REPUBLIC OF TURKEY

ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

IDENTIFICATION GASTRIC EPITHELIAL CELL SURFACE PROTEINS THAT INTERACT WITH *HELICOBACTER PYLORI* OUTER INFLAMMATORY PROTEIN A

ELİF KILIÇ

MASTER THESIS

DEPARTMENT of MEDICAL BIOTECHNOLOGY

SUPERVISOR

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DECLARATION

I hereby declare that this thesis has been written by me based on the data obtained in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analyses have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the original sources in accordance with the publication ethics. I also announce and emphasize that I have not violated any rules secured by patent and copyrights whilst the conduct and writing of this research.

25.12.2020

Elif KILIÇ

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LIST OF ABBREVIATIONS AND SYMBOLS

µl	Microliter
µm	Micrometer
AcOH	Acetic Acid
AlpA/AlpB	Adherence associated proteins
AP-1	Activator protein-1
APS	Ammonium Persulfate
BabA	Blood-antigen binding protein A
BCA	Bicinchoninic acid
BME	β-merkaptoetanol
BSA	Bovine serum albumin
CagA	Cytotoxin-associated gene A
cag PAI	Cag pathogenicity island
CBB	Coomassie brilliant blue
Chis	6XHistidine on C terminal of protein
CT	CT dinucleotide repeats
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
Dnase1	Deoxyribonuclease I
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid

EGFR	Epidermal growth factor receptor
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GSK3ß	Glycogen synthase kinase 3 beta
GST	Glutathione S-transferase
His	Histidine
IL-8	Interleukin -8
IRF1	Interferon regulatoryfactor 1
ISRE	Interferon-stimulated responsive element
LB	Luria Broth
LC-MS/MS	Liquid chromatography–mass spectrometry
LIC	Ligation independent cloning
M	Molar
MetOH	Methanol
Min	Minute
mm	Milimeter
mM	Milimolar
ml	Mililiter
NF-kB	Nuclear factor-kB
OipA	Outer inflamatory protein A
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDK1	PI3K-dependent kinase 1
PFU	Pyrococcus furiosus
pH	Power of Hydrogen
PI3K	Phosphatidylinositol 3-OH kinase
PVDF	Polyvinylidene difluoride
RPMI	Roswell Park Memorial Institute
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SabA	Sialic acid-binding adhesin
SDS	Sodium dodecyl sulfate
STATF	Signal transducers and activators of transcription
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% Tween ® 20
TEMED	Tetramethylethylenediamine
T4SS	Type IV secretion system
UreA	Urease A
UreB	Urease B
U	Unit of enzyme's catalytic activity.
V	Volt
VacA	Vacuolating cytotoxin A

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SUMMARY

Helicobacter pylori (H. pylori) is a pathogenic bacterium that colonizes the mucous layer of the gastric epithelium and infects more than half of the world's population. The attachment of bacteria to gastric epithelial cells is one of the most important steps in bacterial infection. The outer membrane proteins could act as adhesin factors that can interact with specific surface proteins for attachment to gastric epithelial cells in virulence. Outer inflammatory protein A (OipA) is one of the outer membrane proteins of *H. pylori* that induces pro-inflammatory signals and secretion of IL-8 when bacteria attach to the gastric epithelial layer. Although the functions of this protein have been defined, the interaction partner that OipA binds in the gastric epithelial cell layer has not been identified. In this study, it was aimed to identify gastric cell surface proteins that OipA protein interacts. Due to this aim, recombinant OipA protein was produced and gastric epithelial cell surface proteins were extracted from AGS cell line. Then, OipA protein was used as a bait protein to pull down prey proteins from gastric cell surface proteins which interact with OipA. The obtained proteins were analyzed with LC-MS/MS to identify predicted interaction partner. The candidate proteins found in this study are very valuable in terms of being the first in the literature. When these proteins are confirmed by validation experiments, they can form the basis of vaccine studies as vaccine targets. It will also enable the identification of new strategies for the development of novel drug molecules that will target the interactions of the candidate protein and prevent the colonization of bacteria.

Keywords: *H. pylori*, Interaction partner, OipA, Surface protein, Virulence factor

ÖZET

***Helicobacter pylori* İnflamatuvar Protein A (OipA) ile Etkileşime Giren Gastrik Epitel Hücre Yüzey Proteinlerin Tanımlanması**

Helicobacter pylori dünya nüfusunun yaklaşık yarısını enfekte eden midede inflamasyonun oluşmasına yol açan patojenik bir bakteridir. Bakterinin sebep olduğu enfeksiyonda en önemli adımlarından biri bakterinin mide epitel hücrelere ile tutunmasıdır. Bu tutunmada, dış zar proteinleri mide epitel hücrelerine tutunmak için yüzey proteinleri ile etkileşime girebilen adhesin molekülleri olarak rol alır. Dış zar proteinlerinden OipA tutunmada rol alarak mide epitel hücrelerinde pro-enflamatuar sinyallerin oluşmasını ve IL-8 salgılanmasını indükler. Bu proteinin görevleri tanımlanmış olsa da mide epitel hücresinde etkileşime girdiği bağlanma partnerleri tanımlanmamıştır. Bu çalışmada OipA proteininin mide epitel yüzeyinde etkileşime girdiği yüzey proteinlerinin tanımlanması amaçlanmıştır. Bu doğrultuda OipA proteini recombinant olarak üretildi ve AGS hücre hattından mide epitel yüzey proteinleri ekstrakt edildi. OipA ile etkileşime giren mide hücre yüzey proteinlerinden aday proteinleri çekmek için yem proteini olarak kullanıldı. Elde edilen proteinler, olası etkileşim partnerini belirlemek için LC-MS/MS ile analiz edildi. Bu çalışma ile bulunan aday proteinler, literatürde ilk olması açısından da çok değerlidir. Aday proteinler, *H. pylori* enfeksiyonun önlenmesinde büyük önem taşımaktadır. Bu proteinler validasyon deneyleri ile desteklendiğinde aşı hedefi olarak aşı çalışmalarına temel oluşturabilecektir. Ayrıca aday proteinin etkileşimlerini hedef alıp bakterinin kolonizasyonunu önleyecek yeni ilaç moleküllerinin geliştirilmesi için yeni hedeflerin tanımlanmasına olanak sağlayacaktır.

Anahtar Sözcükler: Etkileşim partneri, *H. pylori*, OipA, Virülens faktör, Yüzey proteini

1. AIM OF THE STUDY AND BACKGROUND

Helicobacter pylori, which is estimated to infect approximately half of the world population, has been defined as a type 1 carcinogen by the World Health Organization (WHO) (1). *H. pylori* is a well-recognized human pathogen that infects the stomach and induces inflammation caused by various gastroduodenal diseases such as ulcer, chronic gastritis, mucosa-associated lymphoid tissue lymphoma and gastric cancer (2). In the distribution of *H. pylori* infection, socio-economic conditons, nutrition and urbanization levels have an effect of wide geographical differences between countries (3). People are usually infected with *H. pylori* in childhood. Unless *H. pylori* infection is treated, it continues for life. Most infected people live asymptomatically (4).

The complex interplay between host, environmental and *H. pylori* virulence factors leads to the pathogenesis of infection of *H. pylori* and thus to disease outcome (5). *H. pylori* has developed mechanisms and virulence factors for survival in the human stomach, a very harsh environment where bacteria are difficult to survive (6). The virulence factors are required for *H. pylori* to cause colonization and later inflammation in the gastric mucosa. These virulence factors are responsible for establishing colonization, immune escape from host immune clearance and disesae induction (7).

Adhesion of the bacteria to the gastric epithelium is one of the most important steps in establishing colonization (8). *H. pylori* can attach tightly to receptor of the epithelial cells with adhesin proteins (9). The attachment protects *H. pylori* from gastric peristalsis and emptying while allowing bacterial virulence factor such as cagA and vacA to reach the host (10,11). Outer membrane protein the best studied adhesins,

have been identified as at least 32 which play a role in bacterial adhesion (12,13). The Hop family is the largest class of outer membrane proteins including proteins such as OipA, SabA, BabA, and HopZ (12,13).

Outer inflamatory protein A is an outer membrane protein belonging to the Hop family, encoded by the *oipA* gene (HP0638/*hopH*) (14). The functional status of the oipA protein is determined by the a slipped strand mispairing mechanism based on the CT repeats located at the 5' region of the gene (15). Functional OipA causes gastric inflammation by attachment to gastric epithelial cells and increasing IL-8 production (11). OipA is thought to affect intracellular signaling and alter, regulate host signaling pathways (16). OipA is associsted with neutrophil infiltration, activation of focal adhesion kinase, cytoskeletal reorganization and colonization density (17). Although oipA is responsible for attaching to the gastric epithelial cell, the epithelial cell receptor of OipA is unknown (7).

In this thesis, it is aimed to find possible interaction partner with which OipA protein interacts. Since there is a lack of information in the literature about the interaction partner of OipA, this study is important in terms of being the first. The candidate proteins found are also aimed to contribute to the literature. If the candidate proteins found are supported by validation experiments in future studies, these proteins can be target proteins of vaccine studies that will prevent H.pylori infection. It will even enable the identification of novel targets for the development of new drug molecules that will target protein interactions and prevent colonization of bacteria.

2. INTRODUCTION

2.1 History of *Helicobacter pylori*

The discovery and research of *Helicobacter pylori (H. pylori)* began with the sufficiently advanced microscope resolution sat the end of the nineteenth century (18). Several researchers have reported the presence of spiral microorganisms in the stomachs of animals (19). For humans, in 1889 W. Jaworski was first to describe the spiral organisms in the sediment of gastric washings obtained from humans. Among the other bacteria, he realized, a bacterium with a characteristic spiral appearance which he called it, '*Vibrio rugula'* , and than suggested that this bacterium was the first possible pathogenic role in gastric diseases (20).

In 1982 *H. pylori* was first isolated and cultured by Warren and Marshall in the microbiology lab of the Royal Perth Hospital (21). The first culture obtained from a gastric biopsy of a duodenal ulcer patient was originally called the Campylobacter-Like Organism (CLO), which they thought was a Campylobacter species (20). The ribonucleic acid sequencing studies of the found bacteria showed that it could not be properly included in the genus Campylobacter. the organism was renamed *H. pylori*, which represents a new species of Helicobacter (22). Marshall drank *H. pylori's* culture, developed gastritis, and retrieved the bacteria from the lining of his stomach to show his colleagues that there is a relationship between bacteria and gastritis. Warren and Marshall later demonstrated that antibiotics are effective in treating *H. pylori* infection (21) This discovery was worth the 2005 Nobel prize in physiology or medicine for "the discovery of *H. pylori* and its role in gastritis and peptic ulcer disease" to Robin Warren and Barry Marshall. This information indicated that gastric colonization with *H. pylori* can lead to various gastrointestinal disorders, such as

chronic gastritis, duodenal ulcers (DU) or gastric ulcers (GU), gastric mucosal lymphoid tissue (MALT) and gastric cancer (GC) (19).

2.2 Microbiology of *Helicobacter pylori*

Helicobacter pylori is a gram-negative, spiral-shaped bacterium that infects about fifty percent of the world's population (23). The bacteria is 2.5 - 5.0 μm length and 0.5 - 1.0 mm width (24). *H. pylori* have two to six unipolar sheathed flagella which provide motility and allow rapid movement in the mucous layer on the gastric epithelial cells (25). Although it is generally spiral-shaped, the bacterium may appear as a stick, while coccoid shapes appear after in vitro culture or antibiotic treatment for a long time. These coccoid cannot be cultured and have been proven to represent dead cells (26). The two sequenced *H. pylori* genomes are strain 26695 and J99, include approximately 1.7 M base pairs with a G-C content of 35 to 40%. Both genomes consist of two copies of the 16S, 23S, and 5S rRNA genes (27).

2.3 Epidemiology of *Helicobacter pylori* Infection

Chronic bacterial *H. pylori* infection affects about half of the world's population. (28) The prevalence of *H. pylori* infection in the same country is affected by the socioeconomic status, age, gender, and genetic predisposition of people (29). Among countries, the prevalence of *H. pylori* infection in developing countries is significantly higher than in developed countries (30). Also, it is seen that the acquisition of *H. pylori* is higher in developing countries. Acquisition of *H. pylori* is decreased at a faster rate

in developed countries, possibly due to a faster improvement in hygiene practices (31). *H. pylori* infection is occurred in childhood and continued throughout the life of the individual, especially when the socio-economic state is low and there is no effective treatment in developing countries. However, there is very little acquisition of infection in adulthood (28). The transmission route of *H. pylori* is not fully understood. However, it is thought that new infections occur as an outcome of direct person to person transmission or by environmental contamination. Person-to-person transmission can occur in three possible ways: gastro-oral, oral-oral, and fecal-oral routes, but the dominant conductivity mechanism has not yet been specified (32).

2.4 Pathogenesis of *Helicobacter pylori* Infection

Pathogenesis of *H. pylori* infection can lead to the occurrence of gastrointestinal diseases due to colonization (33). Pathogenesis occurred by colonization is related to bacterial virulence factors, host genetics and immune response, and environmental factors (19). *H. pylori* is the only known organism that can colonize the harsh environment of the human stomach (34). *H. pylori* can survive within the mucous layer by secreting the urease enzyme that breaks down urea into carbon dioxide and ammonia, buffering its environment (35). *H. pylori* use polar flagella to stay in the mucus layer. Polar flagella are required for *H. pylori* colonization with both the ability to swim with movement and the ability to control the direction of movement with chemotactic responses (36). When *H. pylori* move to the mucosal layer lining the gastric epithelium, bacterial adhesins interact with epithelial cellular receptors and *H. pylori* are protected against displacement in the stomach by these interactions (16). *H. pylori* has many known adhesives involved in attachment such as blood-antigen binding protein A (BabA) (37)and sialic acid-binding adhesin (SabA)(38), adherence associated proteins (AlpA and AlpB) (39) and outer inflamatory protein A (OipA) (17). As a final step, *H. pylori* secretes several effector proteins and toxins such as

cytotoxin-associated gene A (*cagA*) and vacuolating cytotoxin A (*vacA*) to cause tissue damage in the host (40). In substance, since *H. pylori* enters the host stomach, four steps are critical and essential for bacteria to generate colonization: Survival in acidic stomach; movement to the epithelial cells with a flagella; binding to host cells by adhesins and receptor interaction; causes tissue damage with toxin release (40).

