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Expression Study of a Recombinant Plasmid containing Dipeptidyl Peptidase-4 Gene in *E. coli*: A Plausible Application for Celiac Disease Patients to Digest Gluten

Yeonjae Lee[1], Ryan Kang[1], Jenna Kwon[1], Kyuhee Jo[1], Jungbin Im[1], Sangwook Jung[1], DongHyun Lee[1], Juhyeon Lee[1], Jeong-Sang Lee[2,*]

[1]*Hankuk Academy of Foreign Studies, Yongin 17035,*
Dep. of Biotechnology and Functional Foods, Jeonju University, Jeonju 55069, Rep. of Korea
**jslee11@jj.ac.kr*

Abstract

Celiac disease (CD) is an immune-mediated enteropathy of small intestine diagnosed in both childhood and adulthood. CD is caused by gluten, which produces gliadorphin during its digestion. The enzyme dipeptidyl peptidase-4 (DPP4) breaks gliadorphin down nevertheless the last tripeptide remains and eventually inhibits DPP4, thus slowing down its process. Therefore, the idea is to produce an additional DPP4 enzyme which is crucial. Consequently, the functional DPP4 gene was cloned into pCDNA3 intermediate (FLAG+DPP4) vector and finally a recombinant plasmid pSB1C3 (Andersons promoters+ FLAG+DPP4) was constructed using synthetic biology. Normally, a DPP4 inhibitor is used as a cure for diabetes. Another important concern was overexpression of DPP4, which might lead to diabetes, accordingly the work was also performed for the regulation of the DPP4 gene expression. In this regard, three types of Anderson promoters (strong, moderate and weak) were utilized to study the control overexpression. This is the first report of idealistic trial for control the exogenous DPP4 gene-expression by molecular biologic tools.

Keywords: *Celiac disease, Vectors, Anderson promoter, Infusion ligation, Synthetic biology*

1. Introduction

Celiac disease (CD) is a gluten-induced autoimmune disorder of small intestine diagnosed in both childhood and adulthood [1]. It is complex multifactorial diseases results from interplay between genetic and environmental factors [2]. Worldwide, the prevalence of CD is about 1%–2%, but appears to be increasing [3]. According to a recent population-based study of pediatric celiac disease of North America, an increasing incidence of CD from 8.1 per 100,000 person-years (2000-2002) to 21.5 per 100,000 person-years (2011-2014) have been reported. In addition, CD prevalence data among children increased

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Corresponding Author: jslee11@jj.ac.kr
Tel: +82-63-220-4660, Fax: +82-63-220-2054
Dep. of Biotechnology and Functional Foods, Jeonju University, Korea

from 2010 (0.10%) to 2014 (0.17%) [4]. If treatment is not given, the symptoms in CD patients include malabsorption, deficiency of iron, deficiency in B vitamins, and reduced quality of life [5]. Moreover, the long-term health effects include Type I diabetes, lactose intolerance, multiple sclerosis (MS), anemia, infertility and miscarriage, osteoporosis, short stature, and neurological conditions like epilepsy and migraines, and also there is enlarged risk of lymphoma [6].CD is characterized by intolerance to gluten (a protein found in wheat, rye, and barley) in genetically susceptible individuals [7]. The protein (basically an environmental factor) is composed of gliadorphins with a large amount of proline as monomer (Fig.1). When people with CD eat gluten rich food, the proline of gliadorphins causes an immune response that targets the small intestine particularly the duodenum. These attacks lead to damage on the villi that stimulate nutrient absorption in the small intestine. The peptide gliadorphin (also known as gluteomorphin) is a byproduct of the digestion of the gliadin element of gluten protein. This gluteomorphin is working as a substrate for dipeptidyl peptidase-4 (DPP4) enzyme, which decomposed into gluten and morphine. The classical symptoms of the disease include nausea, vomiting, and dyspnea in response to intake of gluten.

