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Graduate Institute of Biotechnology

Master Thesis

來自 *Dyella yeojuensis* 的麥膠蛋白酶的同源性表達以及催化活性分析

Homologous expression and catalytic activity analysis of a gliadinase from *Dyella yeojuensis*

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Abstract

Celiac disease (CD) is a chronic autoimmune enteropathy with a prevalence of 1% in western countries. CD is mainly manifested by the autoimmune response against gliadin (one of the components of wheat gluten) in the intestinal digestive system. After gluten is ingested, gliadin only is partially degraded by pepsin due to its high glutamine and proline contents. To date, the only effective treatment method for CD is to follow a lifelong gluten-free diet. Gluten is found in grains of wheat, rye, and barley, so following a gluten-free diet is quite difficult. Recently, a new concept for the treatment of CD is to hydrolyze gliadin peptides with proteases and prevent the gliadin peptide from inducing the immune response. The 0663 gene that was isolated from *Dyella yeojuensis* encodes an acidic gliadin digesting protease (DY_0663). This gene has been introduced into *Escherichia coli* in previous studies, but the recombinant strain has failed to secrete active DY_0663 in quantity. By the transformation of *D. yeojuensis* with pDSK-0663, I have successfully expressed 0663 in the original host. However, further studies about DY_0663 are needed in order to discover its full potential as an oral enzymatic drug, including clarification of cleavage site pattern, identification of protein structure, and modification for better acid tolerance or stronger activities.

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Chapter 1

Intoduction

Foods that contain many different ingredients including proteins, lipids, minerals, and carbohydrates are important in order to provide nutrients and energy for humans. Certain allergenic components of some basic foods consumed negatively daily affect human health. Food allergy is an adverse reaction to a specific food antigen, which is normally harmless in healthy individuals and occurs in a person sensitive to a specific allergen by immunological mechanisms (De Martinis et al., 2020). Food allergies are becoming more extensive worldwide and it is estimated that 4% of the total population is affected by food allergies, posing a serious threat to health in developed countries (Sicherer et al., 2004). Furthermore, there are more research and means that can detect food allergies at the present time (Sampson et al., 2004). Most commonly allergenic foods come from eight main groups of foods that are called the “big 8”. These groups include cow‘s milk, peanuts, chicken eggs, fish, crustacean shellfish, tree nuts, soybeans, and wheat (Hefle et al., 1996; Young et al., 1994).

Wheat (Triticum spp.) is an allergic food that makes up about 90% of the allergic reactions in the foods that we consume daily (Martín-Fernández et al., 2016). Among cereals, wheat is one of the most important essential products in the world and is unique because of its special bread-making properties. Wheat is the most widely grown product worldwide due to being its growth in different climates and high yield, and it is grown on over 220 million hectares of land worldwide (Cianferoni, 2016; Akpinar et al., 2018). Food allergens are generally water/saline soluble proteins. However, wheat contains four different classes of protein allergens: water-soluble (albumin), saline-soluble (globulins), alcohol-soluble (gliadins), and acid-soluble (glutenins) protein allergens (Jin et al., 2019). Gliadins and glutenins, called together gluten, are determining the bread-making properties of wheat and stored together with starch in the endosperm of the seed. However, gluten in wheat has factors that cause some symptoms and allergies in some individuals. The immune system of some individuals against gluten proteins is not strong and can not tolerate wheat consumption (Sharma et al., 2020).

Allergy caused by wheat is manifested by various symptoms such as asthma, abdominal pain, urticaria/angioedema, vomiting, allergic rhinitis, acute exacerbation of atopic dermatitis, and exercise-induced anaphylaxis (Cianferoni, 2016). Consequently, treatment methods should be developed for individuals who are allergic to wheat or are intolerant to certain wheat proteins.

The most widespread wheat-related disorderliness associated with gluten ingestion is celiac disease (CD), which results in significant morbidity in individuals and impaired quality of life, characterized by a specific histological profile and serological, also known as sprue or gluten-sensitive enteropathy (Caio et al., 2019; Sharma et al., 2020). CD is a chronic autoimmune bowel disease with a prevalence of about 1% in Western countries (Moneim et al., 2019). Although it is characterized by inflammation of the small intestine, it is also often associated with various diseases such as pneumonia, lymphoma, osteoporosis, and increased mortality (Al-Toma et al., 2019). Classic symptoms include gastrointestinal problems such as abdominal distention, chronic diarrhea, loss of appetite, malabsorption, and among children failure to grow normally (Fasano et al., 2005). Current diagnosis is based on demonstrating the enteropathy in small intestinal biopsies where histologic investigation shows villous atrophy, the presence of circulating CD-specific antibodies to tissue transglutaminase (tTG) and endomysium (EMA), intraepithelial lymphocytosis, and crypt hyperplasia (Tye-Din et al., 2018). Unfortunately, the only effective treatment method for CD is to follow a lifelong gluten-free diet (Rubio-Tapia et al., 2013).

In this way, food labeling plays a decisive role by providing more information about food ingredients to consumers to determine whether a food contains allergens and therefore hazards can be successfully avoided (van Hengel, 2007). In order to protect the health of those who are sensitive to allergens, food regulations are applied in the USA and the European Union (EU). Within the EU, Directive 2007/68 / EC, EU Regulation No 1169/2011 and Regulation (EU) No 41/2009 are designed to provide mandatory labeling to inform consumers whether food products contain gluten. According to the Codex Alimentarius International Food Standard (CODEX STAN 118, 1979) and Regulation 41/2009 (EC), a product must not exceed 20 mg/kg gluten in foods to be classified as gluten-free. Although there is no safe gluten limit for CD patients, the literature suggests that daily gluten intake should not exceed 50 mg (Catassi et al., 2007).

Wheat grains contain 8 to 15% of protein, of which 10 to 15% are globulin and albumin, and 85 to 90% are gluten. Gluten is a compound mixture composed of two types of proteins, mainly glutelin and prolamin. The name of the latter comes from its rich content in proline and glutamine. Gliadin specifically refers to wheat prolamin (Biesiekierski, 2017; Wieser, 2007).

Prolamin is highly resistant to proteolytic enzymes in the gastrointestinal tract (Shan et al., 2002). In patients with CD, these incompletely degraded toxic peptides are subject to deamination by tissue transglutaminase (Balakireva and Zamyatnin, 2016). This proline-glutamic acid (PE) rich motifs are thought to trigger an autoimmune response and cause many symptoms in patients with CD (Leffler et al., 2015). CD has a genetic predisposition and is highly correlated with human leukocyte antigens HLA-DQ2 or HLA-DQ8 (Sollid and Thorsby, 1993; Tollefsen et al., 2006).

The only treatment for CD today is to follow a strict lifelong gluten-free diet. However to avoid gluten altogether is quite difficult, because gluten-free products sold commercially have been shown to be contaminated with gluten from other grains. This can occur in fields, transportation of grains, storage facilities, or during the grinding process (Collin et al., 2004). Therefore, given these circumstances, there may be a risk that gluten-free grains may become contaminated with gluten. It is recommended by International food standards organization Codex Alimentarius to accept products that do not exceed 20 mg/kg gluten as gluten-free (Terence et al., 2013). Recently, the latest treatment approach for CD is to hydrolyze the gluten consumed with grains by protease-mediated oral route (Bethune and Khosla, 2012; Gordon et al., 2012). Before the gliadin peptide reaches the small intestine, it is digested by a protease in the stomach and hydrolyzes the gliadin peptide without causing an immune response. Thus, there is a need to use enzymes as additives or processing aids in the food biotechnology industry to detoxify gluten or produce non-dietary oral treatments for celiac patients (Moreno et al., 2019).

Here I found a gram-negative bacterium *D. yeojuensis* that produces extracellular medium and utilizes gliadin as nutrients for cell growth. The whole-genome sequence of this *D. yeojuensis* strain was determined by pair-end sequence using the Illumina Miseq system and de novo assembly Identities of the secreted proteinases were confirmed with LC-MS-MS using a self-established WGS database. One of the matched genes, DY_0663, encoding a gliadin digesting serine protease, was cloned by PCR and inserted into different vectors for protein expression. Heterologous expression of DY_0663 in *E. coli* encountered a critical issue, which the expressed DY_0663 couldn't be secreted to medium. Therefore, we took an alternative approach to solve this problem by utilizing *D. yeojuensis* as an expression host. Through transformations of *D. yeojuensis* with pDSK-based vectors containing the cognate promoters of DY_0663, I successfully expressed DY_0663 by extracellular medium. Properties of DY_0663 were examined to give us better understanding about this protease and also to indicate the potential use of DY_0663 in oral therapy for CD patients.

Chapter 2

Materials and Methods

2.1 Preparation of *Dyella yeojuensis*

2.1.1 Selection of gliadin digesting microorganisms

Liquid or solid samples, containing possible gliadin digesting microorganisms, were collected from various sources. Liquid samples were wiped on gliadin agar plates (pH 5.0) using sterile cotton swabs. Gliadin agar plates (pH 5.0) contains 6 g $NaH_2PO_4 \cdot H_2O$, 1.5 g NaCl, 0.2 g KCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.2 g yeast extract, 1 ml 10% SDS, and 15 g agar per liter. Solid samples such as soil, were placed inside 50 ml centrifuge tubes, then added with 25 ml ddH_2O. Tubes were shaken by a vortex machine for 30 minutes, and set aside until solid particles were precipitated. Sterile cotton swabs were soaked with the supernatant, followed by the same protocol as liquid samples. Microorganisms were incubated on gliadin agar plates (pH 5.0) at 28℃ for 5 days. Microorganisms with gliadin digesting ability were able to grow on the plates, and created clear zones around their colonies. Different strains of microorganisms were separated and purified by the streak-plate method.

2.1.2 Antibiotics resistance of *D. yeojuensis*

D. yeojuensis was inoculated with LB medium overnight, and the bacterial broth was diluted 250-fold with flesh LB medium. Antibiotics (ampicillin, chloramphenicol, kanamycin, streptomycin, erythromycin, carbenicillin, cephalosporin, and tetracycline) with indicated concentrations were administered to verify the antibiotics resistance ability of *D. yeojuensis.*

2.1.3 16S rDNA sequencing

To identify the gliadin digesting microorganisms, colony PCR was performed to obtain 16S rDNA gene sequences. PCR reactions contained 37 μL ddH_2O, 5 μL 10× Taq reaction buffer with 20 mM $MgSO_4$, 5 μL 2.5 mM dNTP, 1 μL 10 mM 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3'), 1 μL 10 mM 1492R primer (5'-TACGGTTACCTTGTTACGACTT-3'), and 1 μL 5 U/μL Taq DNA polymerase. Bacterial colonies were removed from the plates using sterilized tips and stirred inside the PCR mixture as a DNA template. The cycling conditions were 94℃ for 5 minutes, (94℃ for 30 seconds, 60℃ for 30 seconds, 72℃ for 90 seconds) ×35 cycles, 72℃ for 90 seconds, then held at 4℃. After PCR reactions were done, DNA products were purified with NucleoSpin Gel and PCR Clean-up Kit (Thermo Fisher). Using 27F primer and 1492R primer as sequencing primers, Sanger sequencing was performed by Biotechnology Center (NCHU, ROC), and identities of gliadin digesting bacteria were confirmed based on the search result with Nucleotide BLAST (NCBI).

2.1.4 Genomic DNA extraction and whole genome sequencing

D. yeojuensis was cultured in 3 ml fresh LB medium overnight, and centrifuged at 5000×g for 10 minutes at 4℃. gDNA of *D. yeojuensis* was extracted from the cell pellet using DNeasy Blood & Tissue Kit (Qiagen). Pair-end sequencing and de novo assembly of gDNA were performed respectively with Illumina Miseq system and CLC Genomics Workbench (Qiagen) by Tri-ibiotech Inc. Predicted ORFs were based on GLIMMER (Gene Locator and Interpolated Markov ModelER) software (Johns Hopkins University), and utilized as a database for protein identification in LC-MS-MS.