2.5 *Helicobacter pylori* Associated Diseases

In previous studies, Marshall and Warren showed a strong correlation between the presence of *H. pylori* and inflammation in gastric biopsy; this observation has been confirmed in numerous studies. As a result of studies, *H. pylori* infection is associated with gastroduodenal diseases (41). *H. pylori* infection is mostly observed in childhood and diseases associated with the infection occur in adulthood. Although *H. pylori* infection usually leads to chronic active gastritis, most infected individuals (~ 85%) stay at asymptomatic level throughout life. In some facts, this gastric inflammation may develop into more serious diseases such as duodenal or gastric ulcers, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, non-cardia gastric adenocarcinoma or gastric cancer. The development of gastric complications is a long process with a multifactor affected by environmental, host genetic, and bacterial virulence factors (42). *H. pylori* infection is the main risk factor in 92% of gastric cancers, which is the fourth most common and second deadliest cancer worldwide, with approximately 740,000 deaths per year (8). Due to the strong relationship between *H. pylori* infection and cancer *H. pylori* infection was described as a type I carcinogen in 1994 by the World Health Organization (43).

2.6 Diagnosis of *Helicobacter pylori* Infection

Diagnostic methods for *H. pylori* infection are divided into two groups as invasive and non-invasive tests. Invasive tests are performed with endoscopic biopsy materials taken from the gastric mucosa (44). Invasive tests involve a rapid urease test (RUT), bacterial culture, and histological analysis of the biopsy samples (44,45). Along with invasive tests, *H. pylori* can be detected using PCR and real-time PCR molecular methods applied to specific *H. pylori* genes (41). Non-invasive tests contain urea breath test (UBT), stool antigen test (SAT), and antibody tests (45). The sensitivity and specificity of each test is now 95-100% with the developing methodologies.

2.7 Treatment of *Helicobacter pylori* Infection

The purpose of *H. pylori* treatment is to completely remove the bacteria. When this happens, reinfection rates are low; so the benefit of the treatment is permanent. So that the therapy to be successful, antibiotics are combined proton-pump inhibitors or ranitidine bismuth citrate. Therefore, triple therapies called antisecretory agent combination with two antimicrobial agents have been thoroughly evaluated and approved by the Food and Drug Administration (FDA). Therapies of *H. pylori* generally consist of two groups, which are classified as first-line therapies and second-line therapies. If the first-line therapy is unsuccessful, second-line therapy should be applied. The reason for the failure of therapies is antibiotic resistance. So, the treatment of *H. pylori* infection is required with newly developed therapeutic approaches and vaccine developments (9).

2.8 Virulence Factors of *Helicobacter pylori*

Helicobacter pylori has an unique set of factors that actively support the successful survival and persistence in the natural hostile ecological niche, the human stomach, throughout the life of the individual unless treated (8). The unique set of factors is called virulence factors what is the ability of a bacterium to induce and develop a disease with a spectrum of severity. So, *H. pylori* is needed virulence factors to cause colonization in the gastric mucosa and subsequent inflammation. These virulence factors are responsible for adhesin, colonization, and activation of the host immune response (46). It has been explained to contain an interaction between virulence factors of *H. pylori* and host genetics and environmental factors. Therefore the virulence factors have been related to gastrointestinal diseases (47).

H. pylori is defined as a highly heterogeneous bacterium (48) that is characterized by high mutational frequency, elevated recombination rate, and exchange of genetic elements (14). Virulence factors have also changed geographically by the genetic mechanism. *H. pylori* virulence factors are determined to have different gene sequences in the formation and progression of gastric diseases in different geographic regions (48).

H. pylori involves about 1600 genes. Some of the potential virulence genes of *H. pylori* have been identified and reported, to date (49). The possibility of identifying additional important pathogenic genes is continued (50). The virulence factors identified were classified into 3 groups according to roles in inflammation on gastric mucosa by Yoshio Yamaoka (11). First group contains virulence factors associated with an escape to a high acidic environment such that urease, flagella, and bacterial

shape. Urease, the essential ingredients of colonization, is called a nickel-containing enzyme, and it requires the efficient acquisition of nickel from the environment for its activity. Urease enzyme takes part in the neutralization of the acidic gastric mucosa (51). Representing about 5-10% of the total protein of the bacteria, urease consists of two subunits [UreA (26 kDa) and UreB (62 kDa)], which also form a larger assembly of 12 catalytic units (52). Flagella consists of three structural elements: a basal body, hook, and filament (51,53). The filament consists of two flagellin protein subunits (FlaA and FlaB) encoded by *flaA* and *flab*. Both proteins are necessary for completed mobility. Flagella helps *H. pylori* to move away from the acidic gastric environment (53). So Urease, flagella motility, and bacterial shape are virulence factors responsible for bacteria escaping from acidic gastric conditions and the formation of permanent infections (54). In addition to all *H. pylori* strains have been proven to have several virulence factors such that flagella and urease enzyme (46).

Second group contains virulence factors associated with colonization of epithelial surfaces such that blood group antigen-binding adhesin (BabA), sialic acid-binding Adhesin (SabA), outer inflammatory protein (OipA), *H. pylori* outer membrane protein (HopQ) and other proteins (54). Adhesion of *H. pylori* to the mucous layer of the gastric epithelium along with these factors, has an important place in the initial colonization and persistence of bacteria in the human stomach (55). BabA is the first well-identified and major adhesin group (8) and binds to the fucosylated Lewis b blood group antigen, found on blood group O (H antigen), A and B antigens which are expressed on gastric human epithelial cells (37,56). SabA is the second most well-identified adhesin group (57), binds to gangliosides with the dimeric sialyl-Lewis x antigen when the expression of sialyl-Lewis x glycosphingolipid (sLex) antigen is increased on the cellular surface (38,58). OipA is involved in bacterial adherence with mucosal damage association with host cell apoptosis , interleukin (IL)-8 induction (59).

The third group contains virulence factors associated with gastric epithelial cell pathogenicity such that cytotoxin-associated gene A (*cagA*), vacuolating cytotoxin A (*vacA*) (54). These virulence factors cause tissue damage and toxin release by an inflammatory response to *H. pylori* (40). CagA is a well-identified oncoprotein that is injected into gastric epithelial cells via a type IV secretion system (T4SS) (7). CagA and T4SS are encoded by cag pathogenicity island (*cag PAI*). CagA causes cellular changes after injected into the cell (60). CagA and T4SS trigger gastric inflammation with an NFκB signal and increased IL-8 secretion (61,62). Therefore, *cag PAI* conduces to gastric carcinogenesis with multiple mechanisms (54). VacA is capable of inducing vacuole formation in eukaryotic cells (63). VacA is acknowledged a multifunctional toxin that produces multiple effects on host cells such as vacuolization and cell necrosis (64,65).

Therefore, none of the virulence factors can be accepted as an independent factor for disease outcome. In fact, when multiple virulence factors occur, there is a greater risk of serious clinical outcomes. Virulence factors have synergistic effects on each other (19).

2.9 Outer Inflammatory Protein A

The outer inflammatory protein A (OipA) is approximately 34 kDa proinflammatory protein which can induce IL-8 (15). OipA is encoded by inflammation-related gene (HP0638/*hopH*) located approximately 100 kb from *cag PAI* on the *H. pylori* chromosome (15,59). A functional *oipA* is connected severe gastric inflammation, high *H. pylori* density, high levels of mucosal IL-8, and the clinical outcome of duodenal ulcer, gastric cancer (59,66).

OipA is classified in the outer membrane protein group. So OipA functions to attach of *H. pylori* to gastric epithelial cells as an adhesin (67,68). The role of OipA in bacterial colonization of the gastric mucosa has been demonstrated in animal studies. (48) In a study, Mongolian gerbils were infected with wild type *H. pylori* 7.13 strain which has functional OipA protein, the infected group developed gastric cancer. Other Mongolian gerbils were infected with the isogenic *oipA* mutant of wild-type *H. pylori* strain 7.13, the infected group was unable to develop gastric cancer (69). These results indicate that (i) *oipA* expression was significantly associated with the presence of gastric cancer, (ii) *oipA* can function as a colonization factor (69,70).

Initially, studies showed that the presence of a functional *cag PAI* is well related to IL- 8 production (71,72) but, some cag-negative strains produced IL-8 from gastric cell lines such as MKN45, AGS, and KATO III. On the other hand, some cag-negative cases are higher than the median IL-8 values of cag-positive cases from gastric biopsies of patients (73). Based on these findings, they indicate that it is effective on another virulence factor other than cag in IL 8 production. Yamaoka et all. discovered that functional OipA is correlated with IL-8 production (15). In contrast, some studies have shown that OipA plays a role in adherence *H. pylori* to host cells and does not affect IL-8 production in gastric cells. Whether OipA directly affects the proinflammatory signal still being discussed (68). The research of Yamaoka et al is found that *oipA* and *cag PAI* are required for the full activation of the IL-8 promoter but *oipA* and *cag PAI* regulate in different pathways on IL-8 production. pathways of *cag PAI* depends on NF-kB; OipA does not depend on NF-kB. OipA is involved in STATF-IRF1-ISRE pathways (74). Also, studies using a mouse model infected with isogenic *oipA* gene mutants also confirmed that *oipA* was associated with the gastric inflammation and CXC chemokine (KC) production (67).

OipA is involved in ß-catenin signaling that leads to open cell-cell junctions and proliferation (69). Previous data is shown that differentness in the cell adhesion complex of the E-cadherin / ß-catenin occurs in gastric cancers related to increased nuclear localization of ß-catenin.(75) So OipA and *cagA* can induce nuclear translocation of ß-catenin and alter β-catenin signaling (69).

Recent studies have indicated that *oipA* induces inflammation and actin dynamics by phosphorylation of multiple signaling pathways with interacting *cag PAI (cagA)* related pathways (49). *cag PAI* and OipA regulate the phosphorylation of Akt in the Thr 308 and Ser 473 regions differently causing an imbalance in downstream proliferation and apoptotic signaling. OipA and CagA induce the inactivation of GSK3ß through the activities of Akt signaling pathways, so GSK3ß→AP-1 and GSK3β→β-catenin pathway may regulate IL-8 production . CagA increases the phosphorylation of the PI3K p85 subunit and PDK1. OipA is contributed to cagA pathways related to the activation of PI3K and PDK1. Therefore, the combination of cag PAI and OipA are required for complete activation of Akt, inactivation of GSK3ß, and activation of PI3K→PDK1 signaling pathways. cag PAI and OipA participate in EGFR-related IL-8 production by the activation of EGFR-PI3K signaling. These signaling pathways are involved in gastric carcinogenesis (76).

OipA plays role in *H. pylori*-induced focal adhesion kinase activation and cytoskeletal reorganization of gastric epithelial cells that effects cell motility and invasiveness. Tabassam et all confirmed that OipA is involved in most of FAK phosphorylation. OipA induces activation of FAK Y397 which is probably a prerequisite for complete activation of FAK and actin fiber formation by intracellular signaling. Activation of FAK is a key regulator of morphological changes caused by H. pylori infection in gastric epithelial cells. The molecular mechanisms by which OipA interacts with host cell receptors are not clear. Tabassam et all thought that

phosphorylation of FAK Y397 may be involved in the interaction between OipA and epithelial cell surface receptors responsible for transmitting the downstream signals causing activation of FAK. OipA is responsible for FAK activation instead of the *cag PAI* or cagA (77).

OipA protein can be expressed from a nonfunctional or functional *oipA* gene. The functional status of *oipA* gene is regulated by a slipped-strand mispairing mechanism based on the number of CT dinucleotide repeats in the 5' region of the gene (15). When a functional protein is expressed, the status of protein is called "on" and the expression of a non-functional protein is called "off." (15,59). Slipped-strand mispairing mechanism is a mutagenic process in phase variation that occurs during DNA replication of repetitive sequences (78). Adding or deleting CT repeats to *oipA* results in a frameshift that directs the phase variation in protein expression (79). Based on previous reports, the number of CT repeats associated with oipA status have been found ranged from 3 to 14 repeats (80,81) If the sequence of *oipA* gene has 6, 9, (3+1), (1+4), (2+6), (2+3), 10 or other CT dinucleodite repats, signal sequence peptide is kept in frame that indicating *oipA* gene is "on" status (15,80,82,83).

In many studies, the prevalence of "on" / "off" status of *oipA* have been investigated according to geographical regions (68). The CT repeat pattern in the signal sequence of *oipA* gene also has geographic characteristics and appears relatively stable in individual patients and families over the years (84). The highest frequency of o*ipA* on status was found in Asian countries such that Japan (100%) (80) and Malaysia/ Singapore (> 85.0%) (85) The prevalence of *oipA* on status was observed in Bulgaria (81.0%) (86), Colombia (79.3%) (87), Portugal (49.6%) (88), USA (45.9%) (89) and Western countries such as Germany (59.0%) (68) and North Italy (60%) (90).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacteria

3.1.1.1 *Helicobacter pylori*

Helicobacter pylori was kindly provided by Prof. Dr. Anne Müller from the Institute for Molecular Cancer Research at the University of Zurich. It is kept in -80°C in our laboratory. *Helicobacter pylori* was primarily grown on Colombia agar plates which consist of Colombia agar, defibrinated horse blood and 200X and 1000X antibiotic cocktails. The ingredients were shown in Table 3.1,Table 3.2 and Table 3.3.

Table 3.1. Components of Colombia agar plates

Component	Amount
Colombia Blood agar	1000ml
Horse Blood	50ml
1000X antibiotic cocktail	1ml
200X antibiotic cocktail	5ml
β-cyclodextrin	5 ml (in DMSO)

Table 3.2. Components of 1000X antibiotic cocktail

Component	Amount
Trimetophrim	100 mg
Amphotericin B	160 mg
DMSO	20 ml

Table 3.3. Components of 200X antibiotic cocktail

Component	Amount
Vancomycin	100mg
Cefsulodin	50mg
Polymixin B	3,3mg
ddH_2O	50ml

The contents of the liquid culture of *Helicobacter pylori* are shown in Table 3.4 to prepare 100 ml. The liquid culture volume can be varied depending on the experiment.