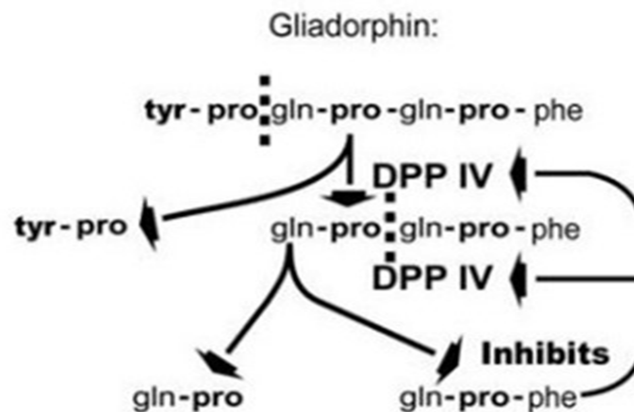

Figure 1. Digestion of Gliadorphin by DPP4.

2. Theory

Ironically, the only treatment available is a permanent gluten-free diet which may lead to malnutrition due to damage in the small intestine that prevents proper absorption of nutrients into the body [8]. DPP4 is a serine exopeptidase that cleaves the N-termini of peptides as well as casomorphin for release of a dipeptide with proline at penultimate position [9]. Such process is repeated twice, removing two dipeptides and leaving tripeptide (gln-pro-phe) with proline in the middle position (Fig.1). The remaining tripeptide ultimately serves as a potent inhibitor of DPP4 leads to inactivation of the enzyme. Therefore, additional input of DPP4 is important in digestion to compensate for the inhibition. In fact, by breaking down casomorphin and gliadorphin, DPP4 essentially embarks on a course of self-destruction hindering many of the functions of this enzyme. Thus, a DPP4 inhibitor is commonly used as agents for the treatment of type 2 diabetes mellitus [10]. The gluten-free products market will be anticipated to be a worth of 7.59 billion USD by 2020 [11]. We learnt that more people are relying on temporary solutions and, thus, a fundamental solution is needed. Considering the importance of the cure for CD patients, the objective of the present

research is to use synthetic biology for making recombinant plasmid for the production of DPP4 gene product and also to optimize its expression by using Anderson promotors of different strength.

3. Experiments

3.1 PCR amplification of DPP4 gene from SPORT6 and purification

As per the requirement of the project, DPP4 gene was amplified from vector pCMV SPORT6 kindly provided from Korea Human Gene Bank, Medical Genomics Research center, KRIBB, Korea. The primers for the amplification of DPP4 gene were designed and consisted of vector sequence (pCDNA), restriction enzyme sequence (RE) and DPP4 gene sequence (Fig. 2). Thus, 5' - pCDNA3 vector sequence (about)10~15mer + BamH1 seq + DPP4 seq - 3' was used as forward primer and 5' - pCDNA3 vector sequence (about)10~15mer + Xho1 seq + DPP4 seq - 3' as reverse primer. The DPP4 thermocycling was performed in triplicate using an automated temperature-control system (TaKaRa Korea Biomedical Inc., Seoul) using primers 5′-TGA CGA CAA GGG ATC CAT GAA GAC ACC GTG-3′ and 5′-TAG ATG CAT GCT CGA GCT AGT GTA AGG AGA AGC ACT-3′ as forward and reverse primers (as described above), respectively PCR reaction mixture (50 µl) consisted of 1 µl of pCDNA3 DNA (100 ng), 2 µl of each (forward and reverse) primer (10 pmoles/ µl), 4 µl of dNTP mix, 1 µl of GXL DNA polymerase, 10 µl of GXL PCR buffer and 30 µl of distilled water.