2.1.5 Protein identification by LC-MS-MS

D.yeojuensis inoculate in 3 ml skim milk medium for 2 days then centrifuged at 16,000×g for 10 minutes at 4℃. Medium supernatant was pipetted to new Eppendorf tubes, mixed with an equal volume of 20% (w/v) trichloroacetic acid, and placed on ice for 30 minutes. Mixtures were latter centrifuged at 20,000×g for 10 minutes at 4℃, then the supernatant was removed. Protein pellets were rinsed with ice-cold acetone twice to remove trichloroacetic acid, and air-dried to evaporate the remaining acetone. Pellets were dissolved with 4× protein sample buffer, and then applied to SDS-PAGE gel electrophoresis. The largest band was cut off from the gel, and then preserved in ddH_2O at -20℃. LC-MS-MS was performed to identify the proteins inside the gel by Biotechnology Center (NCHU, ROC).

2.1.6 Plasmid construction for E. coli expression system

PCR was performed to clone the DY_0663 gene from the gDNA. PCR reactions contains 37 μL ddH_2O, 5 μL 10× Taq reaction buffer with 20 mM $MgSO_4$, 5 μL 2.5 mM dNTP, 1 μL 10 mM DY_0663F primer (5'-CATCTCCATGGAGATGGGAAATCGTATCCTCGCGA-3'), 1 μL 10 mM DY_0663R primer (5'-TGAGGATCCTCAAGGTTCGACGAAGCGCGCGA-3'), 100 μg gDNA, and 1 μL 5 U/μL Taq DNA polymerase. The cycling conditions were 94℃ for 3 minutes, (94℃ for 30 seconds, 60℃ for 30 seconds, 72℃ for 120 seconds) ×35 cycles, 72℃ for 5 minutes, then held at 4℃. The gene was inserted into protein-expressing vector pET-Duet1 via *Nco*I and *Bam*HI cut sites, and ligated with T4 DNA ligase Transformation of *E. coli* DH5α and BL21 (DE3) were achieved with new recombinant plasmids pET-Duet-0663.

2.1.7 Production of recombinant proteins in E. coli Expression system

E. coli BL21(DE3) with or without pET-Duet-0663 was incubated in 3 ml of LB medium overnight. Bacterial broth was diluted with flesh LB medium by fifty-fold, and incubated at 37℃ for 3-4 hours until OD_{600} = 0.8. IPTG (final concentration = 0.5 mM) was added inside the broth, and proteins were induced under 24℃ for 18 hours. Bacterial broth was centrifuged at 18,000×g for 10 minutes at 4℃, and cell pellet with 10% glycerol and phosphate-citrate buffer pH 5.0 as sonicated to release its contents. The protein expression was examined by performing SDS-PAGE electrophoresis and zymogram.

2.2. Chemicals

2.2.1 Preparation of Gliadin solution

2.2.1.1 Crudly extracted gliadin solution (about 6%)

Take high-gluten flour and salt in a 3: 1 ratio and add appropriate water to knead to form a dough, put it in a non-woven bag or soy milk bag, rinse with running water and knead until it becomes a flexible mass (gluten), and wash repeatedly until there was no white starch liquid. Put the above gluten into 70% alcohol and rub it (you can also use a blender to homogenize). Finally, centrifuge the alcohol extract at 5000 × g for 15 minutes. Remove the supernatant and pour it on a plastic film to dry it naturally. The substance is crude extracted gliadin. Take 3 g of crude gliadin and dissolve it in 50 ml 70% alcohol. Use a homogenizer to homogenize gliadin in alcohol. The above solution is called crude extract gliadin solution.

2.2.1.2 Pure gliadin solution

Pure gliadin was purchased commercially from SIGMA®. According to different experiments, different concentrations of pure gliadin solution were prepared. Take appropriate amount of pure gliadin solution and dissolve in 30 mM Glycine-HCl (pH 2.5) buffer solution or 70% alcohol. Use homogenizer to accelerate dissolution.

2.3 Medium

2.3.1 LB (Lysogenic broth) liquid medium

Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Distilled water to	1000 ml

2.3.2 LBA (Lysogenic broth agar) solid medium

Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Agar	15 g
Distilled water to	1000 ml

2.3.3 PDA (Potato Dextrose Agar)

Dextrose	20 g
Potato extract	4 g
Agar*	15 g
Distilled water to	1000 ml

*If solid media is not desired, the mixture should be prepared without adding agar.

2.3.4 PDA medium containing gliadin (pH 4)

Dextrose	20 g
Potato extract	4 g
Agar	15 g
Distilled water to	1000 ml
Na_2HPO_4	10.95 g
$C_6H_8O7 \cdot H_2O$	12.91 g

PDA medium containing gliadin (pH 6)

Dextrose	20 g
Potato extract	4 g
Agar	15 g
Distilled water to	1000 ml
Na_2HPO_4	17.88 g
$C_6H_8O7 \cdot H_2O$	7.78 g

2.3.5 Minimal Medium solid agar containing gliadin (pH 4 and pH 6)

$NaH_2PO_4.H_2O$	6 g
NaCl	1.5 g
KCl	0.2 g
$MgSO_4 \cdot 7H_2O$	1 g
Yeast extract	0.2 g
10 % SDS	1 ml
Gliadin (0.05 mg/ml)	50 ml
Agar	15 g
Distilled water to	1000 ml
Adjust finally to pH 4 and pH 6 with HCl	

2.4 DNA Preparation

2.4.1 Plasmids used in this study

The pDSK-GFPuv plasmid was purchased by AllBio Science and propagated in *E.coli* DH5α. The pDSK-0663 plasmid was made from the pDSK-GFPuv plasmid.

2.4.2 Preparation of plasmid DNA

The pDSK-GFPuv plasmid is a low copy number plasmid. Therefore, the purification of plasmids was performed as follows using chloramphenicol-amplification (2.4.3) and alkaline lysis method (2.4.4).

Table 1. Antibiotics solvents and concentrations

Antibiotic	Solvent	Concentration (mg/ml)
Ampicillin	Water	100
Carbenicillin	Water	100
Cephalosporin	Water	100
Chloramphenicol	Ethanol	30
Erythromycin	Water	100
Kanamycin	Water	50
Streptomycin	Water	20
Tetracycline	Ethanol	10

2.4.3 Chloramphenicol-Amplification of Plasmids

The pDSK-GFPuv plasmid was incubated in LB containing kanamycin (50 μg/ml) at 37°C for 6-8 hours then added chloramphenicol antibiotic (170 μg/ml). Incubate for a further 12 to 16 hours.

2.4.4 DNA Plasmid Isolation Using Alkaline Lysis Method

Pour overnight grown culture to 1.5 ml Eppendorf tube. Centrifugate at 14.000 rpm for 2 min. Discard the supernatant from the Eppendorf tube. Repeat steps 1-3 until the bacterial pellet is as dry as possible. Add 150 μL Solution I, resuspend the bacterial pellet properly by pipetting or vortexing. Add 200 μL Solution II to bacterial suspension and mix by inverting the tube 6-8 times. Add 300 μL Solution III and mix by inverting the tube 6-8 times. Centrifuge at 14.000 rpm for 5 min. Transfer the supernatant into a new 1.5 ml Eppendorf tube max 300 μl. Add an equal volume of isopropanol and invert the tube several times to mix. Incubate in -80°C for 30 min. Centrifuge at 14.000 rpm for 5 min. Discard the supernatant and wash the pellet with 600 μL EtOH 70%. Centrifuge at 14.000 rpm for 5 min. Discard the supernatant and dry the pellet for 10-30 min. Dissolve the pellet in 20-50 μl distilled water pH 8.0. Confirm the plasmid by 1% agarose gel electrophoresis and Nanodrop. Store the plasmid at -20 ° C.

2.4.5 Genomic DNA extraction from *D. yeojuensis*

Use DNeasy Blood & Tissue Kit (QIAGEN®), follow the instructions provided by the company, store the gDNA at -20°C and confirm with 1% agarose gel electrophoresis.

2.4.6 Remove of GFP using restriction enzymes from pDSK-GFPuv

Take x μl plasmid (pDSK-GFPuv) DNA (up to 1 μg), each 1 μl Restriction Enzyme (*Eco*RI/*Pst*I), 2 μl 10X Fast digest Green Buffer (Thermo Fisher Scientific) and distilled water up to final 20 μl, mix by vortex and then incubate at 37℃ for 1 hour. After the reaction, the DNA is purified and used. After the reaction, the DNA was purified by a wizard DNA purification kit (Promega, USA) and used.

2.4.7 Extraction of DY_0663 gene and potential promoter from genomic DNA by PCR

The 0663 gene is 1956 bp long and is encoded as a serine protease in the Dyella genome. Potential promoter of the 0663 gene was found using a web server for the prediction of prokaryote promoter elements and regulons (PePPER, University of Groningen, The Netherlands). The 0663 gene and promoter were obtained from gDNA by PCR. PCR was performed in a reaction mixture (25 μl) of Q5® Hot Start High-Fidelity 2X Master Mix (12.5), 1.25 μl 10 mM Forward primer (5'-ACGCAGAATTCTGCGTTTCCACGGGGTTTGGG), 1.25 μl 10 mM Reverse primer (5'-AAAACTGCAGTCGCTGGCGCACGACGAGAA), 2 μl gDNA, and 8 μl distilled water under the following conditions: 98℃ 30 s, (98℃, 10 s, 72℃, 30 s, 72℃, 1.5 min) × 35 cycles, 72℃, 5 min then held at 4℃. After completion, analyze and confirm by 1% agarose gel electrophoresis. After confirming that it is correct, the DNA was purified by a wizard DNA purification kit (Promega, USA) and used.

2.4.8 DNA Purification (Clean up)

Use the Wizard DNA Purification Kit (Promega, USA), follow the instructions provided by the company. Confirm the DNA with 1% agarose gel electrophoresis and store at -20°C.

2.4.9 Ligation of the DY_0663 gene to the pDSKuv vector

Take Vector DNA (pDSKuv): Insert DNA (0663) = 1: 3 (mole number), 2 μl 10× T4 Ligase Buffer, 2 μl 10 mM ATP and 1 μl T4 DNA Ligase, add distilled water until the total reaction volume is 20 μl and incubate at room temperature for 1 hour or 16°C for 18 hours.

2.5 Protein expression

2.5.1 Preparation of Competent cells

Strains to be made into competent cells were cultured overnight in LB liquid medium. A 50 ml centrifuge tubes were taken, 10 ml of LB liquid medium and 100 μl of culture liquid were added overnight and incubate at 37 ° C until OD_{600} is about ~ 0.6 (about 2 hours). Incubate on ice for 10 min then centrifuge at 5000× g 4°C for 10 minutes to remove the supernatant and add 5 ml of 100 mM cold $CaCl_2$. Incubate on ice for 30 minutes, and centrifuge at 5000× g 4°C for 10 minutes. Remove the supernatant and add 700 μl of 100 mM cold $CaCl_2$. If you want to keep it for later use, add 20% glycerol and store it to -80°C.

2.5.2 Transformation - Heat Shock

Mix 1-5 μl of DNA (usually 10-100 ng) into 100 μl of competent cells in a microcentrifuge tube and gently pipette but do not vortex. Incubate on ice for 30 minutes, put them in a 42°C water bath for 90 seconds, and put back on ice and incubate for 3 minutes, then add 1 ml of LB liquid medium. Incubate at 37°C 200 rpm for 1 hour. Centrifuge at 5000× g 4°C for 10 minutes. Remove 850 μl solution and resuspend pellet. Spread bacteria onto the LB agar plate containing 50 μg/ ml kanamycin and incubate at 37°C overnight.

2.5.3 Preparation and concentration of the protein sample

D. yeojuensis was incubated in 200 ml PDA medium and *D. yeojuensis* transformed strains (C2 and C4) were incubated at 28°C in 200 ml PDA medium containing 50 μg/ml of kanamycin for 3 days. After incubation, the samples were centrifuged at 10000× g for 20 minutes at 4°C. The supernatant was collected and filtered with a 0.22 μm polyethersulfone (PES) vacuum filtration (GeneDireX, USA). Then the filtered supernatant was separated by a 10 kDa ultrafiltration membrane (Biomax, USA) until the final volume become 10 ml (20× concentrated). It was used as a protein sample in later experiments.