Table 3.4. Components of liquid culture for *H. pylori*

Component	Amount
Brucella Broth	90ml
FBS (10% v/v)	10ml
Vancomycin (1000X)	10ul

3.1.1.2 *Escherichia coli*

E. coli DH5α, BL21 (DE3) and *C43 (DE3)* strains were used in transformation and recombinant protein production experiments. *E. coli DH5α, BL21 (DE3)* and *C43 (DE3)* strains were obtain from ATCC. The *E. coli* strains were grown on Luria Broth (LB) agar and Luria Broth liquid medium. LB and agar powder were combined in appropriate quantities in distilled water according to the instructions and autoclaved to prepare the LB agar plate. In the same way, LB powder was mixed with distilled water according to the instructions and autoclaved. Autoclaved 100% Glycerol is required to store bacteria for the long-term. The bacteria culture grown overnight is mixed with autoclaved 100% glycerol in a 1:1 ratio and stored at -80°C.

3.1.2 Commercial Kits

Commercial kits that were used in the thesis study are shown in Table 3.5.

Table 3.5. Commercial kits

Commercial Kit	Supplier Company
Tissue & Cell Genomic DNA Purification Kit	GeneMark
Plasmid MiniPrep Purification Kit	GeneMark
ZymoPURE™ II Plasmid Midiprep Kit	Zymo Research
Hot Start Master Mix	GeneMark
Pfu Master Mix	GeneMark

Table 3.5. Commercial kits (continued)

Plus PCR Clean-Up Kit	GeneMark
Zymoclean™ Gel DNA Recovery Kit	Zymo Research
aLICator LIC Cloning and Expression Kit 1 (untagged)	Thermo Scientific
Pure Proteome™ Nickel Magnetic Beads	MerckMillipore
Pierce™ BCA Protein Assay Kit	Thermo .Scientific
Silver Stain Plus™ Kit	BIO-RAD
Clarify™ Western ECL Substrate	BIO-RAD
Mem-PER™ Plus Membrane Protein Extraction Kit	Thermo Scientific

3.1.3 Primers

Primers that were used in the thesis study are shown in Table 3.6. Primers are were performed designed by NCBI Primer Blast tool. In general, potential primer regions were selected from *oipA* gene of *H. pylori* sequence according to primer design rules. Then *oipA* gene sequence and potential oipA primer regions were entered to NCBI primer blast tool to perform.

Table 3.6. Information of primers

Primer Name	Sequence (5'-3')	Product length (bp)
External OipA fw	CATTAAGCGGTGGTTTTGTG	1093
External OipA rv	AGCCAACTAAAGAGCGGTAA	
Cloning OipA fw	TACTTCCAATCCAATGCAATGAAAAA AGCTCTCTTACTA	964
Cloning OipA rv	TTATCCACTTCCAATGTTATTATTAATG TTTGTTTTTAAAGTT	
pET LIC fw	TCAATGCTTGAAGGAGCGGT	If positive 1434
pET LIC rv	CTCAGCTTCCTTTCGGGCTT	If negative, 500
C His OipA fw	AGAAGGAGATATAACTATGATGAAAAAA GCTCTCTTACT	976
C His OipA rv	GGAGATGGGAAGTCATTAATGATGGTGA TGGTGGTGATGTTTGTTTTTAAAGTT	
N His OipA fw	AGAAGGAGATATAACTATGCACCACCAT CACCATCATATGAAAAAAGCTCTCTTACT	979
N His OipA rv	GGAGATGGGAAGTCATTATTAATGT TTGTTTTTAAAGTT	
Control His fw	TAATACGACTCACTATAGGG	If positive,1400
Control His rv	GAGCGGATAACAATTTCACACAGG	Ifnegative, 530

3.1.4 Antibodies

Antibodies that were used in the thesis study are shown in Table 3.7.

Table 3.7 Antibodies

Antibody	Supplier Company	Experiment
His-Tag (D3I1O) XP® Rabbit mAb #12698	Cell Signaling Technology	Western Blot
Anti-rabbit IgG, HRP-linked Antibody #7074	Cell Signaling Technology	Western Blot

3.1.5 Cell Culture

Cell culture media, solutions, and buffers were used in cell culture are given in Table 3.8.

Table 3.8. Materials of cell culture

Content	Supplier Company
RPMI	Gibco™
FBS, qualified	Gibco™
Penicillin-Streptomycin	Gibco™
0.25%Trypsin-EDTA (1X)	Gibco™
10X PBS Buffer	GeneMark

3.1.6 Antibiotics

Antibiotics were used to grow *Helicobacter pylori* and *E. coli* . The antibiotics were given in Table 3.9.

Table 3.9. Antibiotics

Antibiotic	Supplier Company
Kanamycin	GeneMark
Ampicillin	GeneMark
Trimethoprim	ChemCruz
Amphotericin B	Bristol-Myers Squibb
Vancomycin HCl	Koçak Pharma
Cefsulodin	Koçak Pharma
Polymixin B Sulfate	ChemCruz
β-Cyclodextrin	SIGMA

3.1.7 General biological and chemical materials

General biological and chemical materials that were used in the thesis study are given with the supplier company in Table 3.10.

Table 3.10. General biological and chemical materials with the supplier company

Content	Supplier Company
pET His6 GST TEV LIC cloning vector (1G)	addgene
SspI	New England Biolabs, R0132S
T4 DNA Polymerase	New England Biolabs, M0203S
dNTP (mix)	GeneMark
dCTP	ThermoScientific
dGTP	ThermoScientific
Pfu DNA Polymerase	GeneMark
Taq DNA Polymerase	GeneMark
100bp DNA ladder	GeneMark
1kb DNA ladder	GeneMark
Prestained Protein marker	BIO-RAD, Dual colour (10-250kda) GeneMark (10-180kda)
IPTG	GeneMark
Lactose	SIGMA Life Science
Agarose	NORGEN
50X TAE Buffer	GeneMark
Ethidium Bromide Solution	SIGMA Life Science
Luria-Bertani (LB) Broth	BioShop®
Agar	SIGMA Life Science
Brucella Broth	remel
Columbia Blood Agar Base	OXOID
Glycerol	SIGMA Life Science
B-PER™ Reagent	ThermoScientific
Urea	Merck

Table 3.10. General biological and chemical materials with the supplier company (continued)

Thiourea	Merck
Acrylamide/Bis-acrylamide 30% solution	SIGMA Life Science
Ammonium Per Sulfate (APS)	BioFroxx
Triton® X-100	Merck
Glycine	BIO-RAD
Trizma® Base	SIGMA Life Science
Sodium Dodecycle Sulfate	SIGMA Life Science
4X Laemmli Sample Buffer	BIO-RAD
Bromophenol Blue	BIO-RAD
Brillant Blue G 250	SIGMA Life Science
Ponceau S Solution	SIGMA Life Science
Dry Milk Powder	PINAR
Bovine Serum Albumin (BSA)	SIGMA-ALDRICH
Sodium Cloride	SIGMA-ALDRICH
Sodium Phosphate	SIGMA-ALDRICH
Imidazole	Merck
Hydrocloric Acid Fuming 37%	Merck
Ethanol 100%	Merck
Methanol 100%	Merck
2-Propanol	Merck
Acetic Acid ≥99%	SIGMA-ALDRICH

3.1.8 Equipments

Equipments that used in the thesis, are listed with their supplier company in Table 3.11.

Table 3.11. Equipments with the supplier company

Equipment	Supplier company
Biosafety Class II Cabinet	Thermo Scientific
Thermo Cycler, PCR	BIO-RAD, T100
Water Purification System	Merck Millipore, Milli-Q® Advantage A10
Spectrophotometer	Thermo Scientific
Microplate Spectrophotometer	BioTek, PowerWave XS2
NanoDrop	Thermo Scientific One^{C}
Light Microscopy	Leica DM500
Fluorescent Microscope	ZEISS, AXIO
Imaging System	BIO-RAD, ChemiDoc^{TM} MP
Jar Gassing System	Donwhitley Scientific
pH meter	Thermo Scientific, ORION STAR A211
Observable Real Time Electrophoresis (ORTE)	TIBO, ORTE
Electrophoresis System	MS Measure Science BIO-RAD, Mini Protean
Power Supply	BIO-RAD, Power^{TM} Pac Basic
Mini-Puroteain® Glass Plates	BIORAD, 1mM spacers
Mini-Puroteain® Short Plates	BIO-RAD
TransBlot Turbo^{TM} Transfer System	BIO-RAD

Table 3.11. Equipments with the supplier company (continued)

Ultrasonic Homogenizer	Omni Sonic Ruptor 4000
Microcentrifuges	Thermo Scientific, MICROCL 21R Thermo Scientific, MICROCL 17
Centrifuges	Beckman Coulter™ Allegra 64R Thermo Scientific, SL16R
Vortex	bıoSan Combi-Spin FVL-2400N
Incubator	Thermo Scientific, HERATHERM
Shaking Incubator	Thermo Scientific, MAXQ 4450
Shaker	Witeg
Magnetic Shaker with Heat	Thermo Scientific, CIMAREC
Multi-Rotator	bioSan, MultiBioRS-24
Autoclave	Nüve steamArt
Dry Heat Sterilizer	Nüve FN120
Pipettes	Eppendorf ResearchPlus, Thermo Scientific
Syringe Filter 0.45µm-0.22 µm	sarporius
Pipette Tips	RatioLab®
Serological Pipette Tips	Greiner bio-one
Pipet Controller	Thermo Scientific, Pipet Filler S1
Water Bath	EMCO, ESM-3710
Dry Block Heating Thermostat	BIOSAN, Bio TDB-100
Microwave	SAMSUNG T.D.S
Weighing Machine	UNIBLOC, SHIMADZU UW620H
Fridges	ARCTIKO/ Kirsch

3.2 Methods

3.2.1 Recombinant protein production

3.2.1.1 Cultivation of *Helicobacter pylori*

H. pylori was grown in Columbia blood agar media containing horse blood and selective antibiotics In order to prepare plates, the Columbia agar was dissolved in water according to the proportions specified in the preparation instructions, then autoclaved and equilibrated in a water bath set at 50° C for 1h. 200X and 1000X antibiotic mixtures and freshly prepared β-cyclodextrin wswere added to prevent the growth of bacteria and molds other than *H. pylori.* Horse blood was then added to agar mixture and poured into petri dishes. . To cultivate *H. pylori*, bacteria were spreaded on agar plates and grownat microaerophilic conditions in an automated closed system at 37°C for 3-4 days. The growth and the motility of *H. pylori* was observed under a light microscope. After examination of *H. pylori*, itwas transferred to liquid culture which includesBrucella Broth, Vancomycin and FBS (Table 2.5). *H. pylori* in liquid culture was put into a microaerophilic system at 37° C with shake at 140 rpm for 24 hours. All studies on *H. pylori* were carried out in a laminar flow cabinet in the microbiology laboratory.

3.2.1.2 Amplification of *oipA* gene

To perform ligation independent cloning using pET His6 GST TEV LIC plasmid, oipA specific primers were extended with the cloning sequences which were proposed by the company in the instructions for use. They were designed to transfer the *oipA* gene region of *H. pylori* to the pET His6 GST TEV LIC cloning vector (AddGene) containing GST.

In order to perform ligation independent cloning using aLICator Ligation Independent Cloning and Expression System kit (Thermo Scientific), the primer sequences required to perform the cloning process were designed as specified in the kit instructions. In addition to the primer sequence given in the procedure, the sequence suitable for labeling with the His6 peptide(6xHis) which was not in the vector was added to the primers, to express His peptide on the C terminal of oipA protein. The primer sequences were listed in Table 3.6.

The primer sequences were designed as specified in the kit instructions to perform the cloning process. The primer sequences were added to His6 peptide sequence, which was not included in aLICator LIC Cloning and Expression kit, on the C terminal of oipA protein. The primer sequences were listed in Table 3.6.

To amplify *oipA* gene, genomic DNA of *H. pylori* was isolated from 30ml *H. pylori* liquid culture in this experiment. Genomic DNA was isolated using the gram-negative bacteria-specific protocol proposed by the GeneMark Bacterial DNA

isolation kit. After genomic DNA isolation, the genomic DNA sample was quantified using NanoDrop and stored at -20°C. All studies on *H. pylori* was carried out in a laminar flow cabinet in the microbiology laboratory.

The *oipA* gene sequence was generated using external oipA primers with conventional PCR. Pfu DNA Polymerase is more compatible with the ligation independent cloning method, was used instead of Taq DNA Polymerase. PCR components and reaction conditions were shown in Table 3.12 and Table 3.13.

Table 3.12. PCR assay components

Components	Volume
10X PFU buffer	2,5µl
0.5 mM dNTP	2,5µl
PFU poly.	0,5µl
Forward Primer	0,75µl
Reverse Primer	0,75µl
H. pylori G27 DNA	2µl
DNAse RNAse free Water (up to 25µl)	16,5µl

Table 3.13. PCR reaction conditions

Step	Temperature	Time(min:s)
1. Step	95^{0}C	5:00
2. Step	95^{0}C	0:45
3. Step	59.2^{0}C	1:00
4. Step	72^{0}C	2:00
5. Step	Go to 2. Step	39 cycles
6. Step	72^{0}C	7:00

The *oipA* PCR products were loaded onto 1% agarose gel by using 6X loading dye and Sybr Gold dye. Samples were run at 100V for 50 minutes. Bands were visualized under UV with ChemiDoc (ThermoScientific).

3.2.1.3 Cloning of *oipA* gene

Ligation independent cloning (LIC) was chosen as the cloning method for cloning of *oipA* gene in this thesis study. Both pET His6 GST TEV LIC cloning vector (Addgene) and plate11-Chistag cloning vector were used in LIC optimization studies for cloning of *oipA* gene.