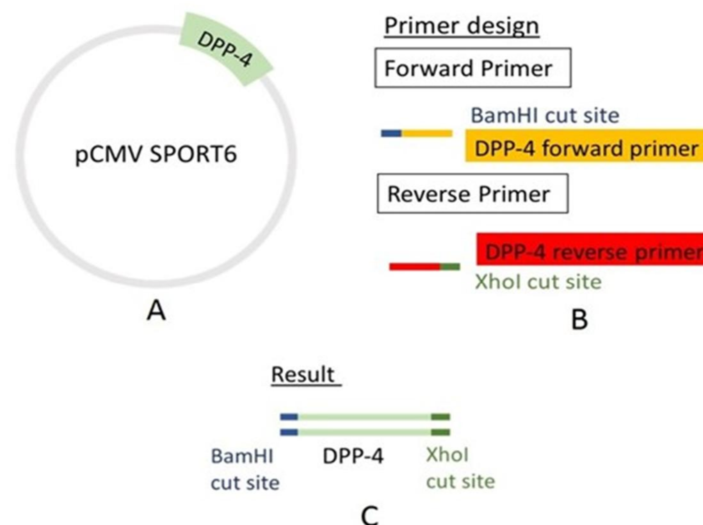

Figure 2. (A) Schematic diagram of the vector pCMV SPORT6 consisted of desired gene DPP-4.
(B) Showing the primers design consisted of BamH1 and Xho1, restriction enzyme sites.
(C) Depicting the digested gene DPP-4 fragment with restriction sites ready for amplification and ligation.

The following PCR conditions were used: 94°C for 5 min, 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 3 min, followed by a final elongation at 73°C for 3 min and amplified DNA samples were stored at 4ºC. PCR products were electrophoresed by using 1% (w/v) agarose

gel (Sigma-Aldrich, Seoul), purified and quantified.

3.2 Preparation of the intermediate vector (pCDNA3) and Infusion ligation

Plasmid pCDNA3 mammalian expressing vector was kindly provided by molecular mechanisms of functional foods laboratory, Jeonju University. The vector (580ng/µl) was digested with BamH1and Xho1. For digestion, 10 µl of DNA, 2 µl of each RE, 3 µl buffer followed by addition of 13 µl of water to make final volume 30 µl. The mixture was incubated at 37ºC overnight. The digestion of the vector was confirmed by agarose gel electrophoresis by loading 27.5 µl of cut vector and 4 µl of 1000 DNA marker and the results (properly cut vector) showed 6300 bp band size on a gel (Fig. 3). After confirmation, the 25 µl vector volume was purified by using DNA extraction Kit (TaKaRa Korea Biomedical Inc., Seoul). Finally, the purified DNA was quantified using Nanodrop (Thermo Fisher Scientific, Seoul) and sequenced. For infusion ligation, a mixture (20 µl) consisted of 5 µl of pCDNA3, 5 µl of insert gene (in step 2.1), 4 µl of infusion ligase (5X) and 6 µl of distilled water was incubated at 50°C for 15 min.

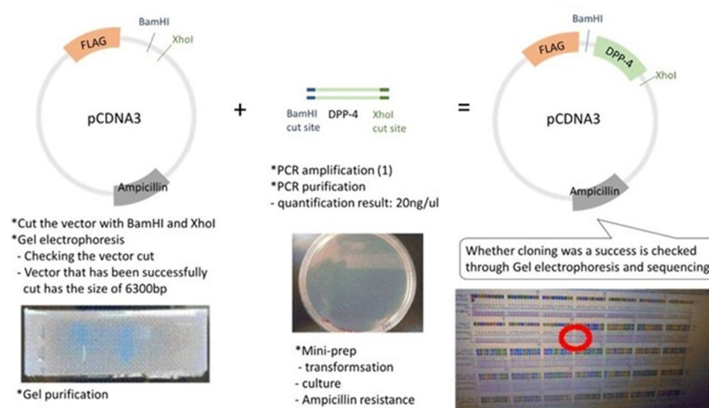

Figure 3. Representation of intermediate vector preparation, purification and checking on an agarose gel electrophoresis and ligation to DPP-4 gene amplified in step 2.1. Finally, bands were confirmed by sequencing.