2.5.4 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Mix with 20 μl protein sample and 5 μl 4× sample buffer (0.38 g Tris base, 0.5 g SDS, 5 ml Glycerol, 2.5 ml 0.4 M DTT or β-ME, 0.05 g Bromophenol Blue, 12.5 ml Distilled water, pH 6.8). Heated at 95℃ for 5 minutes and cooled to room temperature for use. Upload the processed sample to a 10% polyacrylamide gel, and run at a fixed current of 20 mA with the highest voltage in electrophoresis buffer (14.4 g Glycine, 3 g Tris base, 1 g SDS, 1000 ml Distilled water) for 50 minutes electrophoresis. After electrophoresis, remove the running gel and soak in staining buffer (400 ml Methanol, 70 ml Glacial acetic acid, 1 g Brilliant Blue R-250, 2530 ml Distilled water) for 1 hour, and then destain the gel with destaining buffer (1200 ml Methanol, 76 ml Glacial acetic acid, 1724 ml Distilled water) for about 1 hour. (Carola Hunte, et al., 2003).

2.5.5 Zymogram

SDS-PAGE was performed using a 10% acrylamide gel containing 10 mg/ml Gliadin. Mix 20 μl protein sample and 4 μl 5× non-reducing Sample Buffer (125 mM Tris, 4% SDS, 20% Glycerol, 0.01% Bromophenol Blue, pH 6.8, and add ddH_2O up to 50 ml). Add the processed sample to the gel and run at a fixed current of 30 mA with the highest voltage in electrophoresis buffer (14.4 g Glycine, 3 g Tris base, 1 g SDS, 1000 ml Distilled water) for 50 minutes at 4℃. Unpack the gliadin PAGE after electrophoresis and sink the gel in washing buffer (2.5% Triton X-100, 100 mM Tris buffer pH 5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) at 4℃ for 30 min, with a 50 rpm shaking condition (50 ml washing buffer for each gel). Dispose of the washing buffer. Repeat washing step one more time. Transfer the gel to incubating buffer (1% Triton X-100, 100 mM Tris buffer, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) and incubate at 37℃ for 1hour, place on the 50 rpm shaker (50ml incubating buffer for each gel). Stain the gel in Coomassie blue staining buffer for 30 min. Destain the gel in the destaining buffer for 40 min.

2.5.6 Effect of inhibitors on serine protease

The protein sample was treated separately with 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA) and 4 mM dithiothreitol (DTT) then incubate at 28°C for 1 hour. For the result of the serine protease inhibitor test, the activity of the samples on the zymogram gel was evaluated.

2.5.7 Gel filtration chromatography

Take 5 ml of concentrated extracellular medium and pass the sample through a Sephacryl S-300 HR (HiPrep 16/60) column. Sephacryl S-300 HR (HiPrep 16/60) can separate globular protein in the range of 10-1500 kDa MW.

2.5.8 High Performance Liquid Chromatography (HPLC)

Chromatographic analyses of gliadin-derived immunogenic peptides (26-mer and 33-mer) were performed using a C-18 HPLC system (Gilson, USA), including a pump, injector, HPLC column, UV detector, and data analyzer, as well as LCsolution software. The HPLC working conditions were as follows: The column was a C18 column (Supelco column Cat#50538-U) (4.6 × 250 mm, 5 μm). A dual gradient elution system, comprising buffer (A) 0.1% TFA/acetonitrile and buffer (B) 0.1% TFA/water, was implemented as follows: initial, 100% B; 5 min, until 80% A; 20 min, 100% A; 5 min, 100% B. The detection wavelength was set at 230 nm, the flow rate was 1 ml/min, the column temperature and sample temperature was ambient, and the injection volume was 10 μl.

2.5.9 DY_0663 gliadin digesting on Minimal Medium agar plate containing gliadin.

Inoculated *D. yeojuensis* and transformed strains in 5 ml LB and incubate at 28℃ until OD_{600} become 2 then centrifuge at 5,000× g for 5 min. Remove the supernatant and wash bacterial pellet with phosphate-buffered saline (PBS). Centrifuge and suspend in PBS then make 5× serial dilution (OD_{600} 1, 0.2, 0.04, 0.008). Put the sterile filter paper in the LB agar plate containing 10 mg/ml gliadin (pH: 3.4, 4, 5, 6) and add 10 μl of cell dilution. Incubate at 28℃ for 24 hours.

Chapter 3

Results

3.1 Location of 0663 gene in *D. yeojuensis* gDNA

After the *D. yeojuensis* gDNA was sequenced and identified, a 0663 gene with a length of 1956 bp was found that could digest the gliadin. The 0663 gene is a serine protease. Potential promoter of the 0663 gene was found using a web server for the prediction of prokaryote promoter elements and regulons (PePPER, University of Groningen, The Netherlands). The 0663 gene and its promoter were obtained from gDNA by PCR using a designed pair of primer (Figure 1).

3.2 Exogenous gene plasmid construction and protein expression

To confirm DY_0663 is the protease responsible for gliadin digesting, the candidate gene was cloned from gDNA, and inserted to protein expression vector pET-Duet1 through restriction enzyme cut sites (Figure 2A). The newly constructed plasmid pET-Duet-0663 (Figure 2B) was used in the transformation of *E. coli* BL21 (DE3). BL21 (DE3) with pET-Duet1 or pET-Duet-0663 was cultured in LB medium, and administrated with 0.5 mM IPTG under 24℃ for 18 hours. After induction, bacterial broth was centrifuged at 18,000×g for 10 minutes at 4℃, separating into medium and cell pellets. Adding 10% (w/v) glycerol and 50 mM citrate-phosphate buffer pF 7.0 as cell lysis buffer, cell pellets were lysed through sonication. To differentiate soluble and insoluble proteins, cell lysate was centrifuged at 20,000×g for 10 minutes at 4℃, and insoluble proteins formed pellets while soluble ones remained in supernatant. Medium, soluble cell lysate, and insoluble cell debris were examined with SDS-PAGE electrophoresis as shown in Figure 3A. To examine the gliadin digesting ability of DY_0663, insoluble cell debris was also applied to the zymogram under pH 7.0 incubation condition as shown in Figure 3B.

The results indicated that DY_0663 was indeed induced by IPTG, but can't be secreted in extracellular medium, and also existed as an insoluble form inside BL21 (DE3). Although DY_0663 was quite abundant compare to other insoluble proteins, it only demonstrated minor protease activity compared to original proteases produced by *D. yeojuensis*. There are some speculations on this phenomenon. For example, DY_0663 might be in the form of inclusion bodies due to wrong folding, or in the form of integration with membrane protein because lacking essential elements for membrane transportation.

3.3 Selection of plasmid for the extra expression of 0663 gene in *D. yeojuensis*

A good expression vector must have some properties. Unfortunately, not all organisms accept every vector. An expression vector must have a selectable marker, an origin of replication, and a site suitable for inserting a gene such as a multiple cloning site. Therefore, pDSK-GFPuv was used as the expression vector to express more 0663 gene in *D. yeojuensis*. Since the pDSK-GFPuv vector contains the kanamycin resistance gene, it allows the transformed organism to grow in a medium containing kanamycin. In addition, the GFP gene will cause the color of the organism to change, indicating that the organism to which it is transformed successfully expresses this plasmid. Plasmid replicated in *E.coli* (Figure 4A). WT *D.yeojuensis* in LB agar plate containing 50 μg/ml kanamycin (Figure 4B). The pDSK-GFPuv has been successfully transformed to *D. yeojuensis* and it was able to grow up comfortably in LB medium containing kanamycin and showed a visible green color under UV light (Figure 4C). Therefore, pDSK-GFPuv was used as the expression vector to express more 0663 gene in *D. yeojuensis* and later was named pDSK-0663. pDSK-0663 did not show green color because the GFP gene was replaced by the 0663 gene (Figure 4D).

3.4 Expression of DY_0663 gene in *Dyella yeojuensis*

The promotor psbA in the plasmid pDSK-GFPuv was replaced by a gene containing the 0663 promoter in *D. yeojuensis*. To generate pDSK-0663, the fragment encoding the GFP gene in pDSK-GFPuv was removed by enzyme *Eco*RI and *Pst*I (Figure 5A). This plasmid was then used to construct pDSK-0663 by adding the original promoter 0663 gene obtained by PCR from the gDNA of *D. yeojuensis* to the remaining region (Figure 5B). The psbA promoter and GFP gene in the PDSK-GFPuv were removed and the 0663 gene and promoter were added from the *D. yeojuensis* gDNA. Then pDSK-0663 plasmid was inserted into *E.coli* and propagated in LB agar plate containing 50 μg/ml kanamycin. This plasmid was then transformed into *D. yeojuensis*.

3.5 Selection of gliadin digesting bacteria

Liquid or solid samples, containing possible gliadin digesting microorganisms, were collected from various sources. After 1 days of incubation on gliadin agar plates (pH 5.0) at 28℃, microorganisms that can utilize gliadin as sole carbon source and nitrogen source created clear zones surrounding their colonies. *D. yeojuensis*, a gram-negative bacteria possessing such properties and its identity was confirmed by 16s rDNA sequencing. Whole-genome sequence of this bacterium was determined by next generation sequencing technique to gain predicted ORFs data.

To identify extracellular medium produced by *D. yeojuensis*, medium supernatant of the bacterial broth was mixed with 10% trichloroacetic acid, and the resulting protein pellets were applied to SDS-PAGE electrophoresis. The largest band (≈65 kDa), similar to the size on zymogram, was cut from a 10% polyacrylamide gel, and proteins' identities were identified by LC-MS-MS using the gDNA information of *D. yeojuensis* as the database. One of the matched ORF, DY_0663, encoding a serine protease from the S8 family, possibly possesses strong a gliadin digesting ability.

Figure 6A shows the gliadin digesting activity of WT on a plate containing gliadin in pH: 5.0. Figure 6B shows the gliadin digesting activity of WT, C2, and C4 under pH: 5.0. Figures 6C, D, E, and F show the gliadin digesting activity of the extracellular medium (DY_0663) in different pH conditions.

3.6 Antibiotics susceptibility of *D. yeojuensis* and transformed strains

Utilization of homogenous expression of DY_0663 with expression plasmid pDSK-0663 required prior information about antibiotics susceptibility of *D. yeojuensis*. Plasmid pDSK-0663 contained a kanamycin resistance gene and a constitutive plant chloroplast promoter psbA. To select a successful transformation, *D. yeojuensis* must be susceptible to kanamycin. Clarifying antibiotics resistance of *D. yeojuensis* can also be beneficial for safety issues. Most publications introduced *D. yeojuensis* as a soil-dwelling bacterium, thus it should be safe for experimental operation, but there is no information regarding whether it is infectious to humans. Among the tested antibiotics, this *D. yeojuensis* strain is resistant only to cephalosporin C (Figure 7A). Therefore, we can use it as the host of pDSK-0663, and the health risk is considerably manageable. Figure 7B shows the effect of kanamycin of different concentrations on WT. Figure 7C and D shows the effect of kanamycin in different concentrations on C2 and C4. It is resistant to C2 and C4 kanamycin because it contains pDSK-0663 with a kanamycin resistance gene.

3.7 Bacterial gliadin digesting activity

In order to meet the conditions for oral proteases, DY_0663 protease needs to be able to decompose gliadin in the stomach environment (pH 2-4), and the minimum pH activity of DY_0663 must be tested. To assess bacterial gliadin digesting activity, *D. yeojuensis* and transformed strains were incubated at 28°C in LB medium until the culture reached an OD_{600} of 2. After washing the cell pellet with PBS, OD_{600} was adjusted to 1, and 5X serial dilution was made (OD_{600}; 1, 0.2, 0.04, and 0.008) and grown at 28°C under different pH (3.4, 4.0, 5.0, and 6.0) in minimal medium. The results are shown in Figure 8. The enzyme responsible for the digestion of gliadin should have more activity. Therefore, gliadin damage activity has been tested. At pH 4, 5, and 6 a clear active transparent circle was observed, while at pH 3.4 there was no active transparent circle. Therefore, the minimum pH value for WT to digest the gliadin can be considered as pH 4.0. Figure 8A shows the gliadin digesting activity of WT on a plate containing gliadin in pH: 5.0. Figure 8B shows the gliadin digesting activity of WT, C2, and C4 under pH: 5.0. Figures 8C, D, E, and F show the gliadin digesting activity of the extracellular medium (DY_0663) in different pH conditions.