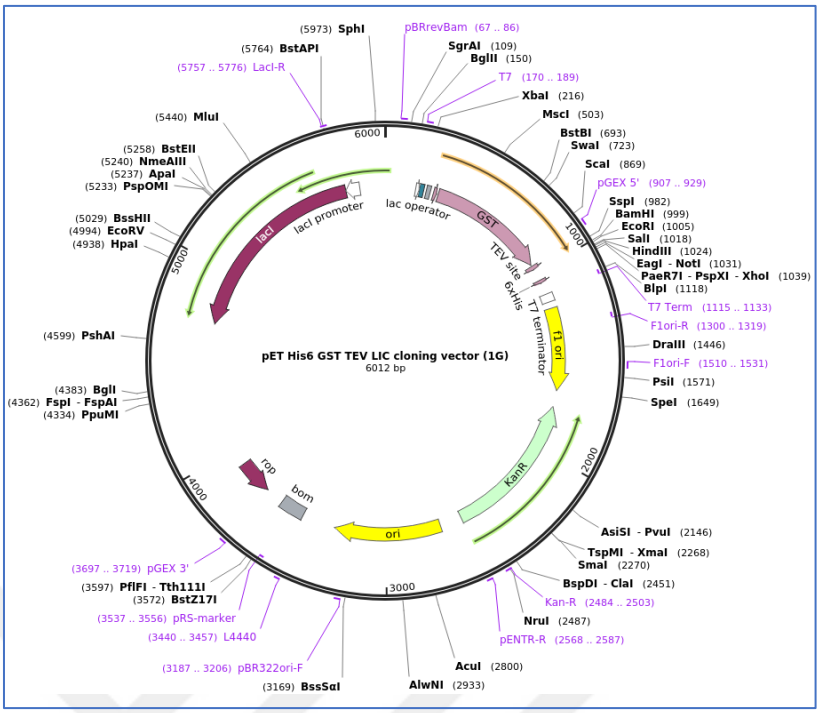

Figure 3.1. The gene map of pET His6 GST TEV LIC cloning vector (91).

In this study, cloning *oipA* gene into pET vector using the LIC method was defined as a cloning strategy 1. Thus pET cloning vector was supplied by Addgene as agar stab-bacteria form. pET cloning vector consists of 6012 bp (Figure 3.1) The pET cloning vector carrying bacteria, were grown on LB agar plate with Kanamycin (50ug/ml) and incubated in an incubator at 37 ˚C overnight. After that, the bacteria were transferred to LB liquid media with Kanamycin (50ug/ml) and incubated with shaking 200rpm at 37 ˚C overnight. Then the pET cloning vector was isolated from liquid culture using miniprep plasmid isolation kit (ZymoResearch) following the protocol recommended by the manufacturer. Plasmid concentrations were measured with NanoDrop (Thermo). To use during the thesis study, the stock culture of bacteria containing pET cloning vector was prepared.The bacteria liquid culture was mixed with autoclaved 50% glycerol in 1:1 ratio and stored at -80˚C.

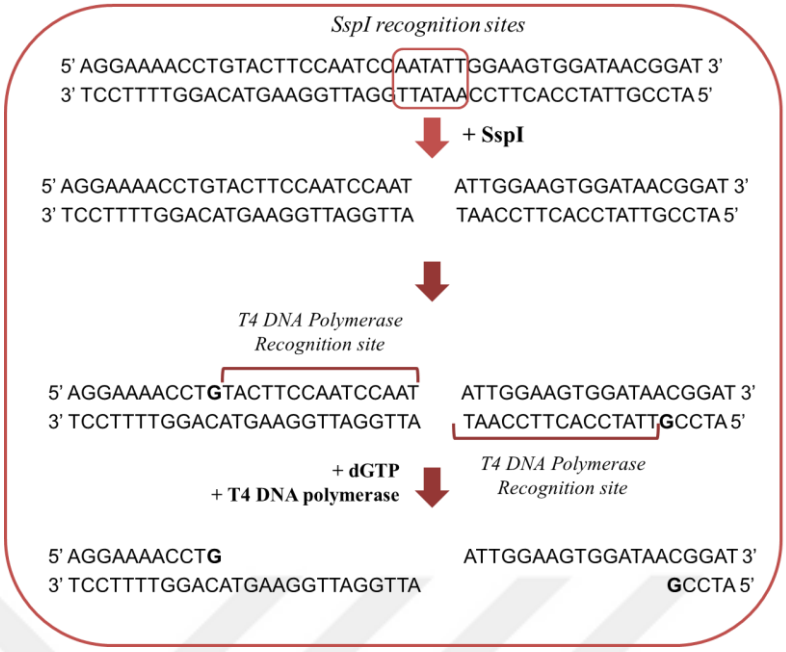

Figure 3.2. Digestion of the vector with SspI enzyme and treatment with T4 DNA polymerase to create the overhangs end.

The pET cloning vector obtained by plasmid isolation is prepared for the LIC method by digestion with SspI enzyme and treatment with T4 DNA polymerase. These preparation steps are summarized in Figure 3.2.

For this, pET cloning vector was digested with restriction enzyme following to LIC steps. In order to perform LIC method, SspI restriction enzyme was recommended by Addgene was the supplier company of pET cloning vector. The vector was treated with SspI enzyme. The reaction was carried out at 37°C for 30 minutes. The reaction was stopped by heating to 75° for 20 minutes. The reaction components were given in Table 3.14.

Table 3.14. SspI enzyme digestion reaction conditions

Component	Volume
SspI Enzyme (NEB)	2 µl
10X NEBuffer	5 µl
Plasmid	2 µg
Distilled water	Up to 50 µl

The vector was cut from 982. base with SspI enzyme and made blunt end and linear. The cut and uncut regions were checked with electrophoresis to confirm the cut and continue the experiment with only linear vectors. For this, The cut and uncut vector were loaded onto 0,8 % agarose gel by using 6X loading dye and Sybr Gold dye. Samples were run at 100V for 50 minutes. Bands were visualized under blue light with ORTE (TIBO). The desired length vector was excised from agarose gel using a lancet. The gel piece was isolated from the Gel DNA Recovery Kit (ZymoResearch). The purify cut vector concentration was measured with NanoDrop. (Thermo)

Table 3.15. T4 DNA polymearease (NEB) reaction components

Components	Volume
10X NEBuffer	5 µl
10mM dCTP for vector	0,5 µl (100mM)
10 mM dGTP for insert	0,5 µl (100mM)
T4 DNA Poly.	1 U/µg
DNA	1 U/µg
Distilled water	Up to 50 µl*

* The reaction volume is changeable depend on the amount of DNA and reaction conditions.

The step of treatment of the pET cloning vector with T4 DNA polymerase is required for the LIC method. (Figure 3.2) The pET cloning vector was treated with T4 DNA polymerase (New England Biolab) enzyme and dCTP with 3 '-> 5' exonuclease activity to form sticky ends suitable for cloning in vector. Reaction components were given in Table 3.15. The reaction was carried out at 12°C for 15 minutes and stopped by heating to 75°C for 20 minutes following to kit instructions. The reaction was set up according to the 1 U T4 DNA polymerase for DNA of 1µg. The reaction was made by the calculating amount of DNA to be used. Optimization studies for T4 treatment step are summarized in Table 3.16.

Table 3.16. T4 DNA polymerase optimization studies.

Reaction conditions indicated in kit instruction
Incubation at 12°C for 15 min
Optimization studies
at 22°C for 30 min
at 25°C for 30 min
at 25°C for 15 min
at 20°C for 30 min

Amplification of *oipA* gene with cloning primers were performed with nested PCR method to prevent non-specific binding of primers to the rest of the sequence. *oipA* PCR product was amplified using external oipA primer, was used as a template DNA for nested PCR. The LIC *oipA* PCR product was generated with cloning oipA primers. The PCR components and conditions were given in Table 3.17 and Table 3.18, respectively.

Table 3.17. PCR with cloning *oipA* primers assay components

Component	Volume
5X PFU Master Mix	5 µl
Forward Primer	0,5 µl
Reverse Primer	0,5 µl
OipA PCR product (1093bp)	3 µl
Distilled water	16 µl

Table 3.18. Cloning *oipA* primers PCR reaction conditions.

Step	Temperature	Time(min:s)
1. Step	94^0C	5:00
2. Step	94^0C	0:45
3. Step	61^0C	1:00
4. Step	72^0C	1:00
5. Step	Go to 2.step	39 cycles
6. Step	72^0C	5:00

LIC *oipA* PCR products were run on 1% agarose gel with agarose gel electrophoresis. LIC *oipA* PCR products were loaded with loading dye and Sybr Gold to stain DNA. And then agarose gel was run at 100V for 50 minutes. After the confirmation of the band, the PCR product (insert) was cleaned with PCR clean up kit (GenemarkBio). The purified insert concentration was measured with NanoDrop (Thermo). LIC *oipA* PCR products were sequenced with Sanger Sequencing to check the accuracy of insert sequence. Sanger Sequencing was carried out as a service purchase.

The preparation steps of *oipA* insert for LIC method were summarized in Figure 3.3. The insert was treated with T4 DNA polymerase (New England Biolab) enzyme with 3' > 5' exonuclease activity to form sticky ends suitable for cloning and dGTP. Reaction components were given in Table 3.15. The reaction was carried out at 12°C for 15 minutes and stopped by heating to 75°C for 20 minutes following to kit instructions. Optimization studies for T4 treatment step are summarized in Table 3.16.

Figure 3.3. Amplification of *oipA* gene with external and cloning primers, treatment of T4 DNA polymerase to form overhang ends.

Prepared vector was mixed with a prepared insert at a molar ratio that was calculated using NEBioCalculator tool(https://nebiocalculator.neb.com/#!/ligation). The mixture was incubated at room temperature for 5 minutes and then 1 ul of 25mM EDTA was added and finally incubated at room temperature for another 5 minutes. The condition is general reaction conditions. Optimization studies for annealing reaction are summarized in Table 3.19.

Table 3.19. Annealing reaction optimization studies.

Optimization Conditions				
1) Incubation at different temperature and time intervals	2) Different vector:insert molar ratio			3) The vector and insert were treated with T4 DNA poly, were incubated at 65°C for 5 minutes and then placed on ice.
at 16°C for 16 hours	1:3	1:6	1:9	
at 25°C for 1 hour	1:4	1:7	1:10	
on ice for 30 minutes	1:5	1:8		

The heat shock transformation method was used to transfer the *oipA* gene cloned vectors to *E. coli DH5α* strain. Competent cell preparation is required for transformation. Competent cells were prepared by $CaCl_2$ and polyethylene glycol (PEG) methods and were used for the thesis study. The preparation steps of the competent cell by $CaCl_2$ are given below.

- Starting Culture: Inoculate 5 ml LB with *E. coli DH5α* strain at 37˚C and 200 rpm for overnight,
- Subculture from starting culture: Add 300 µl starting culture to 30ml LB in 1:100 dilution ratio at 37˚C and 200 rpm until OD600 reaches 0.4,
- Incubate the culture on ice for 30 minutes,
- Centrifuge at 4˚C and 4000rpm for 10minutes,
- Discard the supernatant,
- Resuspend pellet with 20 ml cold 0,1M $CaCl_2$,
- Incubate to resuspended pellet on ice for 30 minutes
- Centrifuge at 4˚C and 4000 rpm for 10 minutes.
- Discard the supernatant.
- Combine pellet by resuspending in 1.5ml 15%glycerol-0.1M $CaCl_2$,

- Add 50 µl aliquots to the chilled microcentrifuge tubes and store at -80˚C.

Preparation of competent cells by PEG method requires Transformation Solution Buffer (TSS) that includes5 g PEG 8000, 1,5 ml 1M sterile $MgCl_2$,2,5 ml DMSO and LB medium up to 50 ml. After preparation TSS buffer pH is adjusted 6.5 and it sterilized using 0,22µm filter and stored at 4˚C. Preparation of competent cell by PEG steps are given below:

- Starting Culture: Inoculate LB with *E. coli DH5α* strain at 37˚C and 200 rpm for overnight,
- Subculture from starting culture: Add starting culture to LB in 1:100 dilution ratio at 37˚C and 200 rpm until OD600 reaches 0.2 – 0.5
- Put the microcentrifuge tube on ice for aliquots.
    - Incubate the culture on ice for 10 minutes.
    - Centrifuge at 4˚C and 3000rpm for 10minutes,
    - Discard the supernatant,
    - Resuspend pellet in cold TSS buffer.
        - TSS volume to use is 10% culture volume.
    - Add 50 µl aliquots to the chilled microcentrifuge tubes
    - Store at -80˚C until further use.

Both competent cells by $CaCl_2$ and PEG were used simultaneously for heat shock transformation that their steps are given below:

- Thaw 50 µl competent cell on ice, carefully.
- Add 2.5 µl annealing mixture to 50 ul competent cells.
- Incubate the cells on ice for 30 minutes.

- Transfer quickly the tube to the water bath previously set at 42˚C. Incubate for 45 seconds and quickly put on ice.
- Add 250 µl LB medium to the tube.
- Incubate at 37˚C and 200 rpm for 60 minutes.
- Spread 100 µl culture on the LB agar containing suitable antibiotics.
- Incubate at 37˚C for overnight.

Cloning results were analyzed from overnight grown colonies with using colony PCR which is used to confirm the presence of the insert DNA for cloned colonies. Colonies were analyzed with pET LIC primers using conventional PCR. Colony PCR contents and conditions are shown in Table 3. 20 and Table 3.21, respectively. Every single colony was picked and resuspended in the prepared PCR mix. The PCR products were confirmed by electrophoresis on 1% agarose gel and stained with TIBO SYBR Gold.

Table 3.20. Colony PCR components for GST-*oipA* gene

Component	Volume
5X Hot Start Master Mix	4 ul
Forward Primer	1 µl
Reverse Primer	1 ul
Distilled Water	Up to 20ul

Table 3.21. Colony PCR conditions

Step	Tempereature	Time(min:s)
1. Step	95^{0}C	3:00
2. Step	95^{0}C	0:30
3. Step	58^{0}C	0:45
4. Step	72^{0}C	1:30
5. Step	Go to 2. Step	38 cycles
6. Step	72^{0}C	5:00

In this study, cloning *oipA* gene into pLATE11 using aLICator LIC Cloning and Expression Kit 1 was defined as a cloning strategy 2. C-His *oipA* PCR products were produced using *H. pylori* genomic DNA as a template DNA and C-His oipA primers in conventional PCR. The PCR components and conditions were given in Table 3.22 and Table 3.23, respectively.