3.3 Transformation and Plasmid isolation

For transformation, Inoue method was used for the synthesis of ultra-competent cells [12]. 100 µl of prepared competent cells were mixed with 20 µl of recombinant plasmid (prepared in step 2.2) and the reaction was put on ice for 30 min. Further, the reaction mixture was incubated at 42°C for 45 s, followed by on ice for 3 min. 900 µl of Luria-Bertani (LB) broth was added in a mixture and incubated at 37°C for 30 min. The mixture was centrifuged at 5000 rpm for 5 min. After centrifugation, 800 µl volume was discarded and 200 µl suspension was plated on ampicillin (100 µg/ml) containing LB agar plate; incubated at 37°C for 16-18 h. One colony from petri dish was grown in LB [13] at 37°C overnight followed by plasmid isolation using plasmid DNA isolation mini Kit (Thermo Fisher Scientific, Seoul) following manufacturer's instructions. The digestion mixture (10 µl) consisted of 3 µl of vector DNA (pCDNA3), 1 µl of each restriction enzyme (BamH1 and Xho1), 1 µl of 10X K buffer and 4 µl of distilled water. Electrophoresis was performed using 10 µl of cut vector and 4 µl of DNA marker for examination of bands. The recombinant plasmid was cut into two bands consisted of a cut vector (6300 bp) and DPP4 gene (2300 bp). The bands were purified as mentioned above and sequenced. For sequencing, primers were designed 100bp before the start of the insert sequence to avoid any error (Fig. 3). The primer sequence and the temperature for forward

and reverse sequencing primers consisted of 5′-AAA CCG GGC CCC TCT GCT-3′ (60.8°C) and 5′-AAG GCA CGG GGG AGG GG-3′ (61.8°C) respectively.

3.4 Construction of backbone plasmid (pCDNA+FLAG+DPP4) and amplification

After confirmation of recombinant plasmid, the next task was to create the backbone plasmid by the final addition of insert gene(anderson+FLAG+DPP4) into pSB1C3. The desired plasmid was created by insertion of Anderson promoter in the backbone through PCR and ligation to the final vector (pSB1C3). For PCR amplification, the primers were designed using the same sequence of DPP4 gene. The reverse primer consisted of three parts (pSB1C3 reverse sequence+ enzyme sequence Pst1+ DPP4 reverse sequence). Therefore, the sequence of the reverse primer consisted of 5′-TTTTGCCGGA CTGCAG CTAGTGTAAGGAGAAGCA-3′ (Tm-51.4°C). Conversely, the forward primer sequence consisted of four parts (vector sequence+ enzyme sequence EcoR1+ Anderson sequence + flag primer sequence). The actual part of primer that binds to the backbone is FLAG sequence. Rest of the sequence acts as an adaptor and Anderson gene does not need amplification using the vector. To check the expression, three kinds of promoters namely A1 (strong), A2 (moderate) and A3 (weak) were used (Table 1).

The same PCR mixture was used for the amplification as described above (section 2.1) except 1 µl pCDNA3 vector DNA (90ng). The amplification was done in duplicates using the same amplification protocol as described above. The PCR was performed, electrophoresed and purification was done.

Table 1. Forward sequences of primers

Forward Primer	Vector sequence	RE[#] gene	Anderson promotor	Tm*	Flag
A1 (strong)	ATGATTTCTG	GAATTC	TTGACGGCTAGCTCAGTCCT AGGTACAGTGCTAGC	55 °C	GACTACAAAG ACGATGACGA
A2 (moderate)	ATGATTTCTG	GAATTC	TTGACGGCTAGCTCAGTCCT AGGTATAGTGCTAGC	55 °C	GACTACAAAG ACGATGACGA
A3 (weak)	ATGATTTCTG	GAATTC	TTGACGGCTAGCTCAGTCCT AGGTACTATGCTAGC	55 °C	GACTACAAAG ACGATGACGA