3.8 SDS-PAGE and Zymogram

After the culture of WT and transformed strains in the PDA medium for 3 days and 7 days, the supernatant was collected and concentrated. SDS-PAGE was performed to determine whether the culture time is related to the produced protease, the most appropriate culture time for the experiment, and protein ratios are shown in Figure 9A. According to this result, it was understood that the culture time did not have a great effect on protease secretion and the minimum culture time for WT was determined as 3 days. The supernatant was collected using 0.22 µm polyethersulfone vacuum filtration and 10 kDa ultrafiltration membrane, and the results are shown in Figure 9B on the zymogram gel. Transformed strains show an additional band under pH 5.0 because of extra DY_0663 secretion. This result shows that there are at least two enzymes that can digest gliadin. One of these enzymes is DY_0663, but the other enzyme is uncertain. This result is enough to prove that 0663 can digest gliadin. Therefore, the 0663 enzyme is one of the enzymes that can digest gliadin.

3.9 DY_0663 Protease Purification

In order to obtain a relative pure DY_0663 protease, the DY_0663 protease was produced in large quantities using optimal expression conditions. The extracellular medium was collected, concentrated, and passed through a Sephacryl S-300 HR (HiPrep 16/60) column. The chromatograms, gliadin digesting activity, active proteins are shown in Figure 10. The protease activity of each fraction can be evidenced by the transparent bands on the zymogram gel Based on the results, the DY_0663 protease can be concentrated and relatively purified by the gel filtration method. Mutant strains showed extra fraction (fraction 19) and this related to 0663. The presence of fraction 19 indicates that transformed strains express the extra 0663 gene (Figure 10A, D, and G). The gliadin digesting activity of the results obtained was tested on zymogram gel. The fraction 19 in transformed strains showed gliadin digesting activity compared to WT (Figure 10B WT, 10E C2, 10H C4). In Figure 10C, fraction 19 does not show active protein in SDS-PAGE, while Figure 10F and I show active protein due to the extra 0663 gene. As shown in Figure 10F and I, fraction 19 shows similar results with the control group (C2, C4). The big band 0663 in Figure 10C, F, and I are thought to form a complex with other proteins.

3.10 Effects of PMSF, EDTA and DTT on DY_0663

The location of DY_0663 on zymogram gel was uncertain. In order to clarify this uncertainty, an inhibitor test was performed. After 1 hour incubation of the extracellular medium solution with 1 mM PMSF, 5 mM EDTA or 4 mM DTT, the protease activity in the solution was examined by zymogram (Figure 11). With this experiment, I found that the second band, with migration equivalent to 48-kDa marker, disappeared due to the treatment of serine protease inhibitor PMSF. In addition, this band was more abundant in the transformed strains. Together, this band should represent DY_0663. Interestingly, the upper band, equivalent to the 63-kDa marker, were insensitive to PMSF, EDTA, and DTT, suggesting that it is neither a serine protease nor a metalloprotease.

3.11 HPLC analysis of 33-mer and 26-mer peptide degradation.

The Extracellular medium was incubated at 37C for 1 hour with 1 mg/ml final concentration of 33-mer or 26-mer peptides to test and confirm the degradation of gliadin peptides by WT and transformed strains. Chromatographic analysis of the collagen peptides was carried out using the C18 column, containing 0.1% TFA / water and 0.1% TFA / acetonitrile buffer and in a pH 4.0 condition. The detection wavelength was set at 230 nm, the flow rate was 1 ml/min, the column temperature and sample temperature was ambient, and the injection volume was 10 µl. Figure 12A shows hydrolytic activity after a 1-hour incubation of the 26-mer peptide of the extracellular medium at 37°C. WT and transformed strains (C2 and C4) digested 26-mer peptide under pH: 4.0 condition. But C2 and C4 showed similar hydrolytic activity with WT. Figure 12B shows hydrolytic activity after a 1-hour incubation of the 33-mer peptide of the extracellular medium at 37°C. WT, C2, and C4 digested 33-mer peptide under pH: 4.0 condition. But the hydrolytic activity of C2 and C4 was not better than WT.

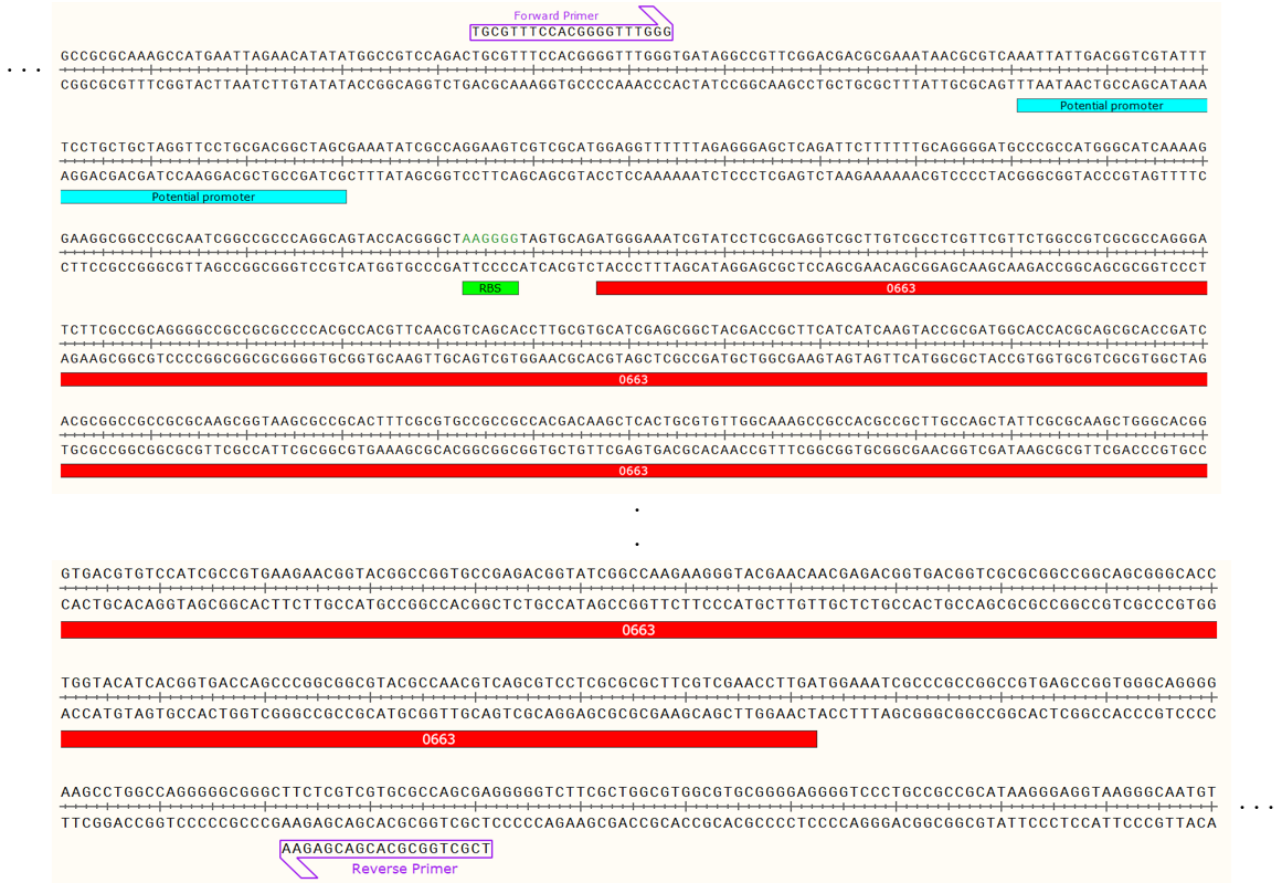

Figure 1: The location of the 0663 gene in the *D. yeojuensis* genome and its potential promoter sequence. The picture was created using SnapGene Viewer and shows parts of 0663 gene (1956 bp), ribosome binding site, potential promoter, and forward and reverse primers used in PCR.

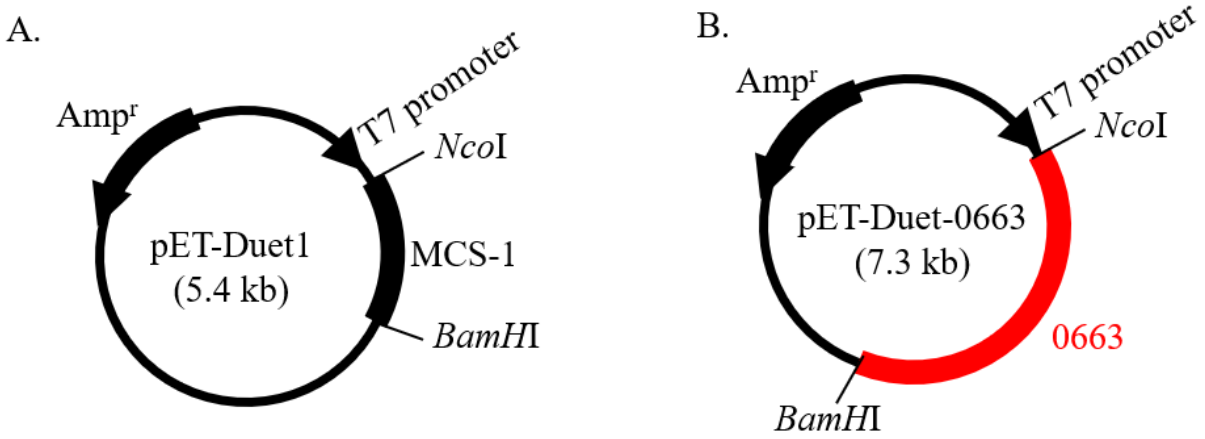

Figure 2. Schematic representation of pET-Duet-0663. pET-Duet-0663 was created by replacing MCS-1 with *DY_0663*, expressing the protein in the *E.coli* system. (A) pET-Duet1 original plasmid. (B) pET-Duet-0663

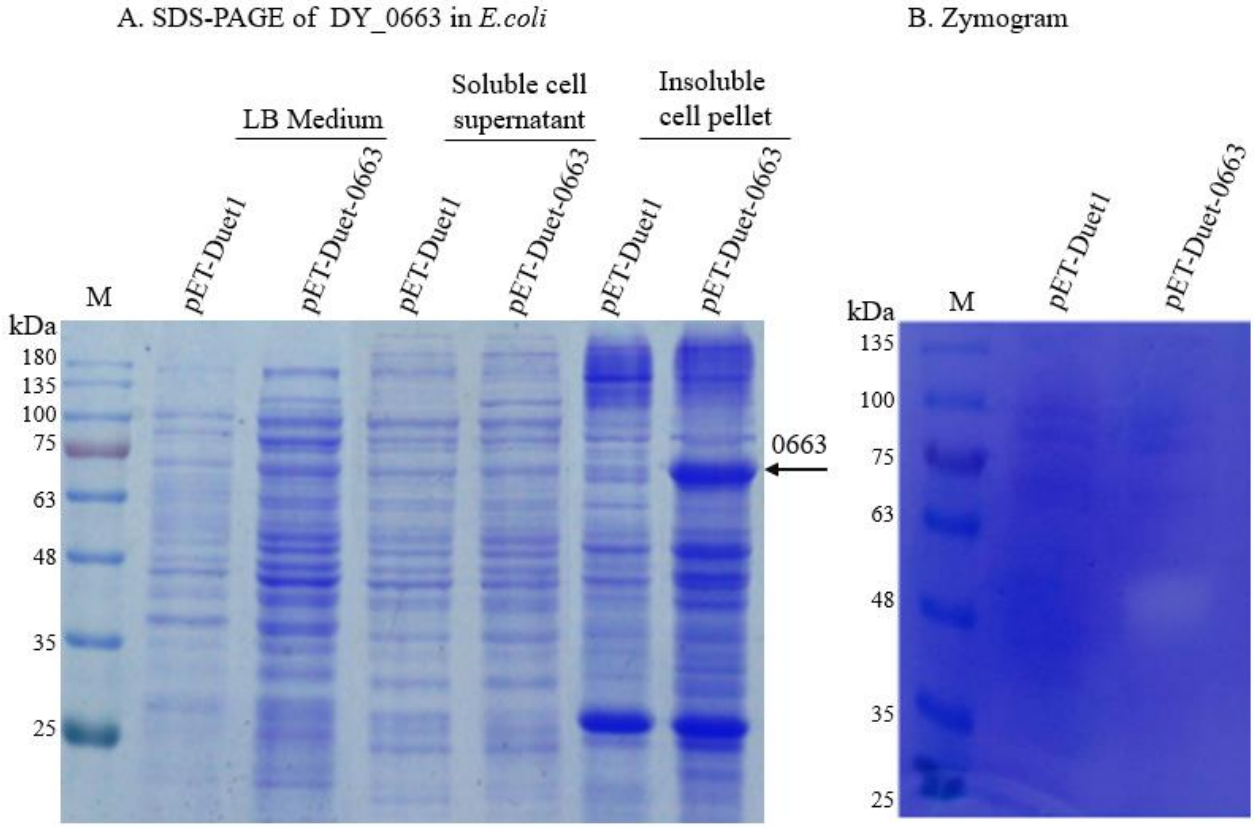

Figure 3. Heterogeneous expression of DY_0663 in E. coli system. (A) SDS-PAGE of DY_0663 induced by 0.5 mM IPTG at 24℃, indicated that DY_0663 existed as insoluble form inside E. coli cells. (B) Zymogram gel of insoluble cell pellet of BL21 (DE3) with pET-Duet1 or pET-Duet-0663.

