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RESEARCH ARTICLE

Molecular cloning of putative L-asparaginase/glutaminase gene from Klebseilla peunomonae MGH 78578

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Abstract

This study is one among a few local studies that have been reported to clone the putative L-asparaginase/glutaminase produced by recombinant gene based on the use of plasmid Ecoli - Nterm 6X HN(PLEICS-01) vector. In this study, a pair of primers was designed to obtain L- glutamin-(asparagine)ase encoding gene from Klebsiella pneumoniae subsp. pneumoniae MGH 78578. The putative L-asparaginase/glutaminase gene encoding gene (KPN-01165) was expressed in E.coli BL21(D3) to make sure the presence of a functional putative L-asparaginase/glutaminase in the cloned DNA. The recombinant L-glutamin-(asparagine)ase gene was 1005 bps which encoding a protein of 334 amino acids. The recombinant enzyme containing a 6xHistidine-tag can easily purified by Ni-NTA chromatography and its molecular weight was determined to be 40 KDa by SDS-PAGE. A putative L-asparaginase/glutaminase catalysed the hydrolysis is not only of L-glutamine, but also of L-asparagine.

INTRODUCTION

L- asparaginase and L-glutaminase from microbial source are amidohydrolase enzyme (EC 3.5.1.38) that catalyze the hydrolysis of L- asparagine (L-asn) and L-glutamine(L-gln) to L-aspartate (L-asp) , L-glutamate(L-glu) respectively and release ammonia (NH_3). L-glutamin-(asparagine)ase have been cloned and expression into different expression vectors by several investigators (Youssef and Al-Omair, 2008 and Ito et al., 2012). Immobilized Metal ion affinity chromatography (IMAC) is a development strategy that uses to purify the histidine-tagged fusion enzyme in only single step, make IMAC an attractive and versatile choice for rapid protein purification with purities over 95% (Cheung et al.,2012).

Both amidase activities are broadly distributed among plants, animal and microorganisms, that include bacteria, yeast, fungi such as E.coli , Pseudomonas sp. , Erwinia sp. (Maria et al., 2004; Jyoti et al., 2011).These activities have attracted much attention with respect to its applications in medical as an anti-leukemic agent and some mast cell tumor (Gomez-fabre et al .,2000), and also as anti-retroviral agent (Roberts and McGregor, 1991). In the other hand both activities play a major role in nitrogen metabolism for both prokaryotes and eukaryotes. Besides the activity of L-glutamin-(asparagin)ase can be used for different industrial purposes for the production of special chemical compounds such as thiamine and glutamine which considered an important **amino acid** contributing to a favorite taste (Kornbrust et al ., 2010; Sarada, 2013), and as aroma enhancing agent (Loeliger, 2000). Other application is act as a biosensor for monitoring the level of glutamine in mammalian and hybridoma cell cultures (Sabu et al., 2000). Clinically, decreasing the level of asparagine and /or glutamine by the administration of bacterial asparaginase and/or glutaminase or by the use their antagonists are considered the common therapy for patients with acute lymphocytic leukemia. In contrast, supplementation with asparagine and glutamine promote a more malignant and less differentiated phenotype in human colon cancer cell lines (Turowski et al.,1994).

This study is aimed to clone the putative L-asparaginase/glutaminase from Klebsiella pneumoniae subsp. pneumoniae MGH 78578 strain into suitable expression host and characterize it , and this was achieved by:

Cloning and expression KPN-01165 into suitable non-pathogenic host (genetically engineered E.coli)

Materials and Methods

Materials

All specific primers used in this study were synthesized by Sigma (UK). Other chemicals were provided from Leicester university (UK).Klebsiella pneumoniae subsp. pneumoniae MGH 78578 strain was obtained from Dr.Kumar Department of Infection, Immunity and Inflammation (III) , DH5a E. coli and Department of (III) Leicester University, E.coli BL21 DE3 was obtained by Novagen (UK).