Table 3.22. C-His *oipA* PCR contents

Components	Volume
PFU master mix	4 µl
Forward primer	1 µl
Reverse primer	1 µl
Water	14 µl

Table 3.23. PCR with C-His *oipA* primers conditions

Step	Tempereature	Time(min:s)
1. Step	95^{0}C	5:00
2. Step	95^{0}C	0:45
3. **Step**	58^{0}C	1:00
4. Step	72^{0}C	2:00
5. Step	Go to 2Step	34 cycles
6. Step	72^{0}C	7:00

C-His *oipA* PCR products were run on 1% agarose gel with agarose gel electrophoresis. C-His *oipA* PCR products were loaded into well of agarose gel with loading dye and Syber Gold to stain DNA. And then agarose gel was run at 100V for 50 minutes. After then, the insert with desired length was cut from gel and isolated with the gel DNA recovery kit (ZymoResearch). The purified insert concentration was measured with NanoDrop.

The amount of insert DNA to use in the LIC reaction must be calculated for aLICator LIC Cloning and Expression Kit1. The C-His *oipA* PCR products were calculated the DNA concentration in pmol/µl using the following formula:

$$\text{Number of base pairs} \times 0.65 = \text{ng/pmol}$$

The amount of purified C-His *oipA* PCR products were determined based on calculated insert DNA concentration. The LIC reaction was carried out following the kit instructions. (Thermo). The gene map of pLATE11 vector in the kit is shown in Figure 3.4. Generation of sticky ends of C-His *oipA* sequence with T4 DNA polymerase is shown in Figure 3.5.

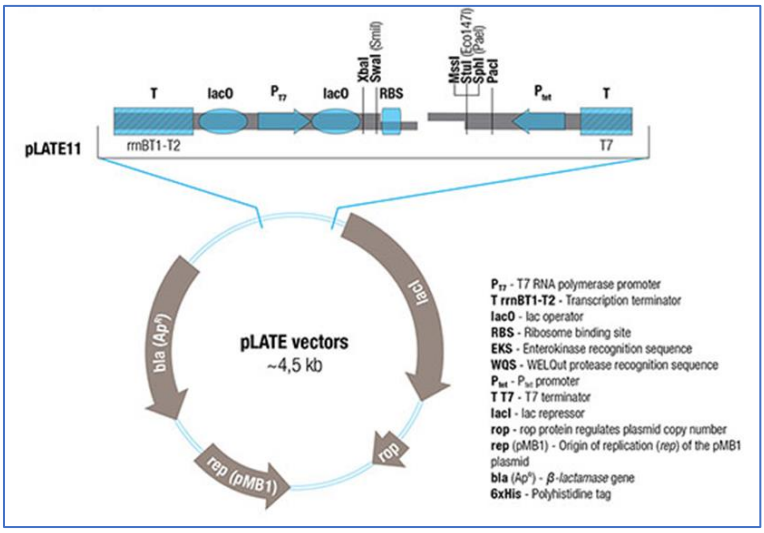

Figure 3.4. Gene map of pLATE11 (92).

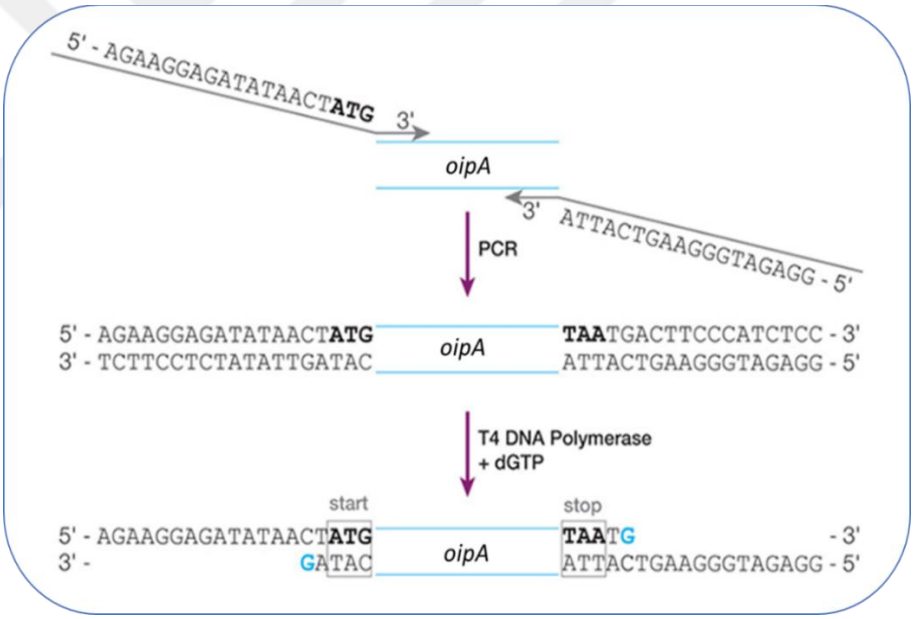

Figure 3.5. Generation of overhangs on the C-His *oipA* sequence in T4 DNA polymerase reaction.

3.2.1.4 Induction of C-His OipA protein for recombinant protein overexpression

Recombinant plasmid including *oipA* gene was transferred to other *E. coli* strain that is required to contain the T7 RNA polymerase gene under the control of the lacUV5 promoter. *E. coli BL21 (DE3), C43 (DE3)* was used for the thesis study. Recombinant plasmid including *oipA* gene was isolated from *E. coli DH5α* containing recombinant plasmid, following the miniprep plasmid isolation kit instructions. (GeneMark) Recombinant plasmid concentration was measured with Nanodrop (Thermo). The recombinant plasmid was transferred to both competent *E. coli BL21 (DE3)* and *C43 (DE3) cell* prepared by $CaCl_2$ method, with heat shock transformation. After transformation, Stock culture was created from recombinant colonies for use in future studies using 50% glycerol in a 1:1 ratio. The expression control plasmid (pLATE31) contained in the kit containing approximately 30kDa of protein was used to check-in each recombinant protein production experiments.

Both *E. coli BL21 (DE3), C43 (DE3)* strains expressing recombinant protein were used for the thesis study. Optimization studies were carried out for induction time and induction temperature. Both *E. coli BL21 (DE3), C43 (DE3)* strains expressing recombinant protein were grown in LB media with antibiotics at 37ºC for 18 hours. After incubation, the grown culture was diluted with LB media in 1:100 ratio and incubated at 37˚C until OD600 reaches 0.6- 0.8. When OD600 value was within the desired range, the uninduced sample was collected from the culture. The final concentration of IPTG was added to the culture medium as 1 mM. After this step, changed parameters are summarized in table 3. 24. At the end of induction, induced samples were collected from each culture and stored at -20˚C for use in protein studies.

Table 3.24. IPTG induction optimization conditions.

Induction Temperature	Induction Time (hour)
37˚C	3 h
30˚C	6 h
25˚C	9 h
20˚C	16 h
16˚C	18 h

Lactose was used for protein expression as an inducer. The both *E. coli BL21 (DE3), C43 (DE3)* strains expressing recombinant protein were used for the thesis study. Optimization studies were carried out for induction time and Lactose concentration. The both *E. coli BL21 (DE3), C43 (DE3)* strains expressing recombinant protein were grown in LB media with antibiotics at 37˚C for 18 hours. After incubation, the grown culture was diluted with LB media in 1:100 ratio and incubated at 37˚C until OD600 reaches 1.0- 1.5. When OD600 value was within the desired range, the uninduced sample was collected from the culture. The induction temperature was kept constant at 25˚C. Changed parameters to induce culture are summarized in table 3. 25. The end of induction, induced samples were collected from each culture and stored at -20˚C for use in protein studies.

Table 3.25. Changed parameters for lactose induction.

Lactose Final Conc.	Induction Time	
30mM	8 h	19 h
15mM	8 h	19 h
3mM	8 h	19 h

3.2.1.5 Total protein extraction with different solutions

The total protein containing recombinant C-His OipA protein was extracted using two different methods. The Bacterial Protein Extraction Reagent - B-PER™ (Thermo Scientific) was used for the first method and thiourea / urea buffer for the second method. In the first method, the total protein containing recombinant C-His OipA protein was extracted from the samples obtained by induction experiments following B-PER™ instructions. All samples were collected as supernatant from culture, soluble protein fraction, and inclusion body – insoluble protein fraction from protein extraction. Insoluble protein fraction was occurred as the last pellet in B-PER™ assay was mixed with additonal B-PER™ solution according to pellet amount. All samples were stored for protein assay.

In the second method, Thiourea/Urea buffer includes 6M urea, 2M thiourea and 2% SDS. Urea and thiourea are chaotropic agents improve to solubilize hydrophobic proteins (93).This buffer should be prepared fresh. The *E. coli BL21 (DE3)* culture which was obtained by IPTG induction experiment was centrifuged at 10.000 g for 10 minutes at 4ºC. The supernatant was discarded. The pellet was dissolved in Thiourea/Urea buffer and incubated for 30 minutes at room temperature. Then the mixture was centrifuged at 16.000 g for 10 minutes. The supernatant was transferred to a new microcentrifuge tube and stored for protein assay. The total protein obtained using thiourea/urea buffer should not be heated above 37°C in SDS PAGE experiments.

3.2.1.6 BCA protein assay

The BCA (bicinchoninic acid) protein assay is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. The Cu^{1+} ion reacts with bicinchoninic acid forming an intense purple-colored reaction product (94). The absorbances are detected at 565nm with increasing protein concentration in a spectrophotometer (Biotech) and the absorbance values obtained compared with the standard curve of BSA protein standard to calculate the protein concentration in mg/ml of sample. The recombinant protein quantification was made by BCA, using bovine serum albumin (BSA) 1mg/ml as standard protein following Pierce™ BCA Protein Assay Kit instructions. Supernatant from culture, soluble protein fraction, and inclusion body – insoluble protein fraction from protein extraction and other protein samples were used for BCA protein assay and their protein concentrations were calculated.

3.2.1.7 Bradford Protein Assay

Bradford protein assay is based on the binding of protein molecules to Coomassie brillant blue under acidic conditions results in a color change from brown to blue. The assay measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex (95). The absorbances are detected generally at 595 nm with increasing protein concentration in a spectrophotometer (Biotech) and the absorbance values obtained compared with the standard curve of BSA protein standard to calculate the protein concentration. Bradford assay does not affect the presence of reducing agents (DTT and beta—mercaptoethanol), metal chelators (EDTA, EGTA) and urea at low concentrations. Protein samples containing incompatibilite with BCA assay are quantificated using Bradfrod assay. For Bradford assay, BSA protein standards were prepared using buffer

containing protein to be measured. 190ul Bradafrod reagent were mixed with 10 ul protein samples in 96 well plate and incubated at room temperature for 5 minutes. Then protein samples prepared were measured the absorbance at 595 nm. Protein concentration was calculated.

3.2.1.8 SDS PAGE

To confirm the presence of proteins extracted, the samples were analyzed by polyacrylamide gel electrophoresis. The content and preparation of 12% (w/v) SDS polyacrylamide gel was given in Table 3.26. The protein samples were diluted with Laemmli Buffer (BioRad) mixture (including 10% beta-mercaptoethanol) in 4:1 ratio, and then heated at 95˚C for 10 minutes. Both the same amount and the same volume of recombinant protein samples were loaded into the SDS PAGE and analyzed. The protein samples were run at 60 - 80 voltages until they come out of the stacking gel and then the voltage was increased to 160.

The most commonly used protein stain is Coomassie Blue staining and is based on Coomassie Brilliant Blue's nonspecific binding to nearly all proteins (96). After running the SDS PAGE, the gel was stained with Coomassie Blue stain for 60 minutes and the stained gel was removed Coomassie Blue stain with Destained Solution (5V H_2O; 4V MetOH; 1V AcOH). SDS PAGE image was visualized using Chemidoc.

For more sensitive detection, silver staining is used as an alternative to Comassie Blue (CBB) staining. Silver staining is approximately 50-100 fold more sensitive than CBB. Silver stainig can detect Silver staining can detect lower nanogram amounts of protein (97). After electrophoresis, staining of SDS PAGE gel with silver was performed following the instructions of the Silver Stain Plus^{TM} kit.

Table 3.26. SDS PAGE; Separating gel, Stacking gel contents

Separating Gel for 10ml		**Stacking Gel for 5ml**	
Components	Volume	**Components**	Volume
Distilled Water	3.2ml	**Distilled Water**	2.975ml
30% Acrylamide Bis-acrylamide	4ml	**30% Acrylamide Bis-acrylamide**	0.67ml
1.5 M Tris HCl pH 8.8	2.6ml	**0.5 M Tris HCl pH 6.8**	1.25ml
10% SDS	0.1ml	**10% SDS**	0.05ml
10% APS	0.1ml	**10% APS**	0.05ml
TEMED	10µl	**TEMED**	5µl

3.2.1.9 Western blot analysis

Protein amounts were equalized to 20 µg, were loaded in 12% SDS-PAGE, and electrophoresis was performed. Samples were transferred from polyacrylamide gel onto the PVDF membrane using a semi-dry transfer system (Biorad) for 1 h at 20V. The membrane was then washed and blocked by incubating in 5% (w/v) milk solution for 1 h under constant agitation in an orbital shaker. The solution is prepared with non-fat dry milk dissolved in 1X TBS at pH 7.4 containing 0.05% (v/v) of Tween 20 (1X TBST). The membrane was washed 3 times for 5 minutes using 1X TBST. The

washed membrane was left overnight at 4˚C with His-tag rabbit antibody, which was selected as the primary antibody and diluted to 1: 2000 with 1X TBST-5% milk solution. The membrane was washed 3 times for 5 minutes using 1X TBST. The washed membrane was left for 1 hour at room temperature with the anti-rabbit IgG antibody selected as secondary antibody and diluted with 1X TBST 5% milk solution to 1: 2500. The membrane was washed 3 times for 5 minutes using 1X TBST. After the washing process, the imaging process started. Increased chemiluminescence horseradish peroxidase (horseradish peroxidase) substrate was used for imaging. Results were taken with UV transilluminator.