RE- Restriction Enzyme site; * Melting temperature

3.5 Transformation using pSB1C3

Plasmid pSB1C3 was kindly provided by iGEM 2017. 100 µl of competent cells (as described in section 2.3) were mixed with 20 µl of recombinant plasmid (prepared in step 2.4) and the reaction was put on ice for 30 min. Further, the reaction mixture was incubated at 42°C for 45 s, followed by on ice for 3 min. 900 µl (9 times the competent cell volume) of LB broth was added in a mixture and incubated at 37°C for 40 min. The mixture was centrifuged at 5000 rpm for 5 min (Centrifuge, Eppendorf, Seoul). After centrifugation, 800 µl volume was discarded and 200 µl suspension was plated on chloramphenicol containing (34 µg/ml) LB agar plate; incubated at 37°C for 16-18 h. Three red colonies were picked and added to 5 ml of LB broth (containing chloramphenicol) and incubated at 37°C for 16 h for plasmid isolation using plasmid DNA isolation mini Kit (Thermo Fisher Scientific, Seoul) following manufacturer's instructions. The plasmid obtained was quantified and was digested with EcoR1 and Pst1 RE. The reaction mixture consisted of 50 µl plasmid (50ng/ µl), 2 µl of each restriction enzyme, 7 µl of buffer followed by 9 µl of distilled water to make final volume 70 µl. The mixture was incubated at 37°C overnight (for complete digestion). The digested product was run on an agarose gel, purified and quantified. The cut vector was obtained with a size of 2070

bp (Fig. 4).

3.6 Infusion Ligation

The purified plasmid was used for the infusion ligation step. The mixture for ligation step consisted of pSB1C3 and PCR amplified-Anderson + Flag and DPP4 (Fig. 5). The total mixture was 20 µl containing 1 µl vector (50 ng), 1 µl insert gene (90 ng), 4 µl infusion ligase (5X), buffer 8 µl and 6 µl of distilled water. The mixture was incubated at 50°C for 15 min.

Ligation was followed by transformation as described previously. The three plates (A1, A2, and A3) were checked for the formation of colonies. The 3 colonies were picked from each plate and were grown in 5 ml of LB broth containing antibiotic chloramphenicol for plasmid isolation. The plasmid digested again with EcoR1 and Pst1 and bands were verified on gel electrophoresis.

3.7 Transfection

The transfection was performed using transfection reagent jetPEI (Polypus). Before transfection, HEK293T cells were removed from the plate containing Dulbecco's modified Eagle's medium (DMEM) supplemented with additional 10% fetal bovine serum (FBS). After washing cells with 5 ml PBS subsequently, 5 ml DMEM was added to the plate for induction of serum starvation. Five micrograms each of plasmid and jetPEI (from Polypus) were separately mixed with 250 µl Opti-MEM media for 10 min and then mixed together for 20 min.

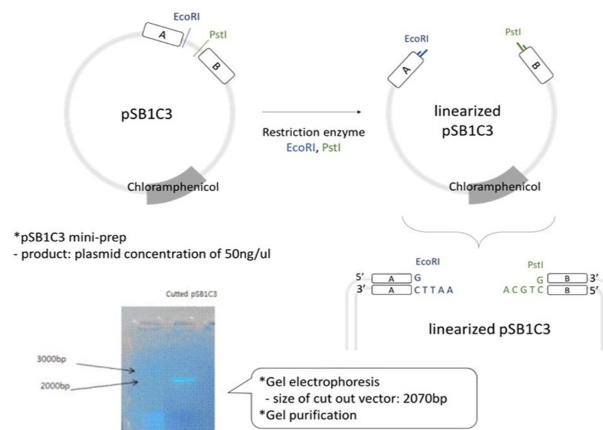

Figure 4. Schematic diagram illustrating the digestion of plasmid pSB1C3 with EcoR1 and Pst1 and agarose gel electrophoresis results revealed a size of 2070 bp.

