the red box.

four time-courses at 4 mM of G-418 Disulfate as an example of many gels. The main aim in these experiments is to find the IC_{50} for this inhibitor (G-418) against the H-Lig ligase protein. **Figure 6 -B-** shows the percentage of ligation products against the G-418 inhibitor in liner scale. The flat line indicate that the inhibitor of G-418 did not inhibit the H-Lig1 ligase protein at any or high concentrations of Geneticin. As it can be seen there is no inhibition, no IC_{50} can be discerned.

In vitro Assay Results.

This section gives the *in vitro* ligation assay result for G-418 Disulfate compound that was tested on H-LIG1 ligase protein to be as novel antibiotic targets. Briefly, the ligation assay was done at the nicked substrate concentration (100 nM of DNA) and 40 nM of ligase H-LIG1 protein. Fortunately, G-418 Disulfate compound (inhibitor) Did NOT inhibit the efficiency of H-Lig1 protein in vitro. **Figure6-A**- shows a 15% (w/v) denaturing gel with



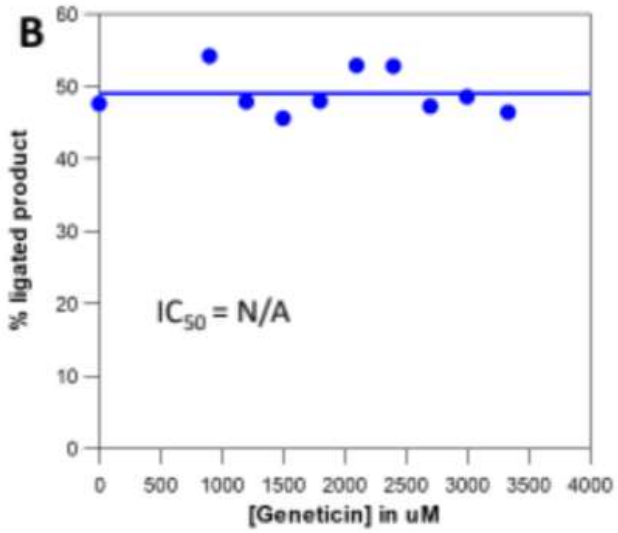


Figure 6-A-. The figure shows the Inhibition of H-LIG1 protein against G-418 Disulfate. lanes 1-4 indicated to the time points of 0.5, 1, 1.5, 3 and 20 minutes to the concentration of 4 mM of G-418 Disulfate (an example of many gels). Figure 6-B- above did not show any inhibition of the inhibitor of Geneticin and the flat line of IC_{50} was flat and did not shows any inhibition.

main objective was to perform together serious trials on LigA protein (in my previous study) [19], and H-Lig1 protein (in this paper) comparing their inhibition underneath experimental situations. Therefore; this will be constructed block compounds to the prospect studies that might be explored or exploited minor molecular compounds libraries as novel inhibitors, which are affected on these two enzymes. The aim here is to test and check if this compound (G-418 Disulfate) will effect on the H-Lig1 protein *in vitro* to state and find the prospective DNA ligases as novel antibiotic targets after I stated that G-418 Disulfate did inhibit the DNA ligase LigA protein in my previous work. Fortunately, it is found that G-418 Disulfate did not inhibit the activity of H-Lig1 (Human DNA Ligase I) protein *in vitro*, which is indicating the promising potential of DNA ligases as novel antibiotic targets.

5. Conclusion

The data of current study indicated that protein production by expression of Eukaryotic DNA ligase Lig I (H-LIG1) in the transgenic *E. coli* bacteria did not inhibit when cultured with G418 Disulfate compound. Compared to the previous study, which showed inhibitory effect of G418 Disulfate on protein expression of prokaryotic DNA ligase, suggesting that DNA ligases exist in both organisms and they are functionally similar but have different structural properties.

Acknowledgment.

4. Discussion

DNA ligases has been discovered by the great resource of Lehman laboratory and other association group for fifty years ago, and this discovery was an important events in the general of biology field [21,22,23,24]. Day by day, the nick or the damage in the DNA can be happened by means of external material with UV or from internal elements consisting of oxidative damage. Damages in the DNA (nicks) always during at some point of the ordinary strategies of DNA in all domains of life. The principle work for all ligases that are to seal or to fix the damage (nick) in one strand of a deoxyribonucleic acid double helix. If the DNA ligases will not repair or fix the nick in the DNA, the cells will be die. Therefore, ligases are very important enzymes for maintaining genomic integrity in the body. As I mentioned above, bacterial ligases are essential as a novel drug objective for antimicrobial treatment, for the reason that eukaryotic ligases (which include DNA ligase I) are extraordinary protein sequences, which are a whole lot larger and more complicated of many subunits [25]. One of most urgent public health problems in the world is antibiotic resistance. The resistance of antimicrobial had been explained as containing doubtlessly catastrophic outcomes for humanity, and early have been represented as “ticking time-bomb” [26]. Since DNA ligase of prokaryotic cell observe No similarity to the eukaryote ligases, DNA ligases of bacterial can be a prospective antibiotic objective, in view that they do not share to prokaryotic cells, as well with different strains of bacteria [27]. In this study, the

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