Isolation of DNA from Klebsiella pneumoniae subsp. pneumoniae MGH 78578 Strain

The DNA extraction method from Gram-negative bacteria was done according to the modify standard phenol/chloroform method (Cheng and Jiang , 2006). The purity and concentrations of the DNA were assessed by using Nano drop by calculating the absorbance (A) value of A260/A280 ratios.

Cloning and overexpression of putative L-asparaginase/glutaminase gene

Pairs of primers were designed in order to detect and purify of KPN-01165 gene by PCR .These are

Forward (F): '5 TACTTCC AATCC ATGAGC AGCC TGGCGTTTAGCGA3'

Reverse (R) :' 5 TATCC ACCTTT ACTGTC AGTAG GTATGGAAATATTGC 3'

The polymerase chain reaction was performed in 25 ml; each reaction mixture was preheated to 95°C for 5 min. Each PCR cycle (35 cycles) was heated for 40 sec at 94 °C for denaturation, 45 sec at 55-60 °C for annealing , 90 min at 72 °C for extension , and final extension at 72 °C for 2 min. The PCR product was analyzed by 1% agarose gel electrophoresis. The PCR product from the 56˚C was pooled and cleaned with PCR purification kit, and then was determined its concentration to be 50 ng/ul by using Nanodrop system. (Sambrook and Russell , 2001).The PCR product was cloned with cloning and expression PLEICS-01 vector ,the cloning reaction used in this work was done according to the In-Fusion Dry Down PCR Cloning Kit as described in Table (1).

Table (1) – Ligation reaction composition

	Test	Control
Reaction No.	Volume / µl	Volume / µl
Purified PCR fragment	2 µl (50 ng)	-
Linearized vector	1µl (50 ng)	1µl (50) ng)
In-Fusion HD Enzyme Premix	2 µl	2 µl
Dnase/Rnase free water	to 10 µl	To 10 µl

The recombinant vector was used to transform competent DH5a E. coli cells. The transformed cells, harboring the recombinant PLEICS-01 plasmid, were grown at 37°C in LB medium supplying with100mg/ml of ampicillin, then the recombinant plasmid was isolated from DH5a E. coli and used to transform E. coli BL21 (DE3).The cells were grown into LB agar plate containing ampicillin and X-gal. The recombinant clones were identified by blue/white selection and grown in LB media sublimated with 100mg/ml ampicillin at 37 °C . KPN-01165 gene was induced by 1 mM IPTG when the absorbance at 600 nm was arrived to 1.5 (Agostinho, 2011).

Determination the molecular weight of putative L-asparaginase/glutaminase

Western blot was a widely accepted analytical technique to detect the specific protein in extract. This method was done after separation the protein by elution the protein from the gel.

Results and Discussion

Isolation of Genomic DNA

Isolation of the genomic DNA of Klebsiella pneumoniae subsp. pneumoniae MGH 78578 strain has been conducted using the Qiagen Kit for DNA extraction, and then separated on a 1% (w/v) agarose gel containing Ethidium Bromide . The genomic DNA that was isolated from Klebsiella pneumoniae showed an approximate concentration of 475 ng/µl with high purity.

Amplification of putative L-asparaginase/glutaminase gene (KPN-01165) from Klebsiella pneumoniae subsp. pneumoniae MGH 78578

The amplified full gene of L- glutamin-(asparagin)ase was approximately 1005 bp in length, which was predicted to encode a polypeptide of 334 amino acids .The optimum temperature for amplifying this gene was 56 ˚C as illustrated in figure (1).