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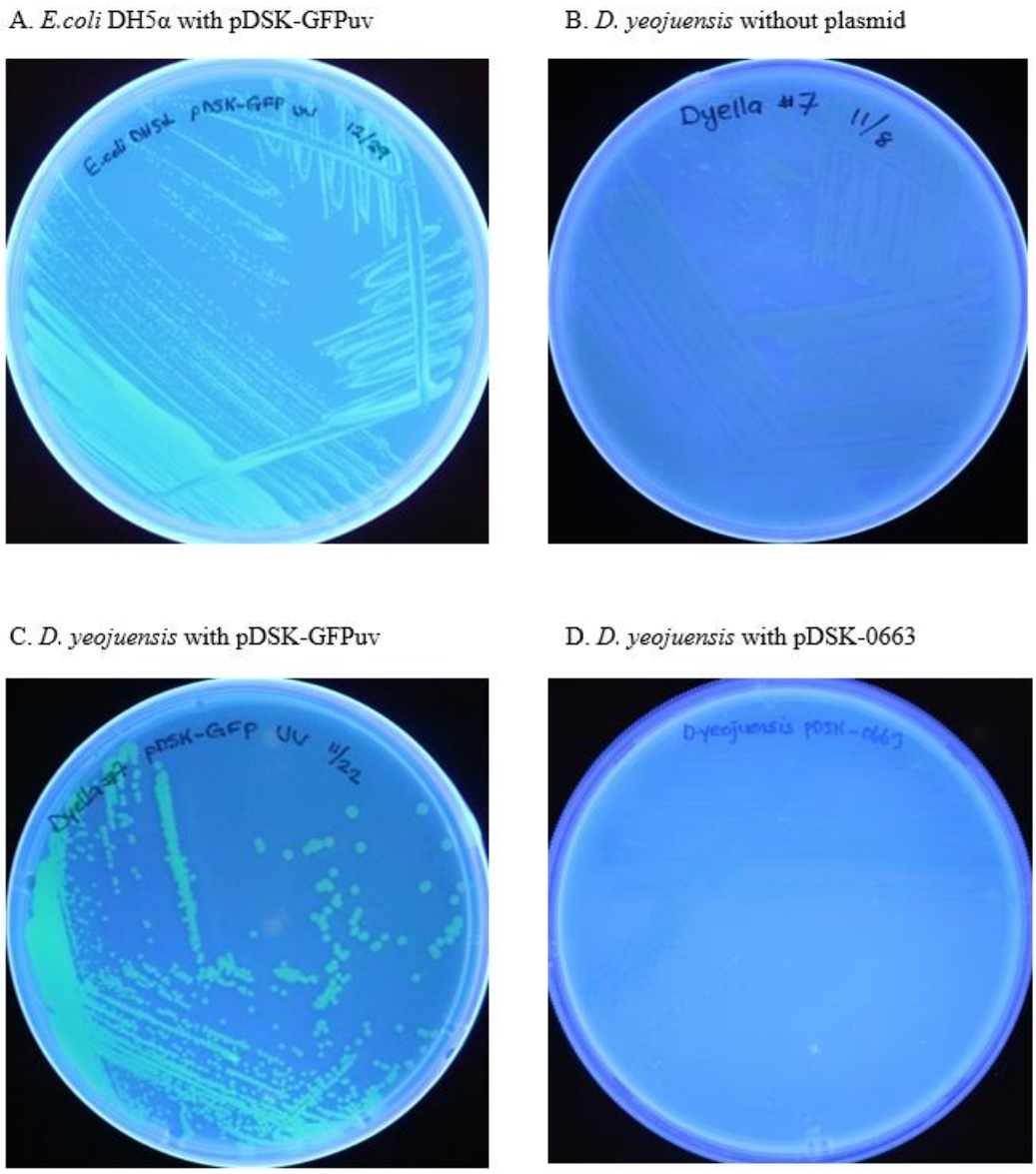


Figure 4: Availability of pDSK-GFPuv as an expression vector. (A) Expression of pDSK-GFPuv in *E.coli*. (B) WT *D. yeojuensis*. (C) The pDSK-GFPuv is manifested in *D. yeojuensis* and visibly changes color under UV light. (D) The expression of pDSK-0663, which occurs after adding the 0663 gene instead of the GFP gene, in *D. yeojuensis*.

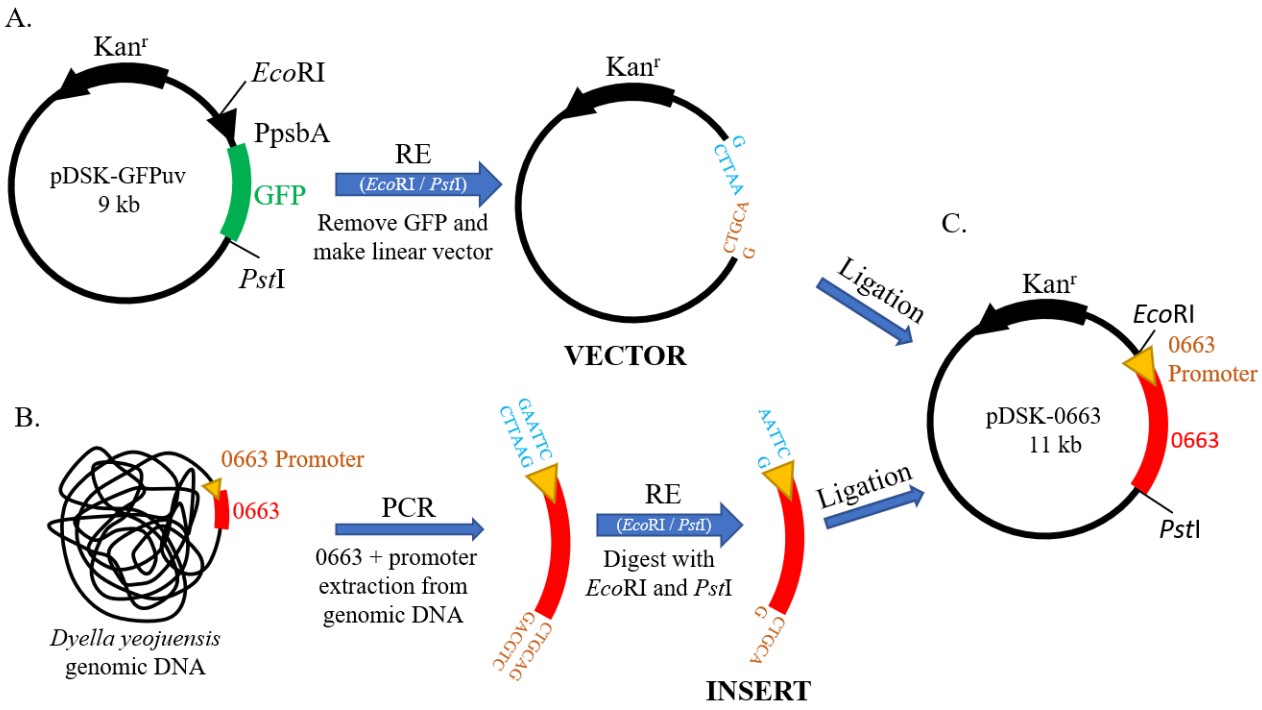


Figure 5. Schematic representation of pDSK-0663. (A) Extraction of the GFP gene from pDSK-GFPuv using restriction enzymes. (B) Cloning of *DY_0663* with an original promoter from gDNA by PCR. (C) pDSK-0663.

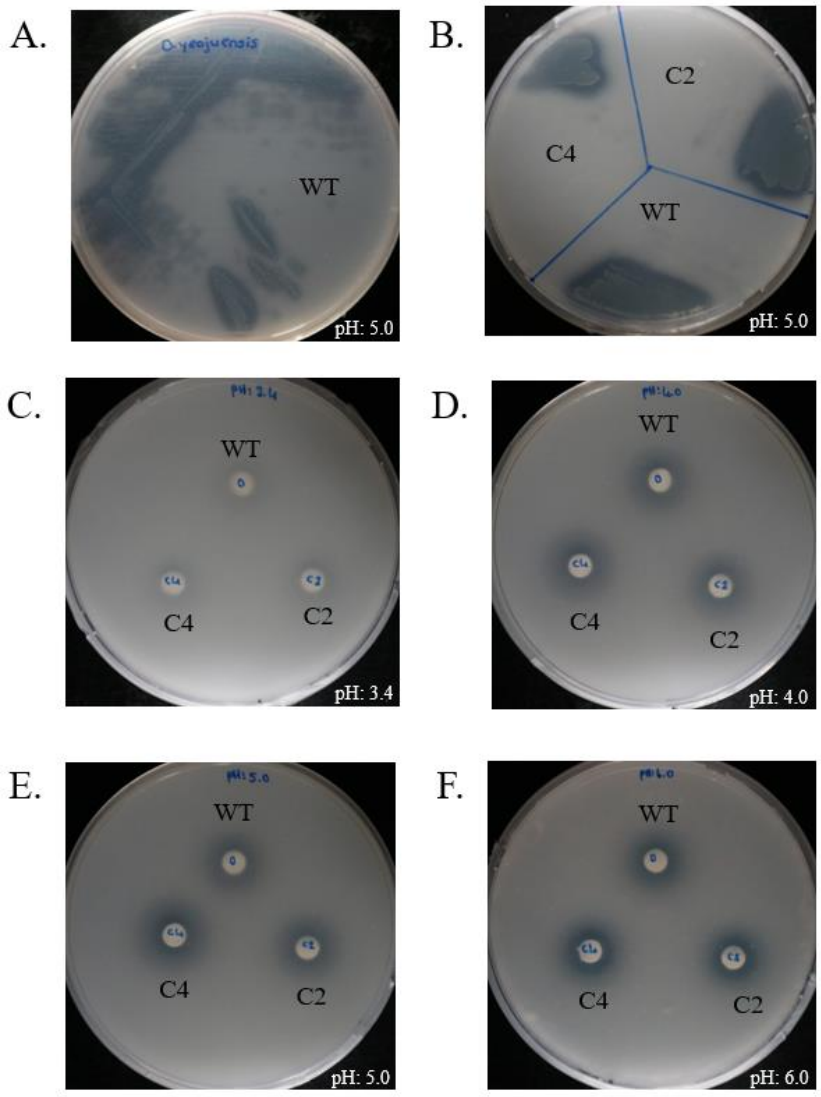


Figure 6. Gliadin digesting activity of WT and transformed strains of *D. yeojuensis* on the plate containing gliadin. (A) The ability of WT to gliadin digesting under pH 5.0 condition. (B) The ability of WT and transformed strains of *D. yeojuensis* to gliadin digesting under pH 5.0. (C) 24-hour incubation of supernatant, at pH 3.4, from 3-day culture of WT and transformed strains of *D. yeojuensis*. (D) 24-hour incubation of supernatant, at pH 4.0, from 3-day culture of WT and transformed strains of *D. yeojuensis*. (E) 24-hour incubation of supernatant, at pH 5.0, from 3-day culture of WT and transformed strains of *D. yeojuensis*. (F) 24-hour incubation of supernatant, at pH 6.0, from 3-day culture of WT and transformed strains of *D. yeojuensis*