For western blot using wet transfer, protein samples were loaded in 12% SDS-PAGE and electrophoresis was performed in the same conditions. Samples were transferred from the gel onto PVDF membrane using a wet transfer system (Biorad) at 4˚C 20V for 16 hours. The next steps were continued as in the semi-dry transfer system.

3.2.1.10 Recombinant protein purification

The protein mixture containing the recombinant protein should be prepared for the purification process, which should be soluble form. Therefore, denaturing conditions must be applied, urea (8M) or guanidine hydrochloride (6M) is used as the denaturing agent, and Tween 20, TritonX-100 is used as a detergent. As a result of the researches, the protocol created below was followed.

A. Total protein extraction

1. Total protein-containing recombinant protein was produced from the bacterial culture in large volume under the optimal induction conditions.
2. Insoluble containing recombinant protein was extracted from the bacterial pellet the B-PER™ (Thermo Scientific) instructions.
3. End of B-PER™ instructions, Insoluble protein mixture pellet was obtained.

B. Inclusion body solubilization

1. Insoluble protein mixture pellet was weighed; the amount of solubility solution containing 2M urea, 500mM NaCl, 2% TritonX-100, and B-PER™ was calculated according to B-PER™ kit instruction and added.

- The solubility solution should be cold.

2. The mixed solution was incubated in the shaker for 30 minutes at room temperature.
3. It was centrifuged at 16.000g for 10 minutes at 4ºC.

- The supernatant was thrown. The pellet remained.

4. The pellet was solved with solubility solution without urea and 3. step was repeated.
5. The pellet mixed with solution 1 containing 20mM Na_3PO_4, 500mM NaCl, 8M Urea and 1mM BME / pH 8
6. The mixed solution was incubated in the shaker for 60 minutes at 30ºC.
7. It was centrifuged at 16.000g for 15 minutes at 4ºC.
8. The supernatant was filtered through a 0.22um / 0.45um filter. The obtained lysate is a dissolved inclusion body and aggregates.

The affinity chromatography method is based on the binding of two molecules, ligand and target protein, in the column matrix on the column. In the protein mixture, the target protein with high binding affinity to ligand binds to the column and those that cannot bind are removed by washing. The target protein is separated from the column with an elution solution containing a higher affinity molecule than the target protein. To purify the C-His OipA protein , the HisTrap Hp column (GE Healthcare), which the His6 peptide is compatible with nickel-containing affinity chromatography method, was used. The recombinant protein was purified by following HisTrap Hp column (GE Healthcare) instructions under denatured conditions. So, the recombinant protein is in primary form (denatured form). For this reason, the recombinant protein must be folded again and reach its bioactive form. The re-folding on the column method was used on the HisTrap column for the re-folding experiment that was based on passing the 6M urea through the column in gradient. Affinity chromatography results were analyzed with BCA, Bradford (Thermo), SDS PAGE, and western blot experiments.

The recombinant protein was purified using PureProteome Nickel Magnetic Beads (MerckMillipore) following this kit instructions under the denatured conditions. Lysis buffer wash buffer and elution buffer were prepared using 8M urea according to kit instructions. Optimization studies were carried out for sample preparation to be purified. Total protein-containing recombinant protein was produced from the bacterial culture in large volume under the optimal induction conditions. The *E. coli* culture in 2 separate falcons was centrifuged at 10,000 x g for 20 minutes. After centrifuge, the supernatant was discarded and pellet in separate 2 falcons was obtained. The pellet 1 was resuspended in denatured conditions with lysis solution (8M urea, 100mM Sodium phosphate, 10mM Tris HCl pH 8) containing 1X protease inhibitor cocktail (brand) recommended by the kit. The Lysate 1 was kept on ice for 30 minutes and centrifuged at 10,000g for 30 minutes 4ºC. Supernatant 1 was transferred to a new falcon and stored to be purified. The pellet 2 was resuspended in B-PER™ containing 0.1% Tween 20, 1X protease inhibitor cocktail (brand), and kept on ice for 30 minutes.

1ml of lysate 2 was taken and stored to be purified. The rest of lysate 2 was centrifugated at 20,000 x g for 15 minutes 4ºC to obtain insoluble protein following B-PER™ kit instructions and called lysate 3. All lysate was used to purify the recombinant protein. Purification using PureProteome Nickel Magnetic Beads, the protocol was given below following kit instructions.

1. Magnetic beads were resuspended by vortexing.
2. 200µl magnetic bead suspension was put into a 1.5 ml microcentrifuge tube.
3. The tube was placed into PurProteome Magnetic stand to collect the beads. Storage buffer was removed with a pipette, carefully.
4. The magnetic beads were resuspended in 500µl lysis buffer containing 8M Urea and incubated with gentle mixing for one minute at room temperature.
5. The tube was placed back into the magnetic stand and removed the buffer.
6. 1 ml of lysate was added to the magnetic beads and incubated with gentle mixing for 30 minutes at room temperature.
7. The tube was placed back into the magnetic stand and removed the lysate
8. The magnetic beads were washed by incubating in 500 µl wash buffer containing 8M urea, with gentle mixing for one minute at room temperature.
9. The tube was placed back into the magnetic stand and removed the Wash buffer.
10. Steps 8 and 9 were repeated two more times.
11. The bound protein was added 100µl elution buffer containing 8M urea and incubated with gentle mixing for two minutes at room temperature.
12. The tube was placed back into the magnetic stand. The eluted protein was transferred into a new microcentrifuge tube.
13. Optional, the elution step could be repeated.

The second method; two pellets obtained from 50 ml *E. coli* culture produced under optimal conditions were used for recombinant protein purification. The pellet 1 was resuspended with 2 ml B-PER^{TM} and added 20 µl 10mg/ml Lysozyme, 2 µl 100% Tween20, 2 µl Dnase1 and 20 µl 100X protease inhibitor cocktail. The lysate1 mixed was kept on ice for 30 minutes. The lysate1 was sonicated on ice. This sonicate cycle was repeated until the lysate became translucent and viscous. The lysate1 was ready to use in sample purification. The pellet 2 resuspended with 2 ml B-PER^{TM} and incubated at room temperature for 15 minutes. The lysate was centrifuged at 20,000 x g for 15 minutes 4ºC. The supernatant was discarded. The Pellet was resuspended with 1.5 ml B-PER^{TM} and added 15 µl 10mg/ml Lysozyme, 1.5 µl 100% Tween20, 2 µl Dnase1 and 15 µl 100X protease inhibitor cocktail. The lysate2 mixture was kept on ice for 30 minutes. The lysate2 was sonicated on ice. This sonicate cycle was repeated until the lysate became translucent and viscous. The lysate1 was ready to use in sample purification. The recombinant protein was purified from lysate 1 and lysate 2 following PureProteome Nickel Magnetic Beads (MerckMillipore) kit instructions. Purification results were analyzed with BCA, Bradford (Thermo), SDS PAGE, and western blot experiments.

3.2.1.11 Passive elution

The purity of the target protein to be obtained from the purification is important for further studies.For that purpose in this study, passive elution was used as a gel recovery technique to increase the purity of the protein. Therefore protein samples obtained from protein purification with magnetic bead system were analyzed by electrophoresis in 12% SDS PAGE. Also prestained protein ladder was used in 12% SDS PAGE for cut the band of interest. After electrophoresis, based on previous western blot results of the OipA protein, the protein band between 35 kda and 25 kda was cut with scalpel from SDS PAGE and placed in microcentrifuge tube. Elution

buffer containing 50 mM Tris-HCl, 150 mM NaCl pH 7.5 , was added to the gel pieces to cover them completely. After protease inhibitor cocktail was added to mixture. The gel pieces were crushed using pestle and incubated overnight at 30°C and 220 rpm. Then, they were centrifuged at 10,000 × g for 10 minutes and supernatant was transferred to a new microcentrifuge tube. Silver staining and western blot were used to verify passive elution assay result.

3.2.1.12 LC-MS/MS of recombinant C-His OipA protein

After protein purification, LC-MS/MS service was purchased to ensure OipA was obtained. Therefore protein samples were analyzed by electrophoresis in 12% SDS PAGE. And then without staining, the protein band was cut from the gel according to the protein ladder and preious western results and placed in a microcentrifuge tube. The gel pieces were analyzed with LC-MS/MS.

3.2.13 Refolding of recombinant protein

The purified recombinant C-His OipA protein was in its denatured form in the end of the purification. It needed to be folded again and have biological activity. The refolding experiment was applied to the pure protein obtained. The refolding experiment was carried out using the dialysis process.

The refolding method was required to 0.1 mM DTT and cold refolding buffer containing 20mM Tris HCl pH 8.5. The volume of the refolding buffer was used up to 200 times the recombinant purify protein volume. The refolding experiment occurred in two steps that in the first step, the refolding buffer and 0.1 mM DTT were used. Recombinant purified protein was transferred to the dialysis membrane which its length was calculated following dialysis supplier company instructions.

The refolding experimental steps using the dialysis method started with the preparation of 1M Tris HCl pH 8.5 solution and 100 mM DTT. A solution containing 20mM Tris-HCl and 0.1mM DTT up to 200 times the volume of protein contained in the dialysis membrane was placed in the beaker. This solution was changed 2 times with an interval of 3 hours. The solution was then replaced with a cold solution containing only 20mM Tris-HCl twice at 3 hours intervals 4ºC, and the protein in the dialysis membrane was centrifuged at 10,000g for 10 minutes 4ºC. Vacuum device (ThermovSpeedVac Concentrator) was used to concentrate on the obtained protein.

3.2.2 The obtaining of human gastric cancer cell membrane proteins

3.2.2.1 Cell culture of human gastric cancer cell line AGS

Human gastric cancer cell line AGS (ATCC® CRL-1739™) was obtained from American Type Culture Collection (ATCC). The AGS cell line was grown in an incubator containing 5% CO2 at 37˚C in the Roswell Park Memorial Institute (RPMI) medium containing 10% Fetal bovine serum (FBS) and 1% Penicillin Streptomycin.

3.2.2.2 Membrane protein extraction from AGS cell line

Membrane protein extraction from around 5x10^6 AGS cells was performed following the MEM-PER™ eukaryotic membrane protein extarction reagent kit instructions. The MEM-PER™ kit is used to extract membrane proteins from mammalian culture using a mild detergent mixture. The procedure is briefly that the mammalian cells are lysed with first detergent, then treated with a second detergent to dissolve membrane proteins. The protein mixture is incubated at 37 ° C to separate hydrophobic proteins from hydrophilic proteins. In phase partitioning, while the hydrophobic fraction containing membrane protein is located at the bottom layer, the hydrophilic fraction is located at top layer. Membran protein obtained was visualized by silver staining on SDS PAGE. It was stored at -80˚C for use in downstream studies. Total protein extraction from AGS cells was carried out following the RIPA lysis and extraction buffer kit instructions. Total protein of AGS cells was obtained to check the membrane protein extraction and visualized by silver stainng on SDS PAGE..

3.2.2.3 Acetone precipitation

The membrane protein obtained with the MEM-PER™ kit was in a solution containing several different detergents. The acetone precipitation method was performed to remove these detergents based kit from membrane proteins. The acetone precipitation is popular and common method for removing undesirable substances, precipitation and concentration of protein (98). For this method, Thermo Scientific's acetone precipitation protocol was applied (99). The procedure is briefly that at least three volumes of ice-cold acetone is added to the protein. It is incubated for at least 1 hour at -20ºC to precipitate the proteins and then centrifuged. In obtained pellet, the residual acetone is allowed to evaporate.The pellet is dissolved in buffer appropriate

for the downstream process. The membrane protein pellet obtained with this protocol was dissolved in 4X Laemmli buffer including BME. The membrane protein precipitated was visualized by silver stainng on SDS PAGE.

3.2.3 The discovery of OipA binding partner

3.2.3.1 Pull down assay

Pull down assay is well established method to identify protein-protein interaction. Pull down assay uses a bait protein bound to beads in a tube to capture protein interaction partners. So, pull down assay is a kind of affinity chromotagraphy in terms of principle of protein-protein interaction (100). For pull down assay in the thesis, refolded C-His OipA protein was used as bait protein; AGS membrane protein was used as a prey protein. Nickel magnetic bead was used to immobilize bait proteins. Pull down assay was made by magnetic bead purification system under the native conditions (Figure 3.6). According to magnetic bead purification kit instruction, Nickel Magnetic bead was activated with equilibration buffer. Refolded C-His OipA was added to magnetic bead to bind and incubated at room temperature for 30 minutes. AGS membrane protein was mixed with refolded C-His OipA-magnetic bead and incubated at room temperature for 30 minutes and at 4°C for 2 hours. After this step pull down assay were continued following magnetic bead purification kit instruction. The samples were collected from every steps and stored. The volume of buffer and magnetic bead are changeable according to volume of protein samples to be used.

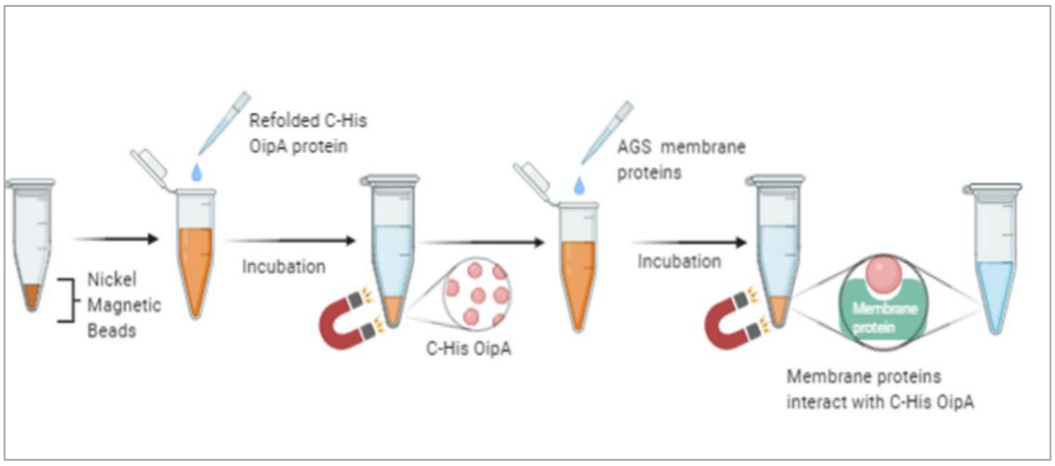

Figure 3.6. Steps of pull down assay using C-His OipA and AGS membrane proteins in the magnetic bead protein purification system.