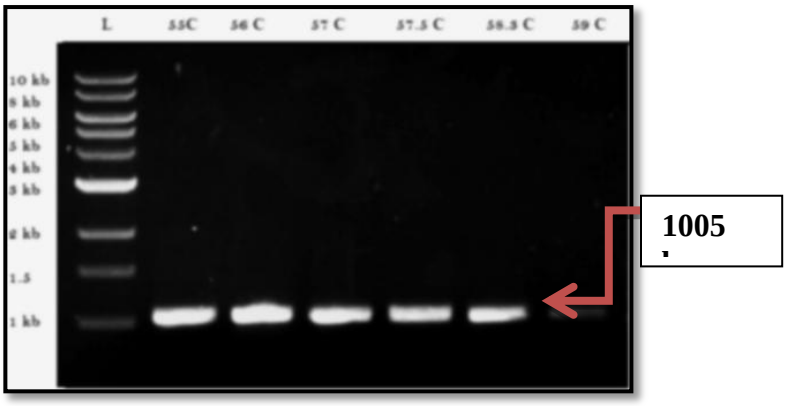

Figure (1): Gel electrophoresis of the amplified KPN -01165 gene of Klebsiella pneumoniae on (1%) agarose visualized under UV after running for 1.15 hr and 110 Volts. L: DNA ladder 10 Kb and L1-L6: Full amplified KPN-01165 gene of putative L-asparaginase/glutaminase at different temperature values range from 55 to 59°C.

Molecular Cloning and Analysis of Clones Containing KPN-01165 Gene

The gene KPN_01165, encoding putative L-asparaginase/glutaminase was cloned downstream to the T7 promoter of the PLEICS-01 vector to allow the sequence of KPN-01165 gene to insert at the C-terminal and 6X His tag at the N-terminal. The recombinant vector was transformed into DH5α competent E.coli cells. Only the positive clones were transformed to the picked and culture on a selective media. The recombinant vector was isolated, and used for the transformation of E.coli BL21 (DE3) competent cells to allow the expression of protein. The purified recombinant plasmid from E.coli BL21 (DE3) competent cells was analyzed on 1% agarose gel. The results in figure (2) showed that the size of the recombinant plasmid was 8635bp, obtained from (7630 bp from the empty PLEICS-01 vector +1005 bp of KPN-01165 gene). The concentration of the recombinant plasmid was approximately 1175-1320 ng/µl.

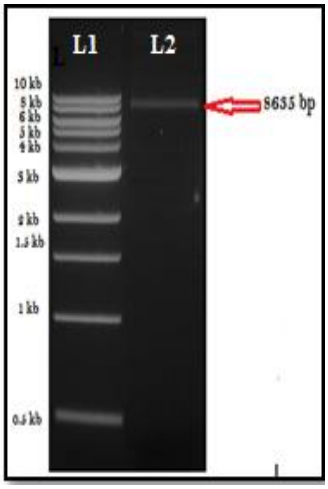

Figure (2): Detection of the recombinant DNA vector obtained from the transformant using 1% agarose gel and by comparing the bands to the 10 Kb DNA ladder marker, visualized under UV after running for 1.15 hr and 110 Volts. L: DNA Ladder 10kb and L1: Recombinant DAN Vector

This can be achieved by using forward and reverse primers for KPN-01165 gene in order to check the PCR product. PCR product represents the KPN-01165 gene with the up and downstream sequences of PLEICS-01 vector, as shown in figure (3).

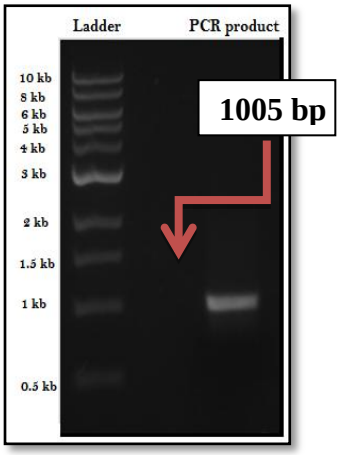

Figure (3): The inserted KPN-01165 gene with a correct orientation to give a PCR product on 1% agarose gel at 110 volt for 1.15 hr. Lane 1: DNA Ladder 10 kb and Lane 2: Amplified the PCR product.