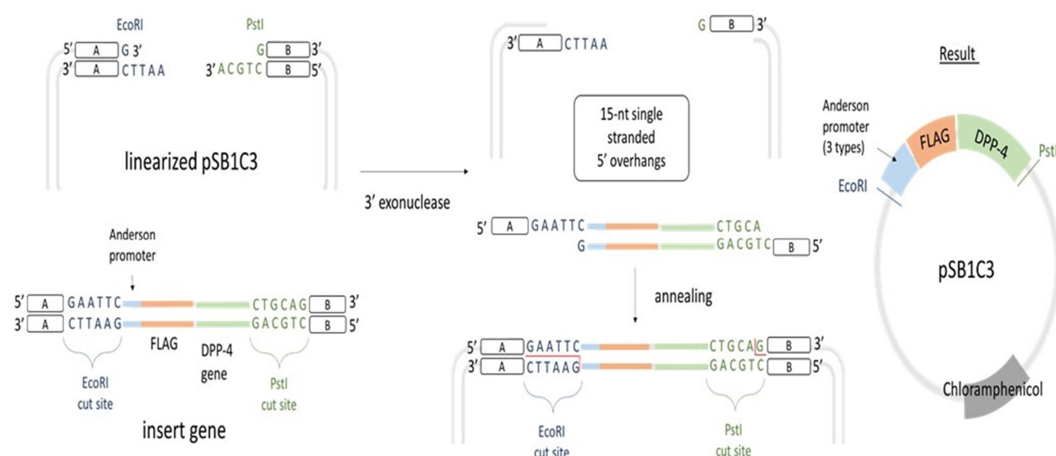

Figure 5. Schematic illustration of the infusion ligation consisted of linearized pSB1C3 and insert gene (Anderson Promotor+ FLAG+ DPP4 gene) and construction of recombinant backbone plasmid.

at room temperature. The mixture was incubated with 293T cells at 37°C in a CO_2 incubator under 5% CO_2. After incubation for 12 ~18 h, the medium was changed with DMEM containing 10% FBS, and the cells were incubated at 37°C for 2 days. The medium was discarded after 2 days of incubation, and 293T cells were washed with ice-cold PBS and collected in a 1.5 ml tube after addition of 500 µl ice-cold lysis buffer. After incubation, the cell lysate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new 1.5 ml vial and analyzed by Western blotting. Actin protein, detected using an anti-beta actin mAb (Sigma-Aldrich, Seoul), was used as a loading control.

3.8 Western Blot analysis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed in a 12% (*v/v*) polyacrylamide gel. The bands were visualized by gel staining with Ponceau S solution (Thermo Fisher Scientific, Seoul), transferred from the gel onto a polyvinylidene fluoride membrane (Sigma-Aldrich, Seoul) for western blotting. The protein-blotted membrane was blocked with skim milk and then reacted with FLAG-DPP4 (Ab) (1/1000) (mAB ANTIFLAGM2 Sigma F1804), followed by incubation with horseradish peroxidase (HRP) conjugated anti-mouse IgG Ab (Santa Cruz, CA, USA). Signal immunoreaction was performed by using solutions 1 and 2 and were incubated with primary and secondary antibodies, respectively. Enhanced Chemiluminescence detection reagent (Thermo Fisher Scientific, Seoul) was used for signal exposure on the film (Kodak, Seoul). An imaging procedure of manual was followed to control the exposing time.

4. Results and Discussion

4.1 Transformation of recombinant plasmid and infusion cloning

Recombinant plasmids were linearized using the EcoR1 and Pst1 restriction enzymes, then transferred into DH5alpha competent cell of *E. coli* strain by electroporation. Positive colonies were randomly picked for identification and colonizing by LB broth within shaking incubator for overnight. After colonizing each clone, each sequence was double-checked using sequencing service. Eventually, we could get the three types

of each colony harboring different Anderson promoter (Weak, Moderate, and Strong).

The results of the PCR amplification and agarose gel electrophoresis showed the presence of gene fragment bands of 2340 bp, which confirmed that the DPP4 gene was successfully integrated into the genome of *E. coli* (Anderson-33 bp+Flag-18 bp + *Eco*R1-6 bp +DPP4-2283 bp) (Fig. 5). After purification, the 87 ng/µl, 92 ng/µl and 94 ng/µl concentrations of the DNA were used for amplification with primers consisted of A1, A2, and A3 respectively. Transformants were examined for the correct insert by using the colony PCR and the DNA bands of the expected size (2340 bp) were confirmed on an agarose gel (Fig. 6).

Results of the transformation of competent *E. coli* cells with recombinant plasmid showed in colonies in three petri dishes A1 (strong Anderson), A2 (moderate Anderson) and A3 (weak Anderson). The recombinant plasmid pSB1C3, purified from *E. coli*, showed two bands after digestion. The upper band of 2070bp representing pSB1C3 and lower band representing DPP4 700 bp (Fig 7). Overnight incubation resulted in complete digestion. Therefore, gel electrophoresis results were showing two strands cut by Pst1 RE.