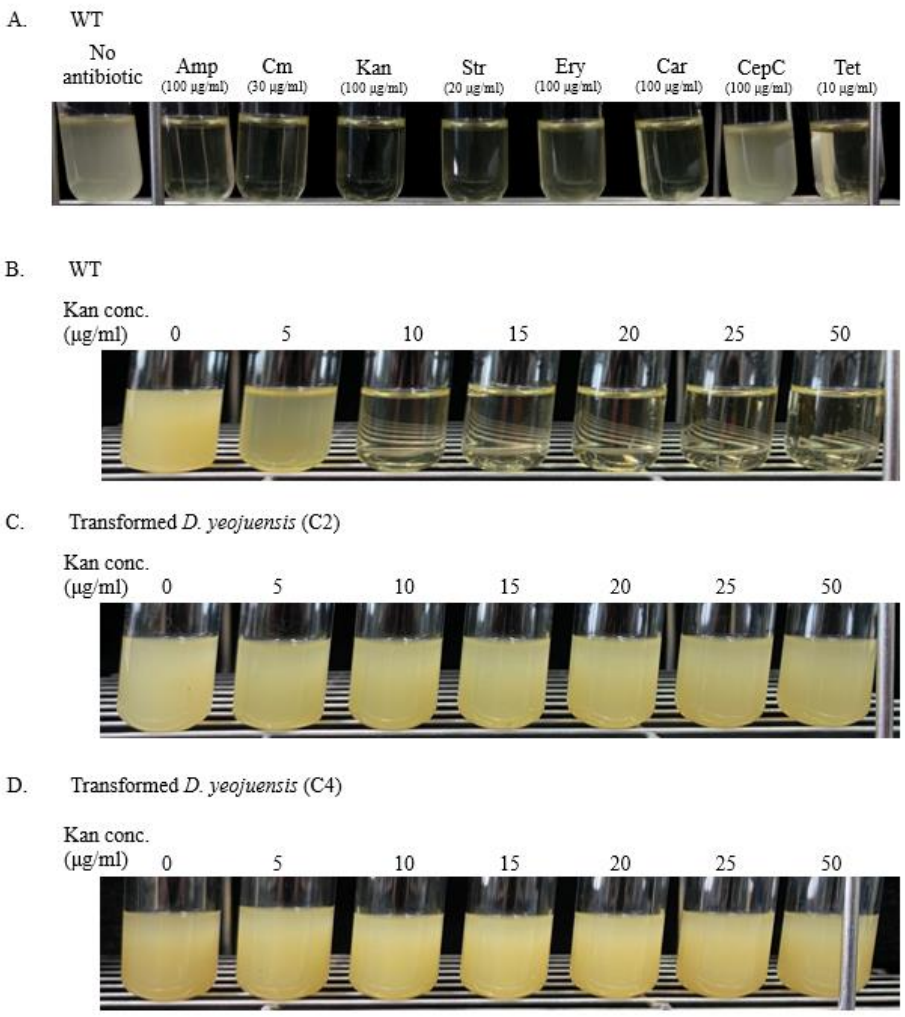


Figure 7. Antibiotics susceptibility of WT and transformed strains. (A) Bacterial culture was incubated in LB medium at 28℃ with various antibiotics, including ampicillin (100 µg/ml), chloramphenicol (30 µg/ml), kanamycin (100 µg/ml), streptomycin (20 µg/ml), erythromycin (100 µg/ml), carbenicillin (100 µg/ml), cephalosporin C (100 µg/ml), and tetracycline (10 µg/ml) for one day. (B) Effect of different concentrations of kanamycin on WT in LB medium. (C) Effect of different concentrations of kanamycin on *D. yeojuensis* transformed strain (C2) in LB medium. (D) Effect of different concentrations of kanamycin on *D. yeojuensis* transformed strain (C4) in LB medium.

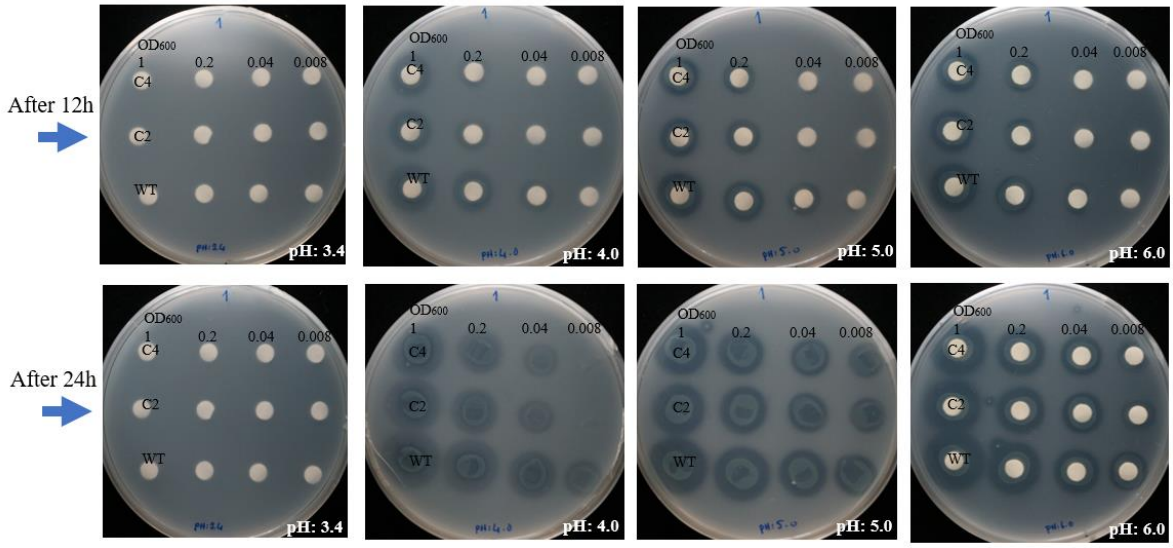


Figure 8: Bacterial gliadin digesting activity. The figure shows the ability of WT and transformed strains C2 and C4 to digest gliadin at different pH and concentrations. At the end of 12 hours and 24 hours, all samples tended to gliadin digesting in pH 5.0 and 6.0. Also, after 24 hours, WT was able to digest the gliadin in pH 4.0.

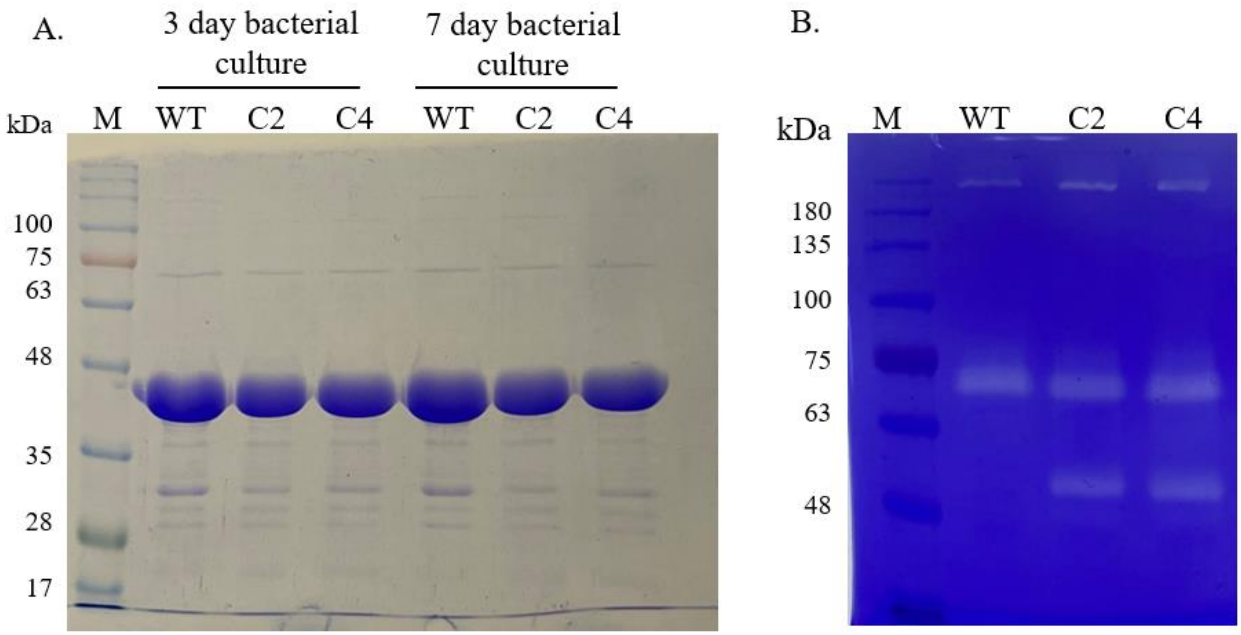


Figure 9. Extracellular medium and gliadin digesting activities of WT and transformed strains. (A) WT, C2, and C4 were incubated in PDA medium at the 3 and 7 days supernatant was collected then proteins were determined by using SDS-PAGE. (B) Zymogram of gliadin digesting activity by WT and transformed strains. Transformed strains show an additional band because of extra DY_0663 secretion.

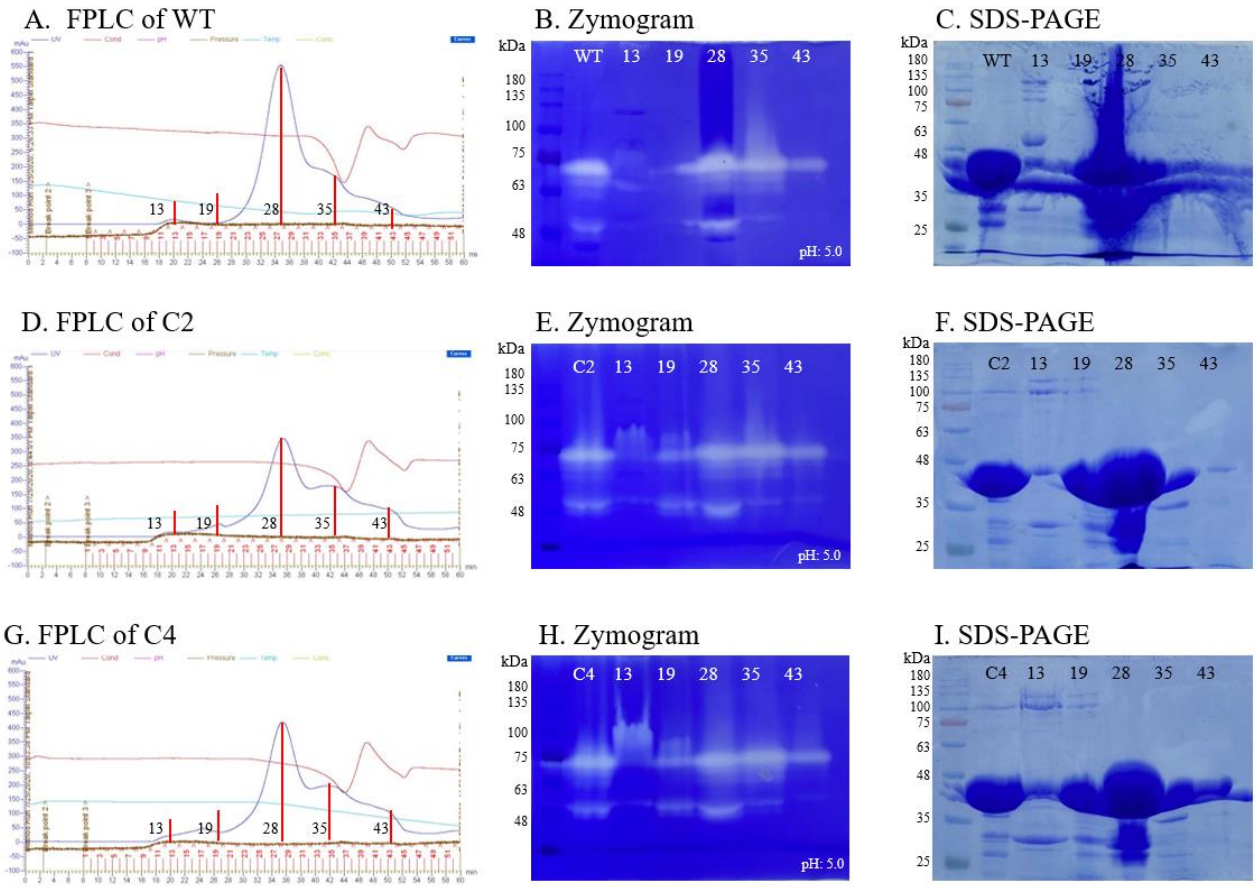


Figure 10. Purification of WT and transformed strains DY_0663 protease (HiPrep 16/60, Sephacryl S-300 HR). The DY_0663 extracellular medium concentrated solution was purified by gel filtration chromatography through a column Sephacryl S-300 HR (HiPrep 16/60). (A) The detection spectrum of *D. yeojuensis* protein OD280 nm. (D) The detection spectrum of DY_0663 C2 protein OD280 nm. (G) The detection spectrum of DY_0663 C4 protein OD280 nm. (B, E, and H) Zymogram gel of the active protein interval. (C, F, and I) 10% SDS-PAGE of the active protein interval. M denotes the Molecular mass marker (GeneDireX, USA).