3.2.3.2 Native PAGE

Protein electrophoresis was performed under native conditions in order not to disturb the interaction of proteins obtained from the pull down assay. For this, the native gel was prepared by omitting SDS from the SDS PAGE preparation recipe indicated in Table 3.26. Running buffer was prepared without SDS. 2X native sample buffer was prepared containing 62.5 mM Tris-HCl, pH 6.8 , 40% glycerol and 0.01% Bromophenol Blue (101). The samples were mixed with sample buffer at a ratio of 1: 1 and separated by native PAGE. It was analyzed by wesrtern blot and silver staining.

3.2.3.3 Blue Native PAGE

The pI of OipA protein is 9.9 and its net charge is usually positive due to the pH of the solutions it contains. OipA protein must be run with a negative charge under native conditions. That's why it was decided to use Blue Native PAGE. Blue native PAGE is a protein discontionus electrophoretic system that provides high resolution separation of membrane protein in a natural conformation, enzymatically active state (102). The principle of separation is the binding of Coomassie blue G250, which provides negative charges to the protein. While migrating to the anode, protein complexes are separated by molecular mass/size (103).

In this thesis study, changes were made to the commonly used blue native PAGE protocol. For this, 10% native polyacrylamide gel was prepared and the recipe was given in the Table 3.27. The Mini-PROTEAN 3 cell system was used for Blue Native PAGE. This system ensures that no two different buffers get mixed with each other. Blue Native PAGE contains two different buffers, anode and cathode. 10X native running buffer was prepared without SDS. 5% (w/v) Coomassie Blue G250 was prepared with distilled water. Anode buffer was formed by diluting 10X native running buffer to 1X. Cathode buffer was prepared by adding 5% Coomassie Blue G250 to the anode buffer at a final concentration of 0.02% 4X sample ponceau S buffer was prepared containing 200 mM Tris HCl, 200 mM NaCl, 40% w/v Glycerol, 0.004% Ponceau S, pH 7,2. 2X native sample buffer was used for this assay. While preparation of protein sample to Blue Native PAGE, protein samples were mixed with sample buffer at desired ratio and 2.5 µl 5% Commassie Blue G250 in 25 µl total volume. The Mini-Protean 3 cell system was set up with native polyacrlamide gel. The inner chamber was filled with cathode buffer and the outer chamber was filled with anode buffer. Prepared protein samples were loaded into native polyacrylamide gel at 4°C.

Blue Native PGE system were run at 70 voltages until they come out of the stacking gel and then the voltage was increased to 130V.

Table 3.27. 10% Native polyacrylamide gel preparation

10% Separating Gel for 10ml		**4%Stacking Gel for 5ml**	
Components	Volume	**Components**	Volume
Distilled Water	4ml	**Distilled Water**	2.975ml
30% Acrylamide Bis-acrylamide	3.3ml	**30% Acrylamide Bis-acrylamide**	0.67ml
1.5 M Tris HCl pH 8.8	2.6ml	**0.5 M Tris HCl pH 6.8**	1.25ml
10% APS	0.1ml	**10% APS**	0.05ml
TEMED	10µl	**TEMED**	5µl

After electrophoresis, native polyacrylamide gel was obtained with CBB stained. and protein bands were visualized. To stain with silver, the obtained gel was destained in destaining solution overnight and then performed silver staining. For western blot, the gel was transferred using optimized wet transfer protocol. Then, the PVDF membrane was washed three times with water, destained with 100% methanol and washed three times with 1X TBST. The western blot was proceed with optimized western blot protocol.

3.2.3.4 LC-MS/MS Analysis

The obtained protein sample from pull down assay was analyzed with LC-MS/MS. The LC-MS/MS results were examined using protein classification tools such that PANTHER, STRING. The network map of LC-MS/MS results was analyzed using CytoScape application.

4. RESULTS

4.1 Recombinant Protein Production

4.1.1 Cultivation of *Helicobacter pylori*

Helicobacter pylori was grown on Columbia agar. Grown bacteria is transferred to *H. pylori* specific liquid culture under optimized conditions. *H. pylori* genomic DNA was isolated from the liquid culture and analyzed with NanoDrop (Thermo). The genomic DNA concentration was given in Table 4.1.

Table 4.1. *H. pylori* DNA concentration

Sample	Concentration ng/µl	A260/280	A260/230
H.pylori DNA	24.6	1.95	2.08

4.1.2 Amplification of *oipA* gene

The *oipA* gene was amplified with external oipA primers using PCR. After PCR *oipA* gene products were visualized on 1% agarose with electrophoresis. The gel results are shown in Figure 4.1.

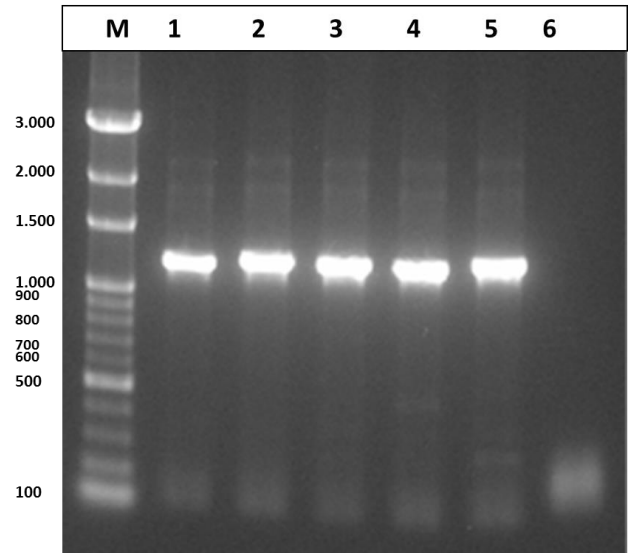

Figure 4.1. The *oipA* gene PCR gel result.

Lane M, 100bp-marker (GeneMark), Lane 1, 2, 3, 4 and 5 *oipA* PCR product (1093bp), Lane 6 negative control.

4.1.3 Cloning of *oipA* gene

4.1.3.1 Cloning strategy 1

Cloning strategy 1 is defined as the strategy that includes the cloning of oipA PCR fragment (964bp) into pET vector by LIC method. Bacteria containing pET vector was produced on LB agar with kanamycin and transferred to LB media with kanamycin. After producing the bacteria, pET vector was obtained by using a miniprep plasmid isolation kit (GeneMark). The isolated pET vector DNA concentrations were measured with Nanodrop (Thermo). NanoDrop results are given in Table 4.2.

Table 4.2. pET vector DNA concentrations

Sample	Concentration ng/µl	A 260/280	A260/230
pET vector 1	193.3	2.03	1.56
pET vector 2	126.6	1.95	1.61
pET vector 3	110.1	1.92	1.32

SspI restriction enzyme digestion were done for 1 µg DNA from each pET vector (1-2-3) DNA samples. Digested pET vectors were visualized with agarose gel electrophoresis by using 0.8% agarose. Gel results are shown in Figure 4.2. According to gel results, pET vector 2 and 3 DNA digestion were better than the pET vector 1 so digested pET vector 2 and 3 DNAs were isolated from the gel with the Gel recovery kit (GenemarkBio) and after measured with NanoDrop (Thermo). NanoDrop results are given in Table 4.3.

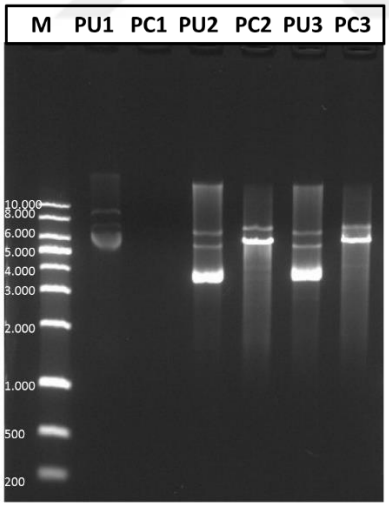

Figure 4.2. Digested pET vectors gel result.

PU is a representative uncut plasmid, PC is cut plasmid with SspI. PU1 is uncut pET vector 1, PC1 is cut pET vector 1, PU2 is uncut pET vector 2, PC2 is cut pET vector 2, PU3 is uncut pET vector 3, PC3 is cut pET vector 3.

Cloning *oipA* sequences were amplified with Nested PCR, which *oipA* PCR products were used as a templated DNA. The PCR products were cleared from non-internal dNTPs, primers, DNA polymerase and contaminating DNA using the PCR clean up kit (GeneMark). The PCR results are shown in Figure 4.3 and cleared PCR products are given in Table 4.3.

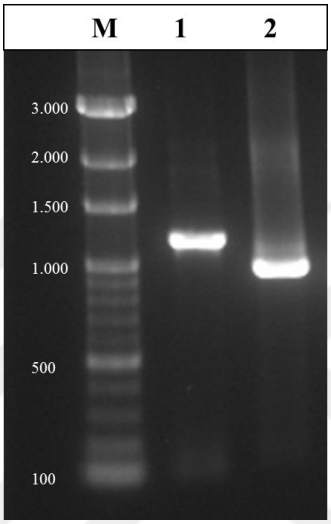

Figure 4.3. Nested PCR gel result.

Lane M is 100bp marker (GeneMark), Lane 1 is *oipA* PCR product with using external oipA primers(1093bp), Lane 2 is cloning *oipA* PCR product with using cloning oipA primers (964bp).

Table 4.3. DNA concentrations of purify cut pET vectors and cloning oipA PCR products with Nanodrop.

Sample	Concentration ng/µl	A260/280	A260/230
Cut pET vector 2	12,3	1,94	0,88
Cut pET vector 3	9,2	1,90	0,27
Cloning oipA PCR product	26,5	1,85	0,55

SspI digested pET vector DNA and cloning *oipA* insert were separately treated with T4 DNA polymerase (NEB) under different reaction conditions specified in Cloning of *oipA* gene (M&M 3.2.1.3). Then plasmid and insert were annealed under different reaction conditions. The 2.5 µl of annealed vector and insert mixture were transferred to competent *E. coli DH5α* prepared both by $CaCl_2$ and PEG simultaneously. The uncut pET vector was used as a transformation positive control and the cut pET vector was used as a transformation negative control at each experiment to check transformation. At the end of the cloning process, colonies existed at positive control and negative control and assay samples. The colony PCR was made using pET LIC primers from obtained colonies. According to colony PCR results, the recombinant oipA sequence (1434bp) was not observed and 500bp of pET plasmid sequence as a negative result was observed on the gel. Transformation with competent cells prepared by the $CaCl_2$ method was obtained more effective results. Successful results could not be obtained with the Cloning Strategy 1.

4.1.3.2 Cloning strategy 2

Cloning strategy 2 was included C-His *oipA* insert and pLATE11 according to aLICator LIC Cloning and Expression kit. C-His *oipA* insert (976bp) was amplified with C-His *oipA* primers using conventional PCR. The PCR results were performed with agarose gel electrophoresis that the gel result is shown in Figure 4.4.

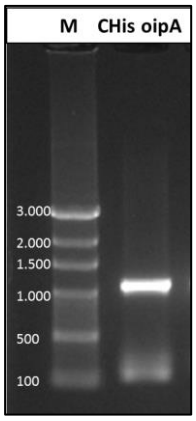

Figure 4.4. Chis *oipA* PCR products gel result.

Lane M, 100bp marker (GeneMark), Lane Chis oipA, PCR product (976bp).

C-His *oipA* PCR products were purified from agarose gel and measured as a 19ng/µl concentration with NanoDrop (Thermo). The amount of insert to be used was calculated following the formula in the kit which is "number of base pairs × 0.65 = ng/pmol" and the result of the equation was;

976bp x 0.65=ng/0.1pmol → 63.44ng C-His oipA DNA

63.44 ng C-His *oipA* PCR product was required to T4 DNA polymerase reaction. 3.4 µl C-His *oipA* PCR product was used. The control PCR fragment was used as a positive control to check the T4 DNA polymerase reaction. T4 DNA polymerase treatment and annealing reactions were carried out according to the kit instructions. pLATE31 Was a positive transformation control in the experiment. The samples were transferred to competent *E. coli DH5α* prepared by $CaCl_2$.

After transformation, colonies were observed in LB-AMP agar plates that belongs to positive control insert and pLATE31. But the plates that inoculate by the C-His *oipA*-pLATE11 transformation was not contain any colony. Colony PCR was made to the colonies seen on the control plate to determine which of the experimental steps could be associated with the transformation problem of the C-His *oipA*-pLATE11. Obtaining the expected size of the product (898 bp) as a result of PCR showed that

there were no problems in the T4 DNA polymerase treatment, annealing reaction, and gene transfer experimental steps. As optimization of the experimental steps, different vector: insert molar ratios were tried and the desired result could not be obtained. Thereupon, a detailed literature review was made. An article encountered as a result of the literature review gave an idea that the problem experienced in the experimental procedure may be caused by secondary structures that may occur in the DNA fragment of the insert sequence to be transferred. As stated in the article, occurred secondary structures may affect the activity of T4 DNA polymerase and the problem can be solved by trying different T4 DNA polymerase activity temperatures (104). Based on this information, the C-His *oipA* and control PCR fragments were analyzed by UNAfold "Unified Nucleic Acid Folding" program in a computer environment to eliminate cloning problems. (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form). The folding profiles of the insert DNA fragments are shown in Figure 4.5.