The DPP-4 sequence contains two Pst1 sites (1020-1025 and 1794-1799). Accordingly, the additional presence of the particular site that restriction enzyme is likely to mistakenly recognize, may lead to decomposition of DPP-4. In addition, 3A method was used to insert Anderson promoter to the vector, was not found successful. Therefore, infusion cloning method was chosen.

Additionally, the DNA sequence of the A1 after purification was determined to be correct and similar for the rest two promoters. Therefore, in the present study, we successfully constructed recombinant *E. coli* consisted of DPP4 gene.

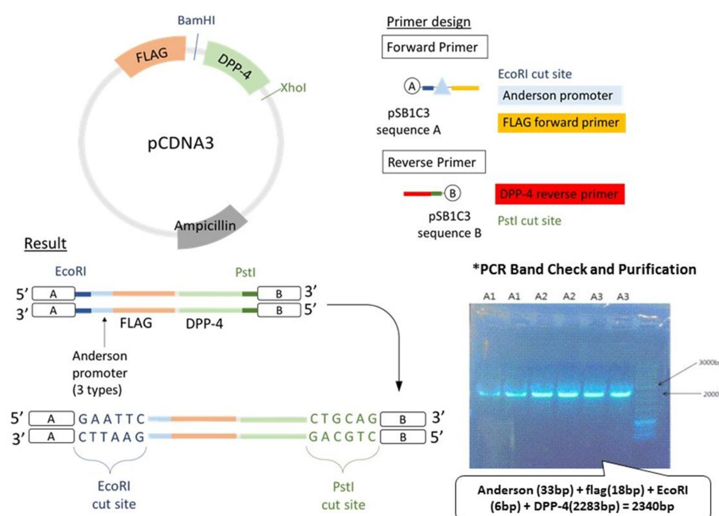

Figure 6. The recombinant structure of the plasmid and the transformation result for the amplification of DPP-4 gene showing a band of 2340 bp. Lanes 1-2 representing A1 (recombinant plasmid with strong promotor); lanes 3-4 representing A2 (with medium promotor); lanes 5-6 representing A3 (with weak promotor) ;(from lane1 to 6, duplicated) lane 7 1000bp DNA Marker.

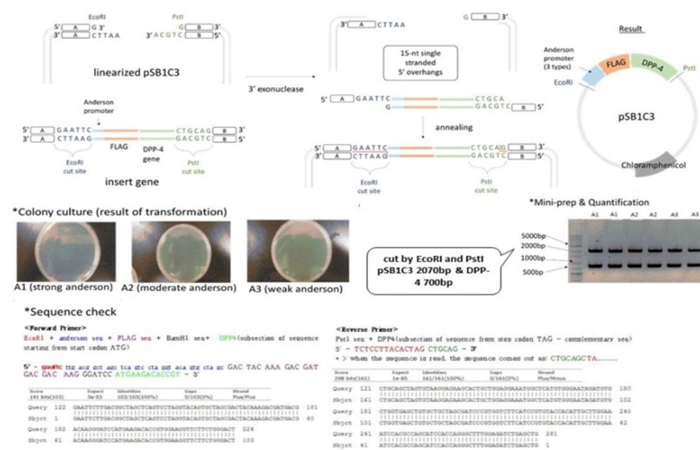

Figure 7. The figure is depicting the construction of the recombinant plasmid using infusion cloning method.

4.2 Expression of DPP4 protein

To confirm the expression of a DPP4 protein, the supernatant containing the desired protein was analyzed with SDS-PAGE and western blot analysis. As shown in the fig.8, two bands can be observed. The control beta-actin protein expression observed at 55kDa has almost similar band size for weak, moderate, and high promoters. However, for the Flag-tagged target gene expression with the Anderson promoter, it can be observed that the weak promoter has the least expression, moderate in between weak and strong, and high expression was observed from the strong promoter. Because each people express a different degree of proteins in their gut bacteria, they all require different amounts of enzyme to bind to its substrate. Specifically, thus, we added three types of Anderson promoter each with different strength to regulate the expressivity of the gene and optimize the amount of expressed gene.