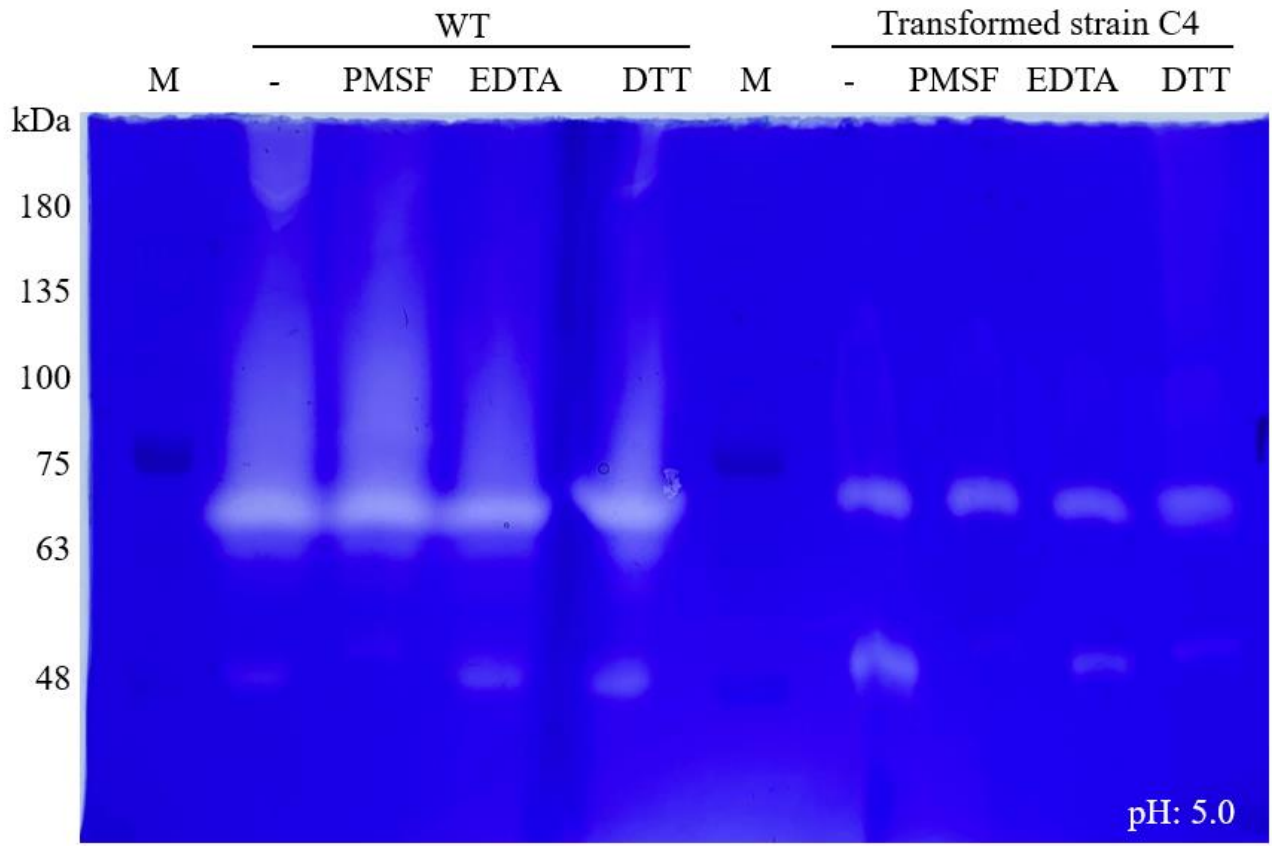
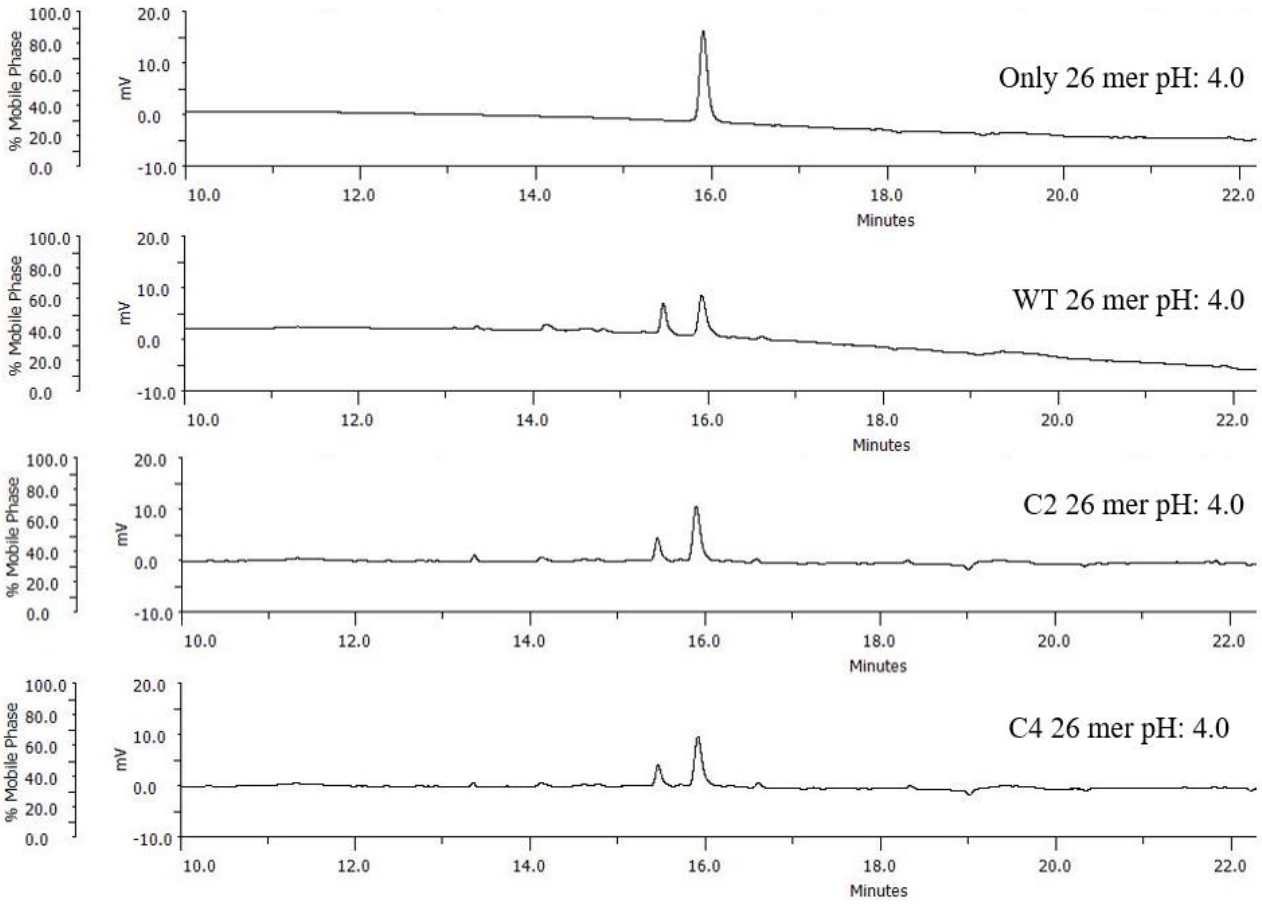


Figure 11. Effect of protease inhibitors on extracellular medium. WT and transformed strain C4 appear to have an effect on proteases in the presence of 1 mM PMSF, 5 mM EDTA or 4 mM DTT. After 1 hour of incubation, PMSF significantly inhibited serine protease in the second band.

A.


% Mobile Phase
100.0
80.0
60.0
40.0
20.0
0.0
mV
20.0
10.0
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-10.0
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12.0
14.0
16.0
18.0
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Minutes
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WT 26 mer pH: 4.0
C2 26 mer pH: 4.0
C4 26 mer pH: 4.0

B.

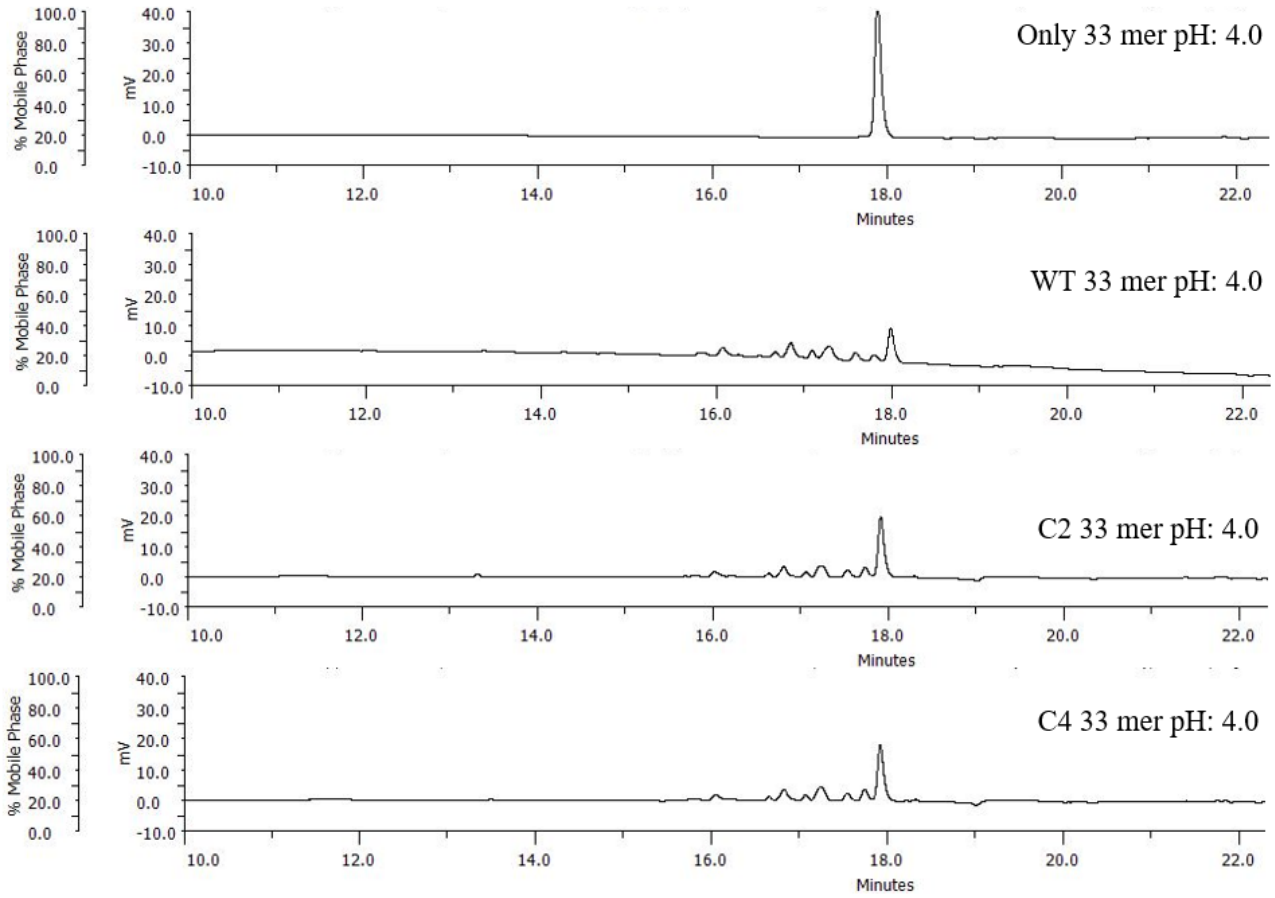

Figure 12. HPLC analysis of 33-mer and 26-mer peptides degradation by proteases secreted by *D. yeojuensis* and transformed strains. (A) The hydrolytic activity of *D. yeojuensis* and its transformed strains against 26-mer. (B) The hydrolytic activity of *D. yeojuensis* and its transformed strains against 33-mer.