Expression, Purification and Characterization of Fusion Enzyme

Gene expression was tested and optimized for the enzyme encoded by KPN-01165 gene after being induced with 1mM IPTG. The expression was done under the control of T7 promoter of pLEICS-01 vector in BL21 (DE3) E.coli cells. Recombinant enzyme was visible in all prepared samples around 40 KDa range (figure 4). This result implied that the fusion enzyme was in an insoluble fraction. It further indicated that all the pellet samples resulting from the extraction of BL 21 (DE3) E.coli cells harboring the recombinant vector (pLEICS-01 vector + KPN-01165 gene) were prepared assuming that the fusion enzyme was in an insoluble fraction of the cells lysate extraction. Generally Recombinant protein in several host systems was accumulated intracellularly in insoluble aggregates. The protein in this case was inactive and denatured, such protein called **inclusion bodies.**

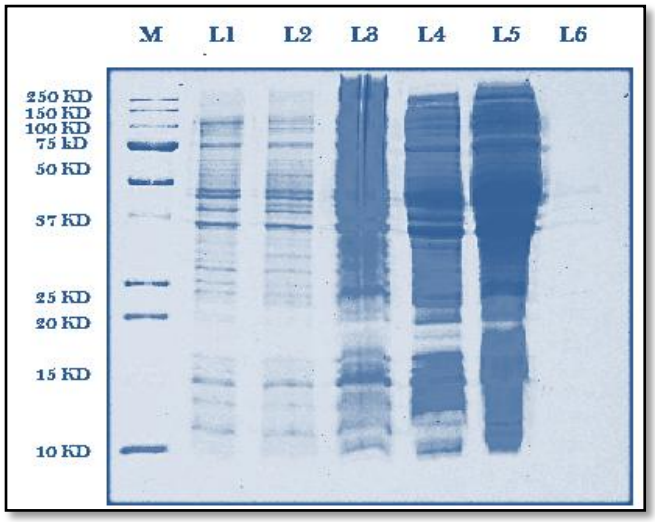

Figure (4): SDS-PAGE profile of total cell lysate from samples after expression

M: Molecular weight of the protein marker from (250-10)kDa ; L1: total extract of the un induced cells with IPTG as control; L2: total extract of the cells induced with 1mM IPTG; L3: total cell lysate after sonication; L 4:pellet extract of the cells after extraction; L5: pellet of the cells after column; L6: cells extract of the cells after wash.

Determination the Molecular Weight of L-glutamin-(asparagin)ase

As the intrinsic charge differences between proteins are masked by the SDS, separation of proteins is due to differences in size , hence the method can be used to determine molecular sizes. A linear relationship between mobility and log MW obtains over a molecular weight range dependent upon the gel pore size. The gel can thus be standardized with proteins of known molecular weight and subsequently used to estimate the molecular weights of unknowns. The purified enzyme was analyzed under denaturing conditions using 10 % SDS-PAGE. Analysis results indicating that the L-glutamin-(asparagine)ase was purified till homogeneity with a single band upon staining by

silver stain and also shown that band had a molecular weight was approximately 40 KDa. The size of the fusion enzyme was more than the original size of the enzyme. All that due to the sequence of enzyme was fused to the C-terminal His tag of the pLEICS-01 vector (Figure 5).

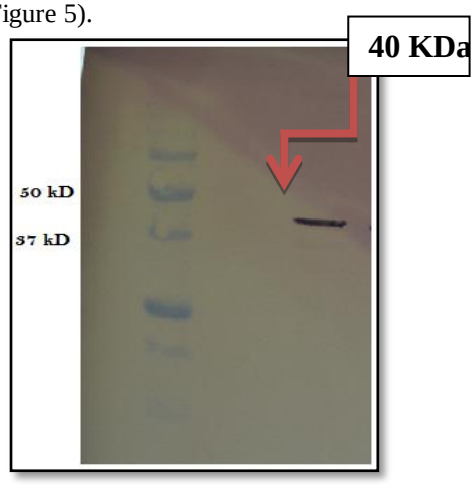

Figure (5): Determination the molecular weight of purified enzyme using western blot

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