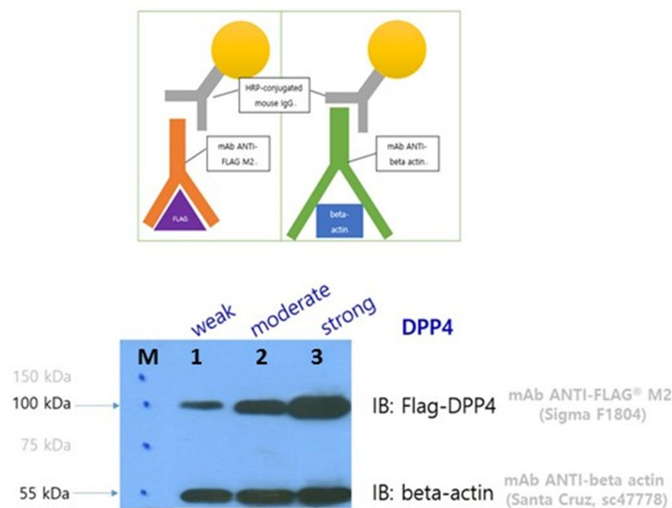

Figure 8. Detection of the recombinant peptide from *E. coli* using western blot analysis. Upper horizontal lane (100kDa): Western blot with the anti-FLAG antibody. DNA marker (lane M); purified peptide from the recombinant *E. coli* with weak promotor (lane1); with moderate

promotor (lane 2); and with strong promotor (lane 3). Lower horizontal lane (55 kDa): Western blot with anti-beta antibody acted as control during the experiment.

A band of 100kDa can be seen in three lanes but significantly distinguishable band sizes. This demonstrates that our recombinant plasmid successfully functions inside the mammalian cell (HEK293T), not only producing DPP4 but also regulating the amount of its expression. We utilized FLAG octapeptide DYKDDDDK (where D=aspartic acid, Y=tyrosine, and K=lysine) for detecting the DPP4 expression [14]. Like Flag-tag, molecular tagging is used as an alternative convenient way for detecting the target protein expression by western blot analysis, localization by immunofluorescence and collecting by affinity chromatography, and so on. HA-tag and Myc-tag, His-tag can be the good examples for molecular tagging.

A functional expression and assay system constructed using synthetic biology can act as an important tool for the production of desired protein on large scale. It has been reported that life-long adherence to gluten-free-diet (GFD) is the only treatment available for CD patients [8]. However, it has also been mentioned that GFD can provide protection against some problems but not all [15]. Therefore, we emphasize the ideal application of the developed recombinant vector for the production of DPP4 protein and utilize further in food products specifically targeting the CD patients.

To our knowledge, this is the first report of idealistic trial for control the exogenous production of DPP4 using synthetic biology. In the future, after the synthesis of the recombinant plasmid and checking the relevant parameters, TNCR Korea will be designing the new gut bacteria and will use further in probiotic yogurts to be safely consumed by gluten intolerance patients. The use of “Enteric-coating technology, i.e., probiotic capsules ensure the survival of component bacteria through the passage of gastrointestinal tract. Additional research on *in vitro* evaluation of acid and bile salt resistance of the bacterial strain would further expand the research zone.

5. Conclusion

In conclusion, we successfully developed a modified plasmid using synthetic biology with the transformation of *E. coli*, which secretes DPP4 with optimized promoter conditions. Importantly, we clearly demonstrated that infusion cloning worked for the construction of the desired vector and confirmed the expression of the protein. Therefore, this could be a useful approach for CD patients. The future work will be targeted by designing an industrially important probiotic strain harboring designed recombinant plasmid and their utilization for the production of yogurt or Probiotic capsules for gluten sensitive persons. Before considering the actual potential of this research, many relevant ethical concerns need to be taken care of. Nevertheless, this is the first report of the idealistic trial of the exogenous DPP4 gene-expression by molecular biologic tools.

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