Chapter 4

Discussion

Currently, the only treatment for patients with celiac disease (CD) is to implement a strict gluten-free diet; however, a lifelong gluten-free diet can be very expensive and challenging. Many patients' intestines are unable to heal completely and continue to suffer from the pain caused by the symptoms. The most common cause is the inability to fully implement a gluten-free diet. Despite the best efforts of patients, gluten accidentally ingested through cross-contamination can still harm their health. At this stage, many companies are developing drugs to treat CD, hoping to reduce the burden of the life of patients with CD and improve long-term health problems.

Here I wish to establish a new oral enzymatic therapy for CD, eliminating immunogenic peptides from gliadin before reaching the duodenum. There are other publications with similar solutions, but utilizing different enzymes or methods. Kuma030 is a mutated kumamolisin from *Alicyclobacillus sendaiensis*, obtaining high gluten-degrading activity and protease cleavage site between proline and glutamine (Liu et al., 2019). Proline and glutamine are major amino acids in 33-mer peptides from α gliadin and 26-mer peptides from γ-gliadin, indicating its potential as a new treatment for CD. ALV003 is a new preclinical drug containing two enzymes, SC-PEP from *Sphigomonas capsulate* and EP-B2 from wheat grains, both of them required for better pyrolytic activity against gliadin-derived peptides (Tye-Din et al., 2010). For practical applications of these enzymes, one of the problems is overcoming decreased activity under extremely low pH conditions similar to the human stomach. Some tackle this issue by pharmaceutical modifications, protecting them from malfunction under low pH and damage caused by pepsin. Subtilisins-A is a serine protease possessing gliadin digesting activity at neutral pH, which significantly underperform at acidic conditions (Darwish et al., 2019). Two modification techniques, PEGylation and Polylactic glycolic acid (PLGA) microencapsulation, prevent Subtilisins-A damaged by acidic exposure or autolysis, and improve its detoxification efficiency. In future studies, the proteolytic activity of DY_0663 can also be improved following similar principles.

Introducing mutations to DY_0663 through rational design or random manner might change its structure, thus enhancing its acid tolerance or substrate specificity. Applying PEGylation and microencapsulation to DY_0663 are also good options, because they are considered safe as chemicals approved by FDA. Subtilisins-A and DY_0663 are both serine proteases in the S8 family, so it is possible that both share the same applicability. There are also other experimental approaches focusing on reducing gliadin toxicity, preventing gliadin-induced intestinal permeability, or desensitizing celiac patients through tolerogenic vaccines (Maiuri et al., 2019).

Chapter 5

Conclusion

Although the traditional treatment for CD is to follow a gluten-free diet, oral enzymatic therapy as an alternative method has been recommended. I believe that DY_0663 is a candidate for such treatment. In this thesis, DY_0663 protease was proven to be efficient at digesting gliadin. I tried to express the DY_0663 thorough *E. coli* system, but such a method was unfavorable. Instead, *D. yeojuensis* was chosen as the host for pDSK plasmid containing the DY_0663 gene and its original promoter. Since they were originated from *D. yeojuensis*, the expression and extracellular medium of DY_0663 was successful. Both WT and transformed strains proved to have gliadin digesting activity and transformed strains showed similar activity compared to WT. The extracellular medium of DY_0663 has also been confirmed to digest 26-mer and 33-mer peptides under acidic conditions similar to the human stomach.

In the future, further studies about DY_0663 will be recommended to discover its full potential as an oral enzymatic drug, including clarification of cleavage site pattern, determination of protein structure, and modification for better acid tolerance or stronger activities. *D. yeojuensis* can also act as the expression host for other proteases from *D. yeojuensis*, since DY_0663 may not be the only one that can digest gliadin.

References

Akpinar, B. A., Biyiklioglu, S., Alptekin, B., Havránková, M., Vrána, J., Doležel, J., ... & Budak, H. (2018). Chromosome‐based survey sequencing reveals the genome organization of wild wheat progenitor Triticum dicoccoides. *Plant biotechnology journal*, *16*(12), 2077-2087.

Al-Toma, A., Volta, U., Auricchio, R., Castillejo, G., Sanders, D. S., Cellier, C., ... & Lundin, K. E. (2019). European Society for the Study of Coeliac Disease (ESsCD) guideline for coeliac disease and other gluten-related disorders. *United European gastroenterology journal*, *7*(5), 583-613.

Balakireva, A. V., & Zamyatnin, A. A. (2016). Properties of gluten intolerance: gluten structure, evolution, pathogenicity and detoxification capabilities. *Nutrients*, *8*(10), 644.

Bethune, M. T., & Khosla, C. (2012). Oral enzyme therapy for celiac sprue. In *Methods in enzymology* (Vol. 502, pp. 241-271). Academic Press.

Biesiekierski, J. R. (2017). What is gluten?. *Journal of gastroenterology and hepatology*, *32*, 78-81.

Caio, G., Volta, U., Sapone, A., Leffler, D. A., De Giorgio, R., Catassi, C., & Fasano, A. (2019). Celiac disease: a comprehensive current review. *BMC medicine*, *17*(1), 1-20.

Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., ... & Pianelli, G. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *The American journal of clinical nutrition*, *85*(1), 160-166.

Cianferoni, A. (2016). Wheat allergy: diagnosis and management. *Journal of asthma and allergy*, *9*, 13.

Collin, P., Thorell, L., Kaukinen, K., & Mäki, M. (2004). The safe threshold for gluten contamination in gluten‐free products. Can trace amounts be accepted in the treatment of coeliac disease?. *Alimentary pharmacology & therapeutics*, *19*(12), 1277-1283.

Darwish, G., Helmerhorst, E. J., Schuppan, D., Oppenheim, F. G., & Wei, G. (2019). Pharmaceutically modified subtilisins withstand acidic conditions and effectively degrade gluten in vivo. *Scientific reports*, *9*(1), 7505.

De Martinis, M., Sirufo, M. M., Suppa, M., & Ginaldi, L. (2020). New perspectives in food allergy. *International Journal of Molecular Sciences*, *21*(4), 1474.

Fasano, A. (2005). Clinical presentation of celiac disease in the pediatric population. *Gastroenterology*, *128*(4), S68-S73.

Gordon, S. R., Stanley, E. J., Wolf, S., Toland, A., Wu, S. J., Hadidi, D., and Siegel, J. B. (2012). Computational design of an α-gliadin peptidase. *Journal of the American Chemical Society, 134*(50), 20513-20520.

Hefle, S. L., Nordlee, J. A., & Taylor, S. L. (1996). Allergenic foods. *Critical Reviews in Food Science & Nutrition*, *36*(S1), 69-89.

Jin, Y., Acharya, H. G., Acharya, D., Jorgensen, R., Gao, H., Secord, J., ... & Gangur, V. (2019). Advances in molecular mechanisms of wheat allergenicity in animal models: a comprehensive review. *Molecules, 24*(6), 1142.

Koerner, T. B., Cleroux, C., Poirier, C., Cantin, I., La Vieille, S., Hayward, S., & Dubois, S. (2013). Gluten contamination of naturally gluten-free flours and starches used by Canadians with celiac disease. *Food Additives & Contaminants: Part A*, *30*(12), 2017-2021.

Leffler, D. A., Green, P. H., & Fasano, A. (2015). Extraintestinal manifestations of coeliac disease. *Nature Reviews Gastroenterology & Hepatology*, *12*(10), 561.

Liu, H., Fan, X., Song, H., Hu, X., Zhang, G., Yu, C., & Yi, L. (2019). Efficient production of gluten hydrolase Kuma030 in E. coli by hot acid treatment without chromatography. *Enzyme and microbial technology*, *129*, 109356.

Maiuri, L., Villella, V. R., Raia, V., & Kroemer, G. (2019). The gliadin-CFTR connection: new perspectives for the treatment of celiac disease. *Italian journal of pediatrics*, *45*(1), 40.

Martín-Fernández, B., Costa, J., Oliveira, M. B. P., López-Ruiz, B., & Mafra, I. (2015). Screening new gene markers for gluten detection in foods. *Food Control*, *56*, 57-63.

Moneim, J., Asad, H., Butt, E., Foridi, J. S., Khan, Y., Patel, S., ... & Thakar, R. (2019). The use of an invitational letter to increase the vaccine uptake of patients with coeliac disease. *Primary health care research & development*, *20*.

Moreno Amador, M. D. L., Arévalo-Rodríguez, M., Durán, E. M., Martínez Reyes, J. C., & Sousa Martín, C. (2019). A new microbial gluten-degrading prolyl endopeptidase: Potential application in celiac disease to reduce gluten immunogenic peptides. *PloS one*, *14*(6), e0218346.

Pazzirota, T., Martin, L., Mezcua, M., Ferrer, C., & Fernandez-Alba, A. R. (2013). Processing factor for a selected group of pesticides in a wine-making process: distribution of pesticides during grape processing. *Food Additives & Contaminants: Part A*, *30*(10), 1752-1760.

Rubio-Tapia, A., Hill, I. D., Kelly, C. P., Calderwood, A. H., & Murray, J. A. (2013). American College of Gastroenterology clinical guideline: diagnosis and management of celiac disease. *The American journal of gastroenterology*, *108*(5), 656.

Sampson, H. A. (2004). Update on food allergy. *Journal of allergy and clinical immunology*, *113*(5), 805-819.

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., ... & Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. *Science*, *297*(5590), 2275-2279.

Sharma, N., Bhatia, S., Chunduri, V., Kaur, S., Sharma, S., Kapoor, P., ... & Garg, M. (2020). Pathogenesis of celiac disease and other gluten related disorders in wheat and strategies for mitigating them. *Frontiers in Nutrition*, *7*.

Sicherer, S. H., Muñoz-Furlong, A., & Sampson, H. A. (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology*, *114*(1), 159-165.

Sollid, L. M., & Thorsby, E. (1993). HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology*, *105*(3), 910-922.

Tollefsen, S., Arentz-Hansen, H., Fleckenstein, B., Molberg, O., Ráki, M., Kwok, W. W., ... & Sollid, L. M. (2006). HLA-DQ2 and-DQ8 signatures of gluten T cell epitopes in celiac disease. *The Journal of clinical investigation*, *116*(8), 2226-2236.

Tye-Din, J. A., Galipeau, H. J., & Agardh, D. (2018). Celiac disease: a review of current concepts in pathogenesis, prevention, and novel therapies. *Frontiers in pediatrics*, *6*, 350.

Tye-Din, J. A., Anderson, R. P., Ffrench, R. A., Brown, G. J., Hodsman, P., Siegel, M., Botwick, W., & Shreeniwas, R. (2010). The effects of ALV003 pre-digestion of gluten on immune response and symptoms in celiac disease in vivo. *Clinical immunology (Orlando, Fla.)*, *134*(3), 289–295.

van Hengel, A. J. (2007). Food allergen detection methods and the challenge to protect food-allergic consumers. *Analytical and bioanalytical chemistry*, *389*(1), 111-118.

Wieser, H. (2007). Chemistry of gluten proteins. *Food microbiology*, *24*(2), 115-119.

Young, E., Stoneham, M. D., Petruckevitch, A., Barton, J., & Rona, R. (1994). A population study of food intolerance. *The lancet*, *343*(8906), 1127-1130.