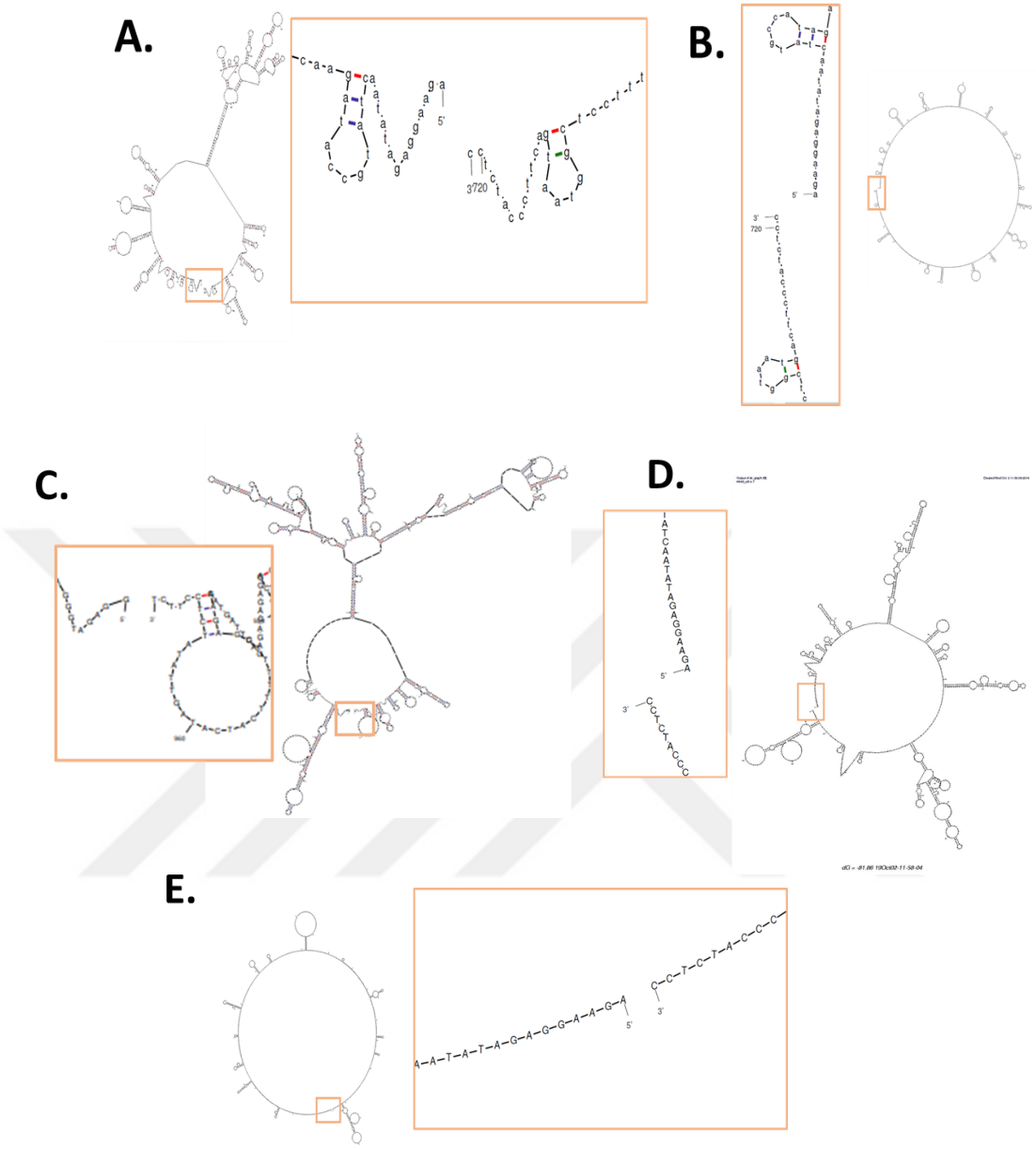

Figure 4.5. UNAfold analysis of C-His *oipA* and control gene sequences at different temperatures.

A is control sequence at 25˚C, **B** is control sequence at 50˚C, **C** is C-His *oipA* sequence at 25˚C, **D** is C-His *oipA* sequence at 37˚C, E is C-His *oipA* sequence at 50˚C.

The experimental procedures were revised, and T4 DNA polymerase experiments were optimized by trying different temperatures and times that were at 25˚C for 5 minutes, at 37˚C for 2 minutes and at 50˚C for 30 seconds. As a result of the optimization experiments, 1: 7 vector: insert molar ratio and T4 DNA polymerase activity were found to be successful at 50˚C for 30 seconds. After transformation,

colonies were observed in the control and test LB-AMP agar plates. The desired size of C-His *oipA* product was obtained as a result of the colony PCR (1154bp). Gel results of the products are shown in Figure 4.6. Plasmid isolation was made from the obtained colonies and PCR was made with the control primers included in the kit. PCR results were confirmed that the *oipA* gene was cloned and the sample was sent for the Sanger sequencing analysis.The Sanger sequencing result of cloned C-His *oipA* is shown in Figure 4.7

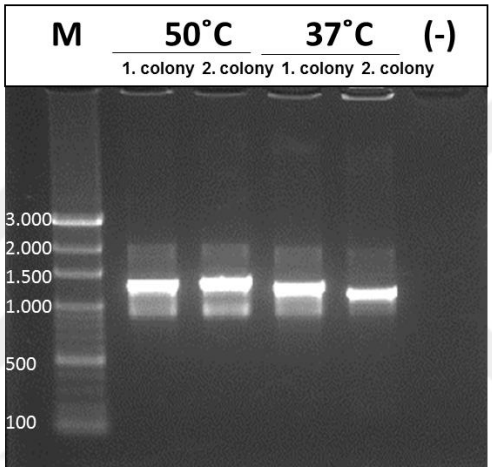

Figure 4.6. The gel result of products obtained with Colony PCR after cloning.

Lane M is 100bp marker (GeneMark), Lane 2 and 3 are that colonies obtained as a result of cloning performed at 50˚C, Lane 4 and 5 are that colonies obtained as a result of cloning performed at 37˚C, Lane 6 is a negative control for colony PCR.

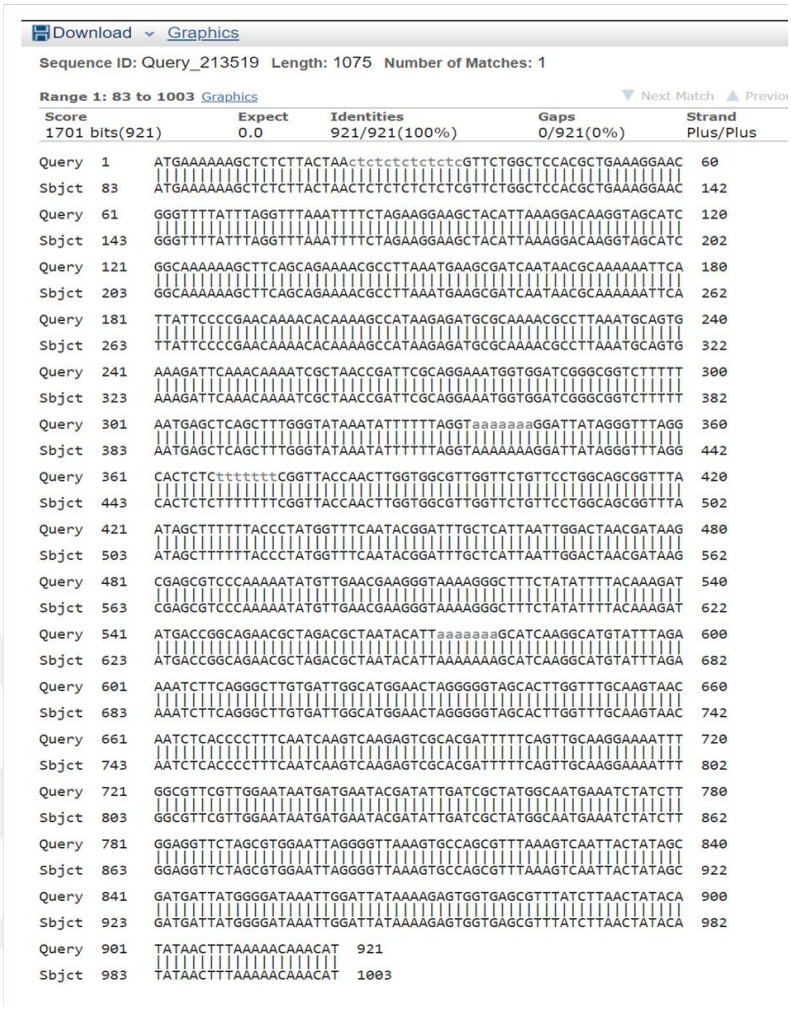

Figure 4.7. Sanger sequencing results of C-His *oipA* after cloning.

Query is *H. pylori* G27 *oipA* sequence, Sbjct is C-His *oipA* sequence obtained after cloning.

4.1.4 Induction for recombinant protein overexpression

The following protein experiments were continued with OipA protein-tagged 6XHis on C terminal. So the recombinant pLATE11-C-His *oipA* plasmid was used for recombinant protein production. The signal peptide sequence of the C-His OipA protein recombinantly produced in *E. coli* is truncated. Therefore, while normally the molecular mass of OipA protein is about 34 kdA, the recombinant C-His OipA is about 32kDa.

E. coli BL21 (DE3) and *C43 (DE3)* strains were used to find the most effective result of recombinant protein production. The recombinant plasmid which obtained by cloning was transferred to both of *E. coli BL21 (DE3)* and *C43 (DE3)* competent cells prepared by $CaCl_2$ method to produce recombinant protein. The successful result of the transformation was achieved. After transformation, stock culture was made from obtained colonies for further studies.

E. coli BL21 (DE3) and *C43 (DE3)* strains were used to produce recombinant protein. Lactose and IPTG induction methods were performed to overexpression. Optimization studies were carried out with different induction methods and host strains. There is no detailed information about the structure of OipA protein in the literature so when the OipA protein was produced, it was not known whether, it was in the soluble, insoluble, or extracellular protein fraction. Therefore after the induction, extracellular supernatant and both soluble and insoluble fraction of the protein were collected and analyzed by SDS PAGE and western blot.

In the first try, recombinant C-His OipA protein was expressed in *E. coli BL21 (DE3)* strain at different temperatures and times by adding 1 mM final concentration of IPTG, according to IPTG induction method. The samples were taken from induced cultures at 37 ˚C for 3 hours, at 30 ˚C for 6 hours, at 25 ˚C for 9 hours and 20 ˚C for 16 hours. The same treatments were done for the control protein (30kDa). The samples which was included extracellular supernatant and total protein, were extracted total protein with B-PER™. The two-group protein was quantified with BCA protein assay. According to the protein concentration results, protein amounts were equalized to 20 µg and then performed on 12% SDS PAGE and western blot. (Figure 4.8).

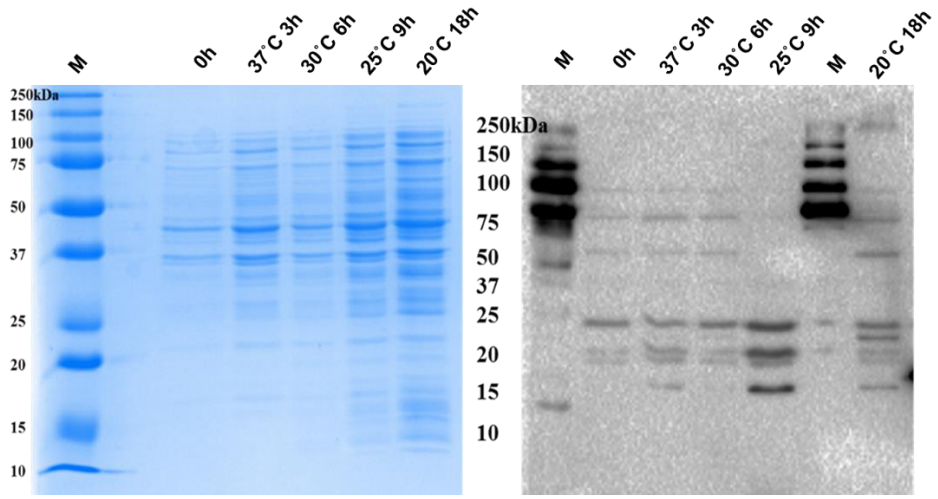

Figure 4.8. SDS PAGE and Western Blot of IPTG induction of C-His OipA expression with different temperatures and times.

M is pre-stained protein marker (Bio-Rad), 0.h is total protein from pre-induction control, 37°C 3h is the total protein taken at the end of induction at 37°C for 3 hours, 30°C 6h is the total protein taken at the end of induction at 30°C for 6 hours, 25°C 9h is the total protein taken at the end of induction at 25°C for 9 hours and, 20°C 18h is the total protein taken at the end of induction at 20°C for 18 hours.

At the end of the first try, extracellular protein samples from both control and C-His OipA group were analyzed with SDS PAGE and protein bands were not observed in the gel. When Control total protein samples were examined, overexpression was detected by IPTG induction for control protein in the 30kDa band. The optimal result could not be obtained for the C-His OipA protein, which has a molecular mass of about 32 kDa in the gel.

In the second try, *E. coli BL21 (DE3)* and *C43 (DE3)* strains expressing C-His OipA were used to IPTG induction at 37°C for 4 hours. Before adding IPTG (1mM), the 10 ml uninduced samples were taken from both groups and centrifuged at 10,000 g for 10 minutes. Supernatant and pellet were stored at -20°C. After 4 hours of IPTG induction at 37°C, the 10 ml induced samples were taken from both groups and centrifuged at 10,000 g for 10 minutes. Supernatant and pellet were stored. The both uninduced and induced pellets were resuspended in 500 µl B-PER™, final

concentration of 0.1mg/ml Lysozyme and 0.1% Tween20 were added and incubated on ice for 30 minutes. Each protein mixture was sonicated on ice. The samples were taken from each protein mixture as a total protein. Each protein mixture was centrifuged at 4˚C and 20,000 g for 15 minutes. The supernatant was stored as a soluble protein fraction. The obtained pellets were resuspended in 200 µl B-PER^{TM}, final concentration of 0.1mg/ml Lysozyme, and 0.1% Tween20 were added and incubated on ice for 30 minutes. The mixture was not applied to sonication since the mixture was not in viscous form. The obtained protein mixture was called as an insoluble protein fraction. All samples that were supernatant, total protein, soluble and, insoluble protein fraction were analyzed with SDS PAGE and western blot. (Figure 4.9)

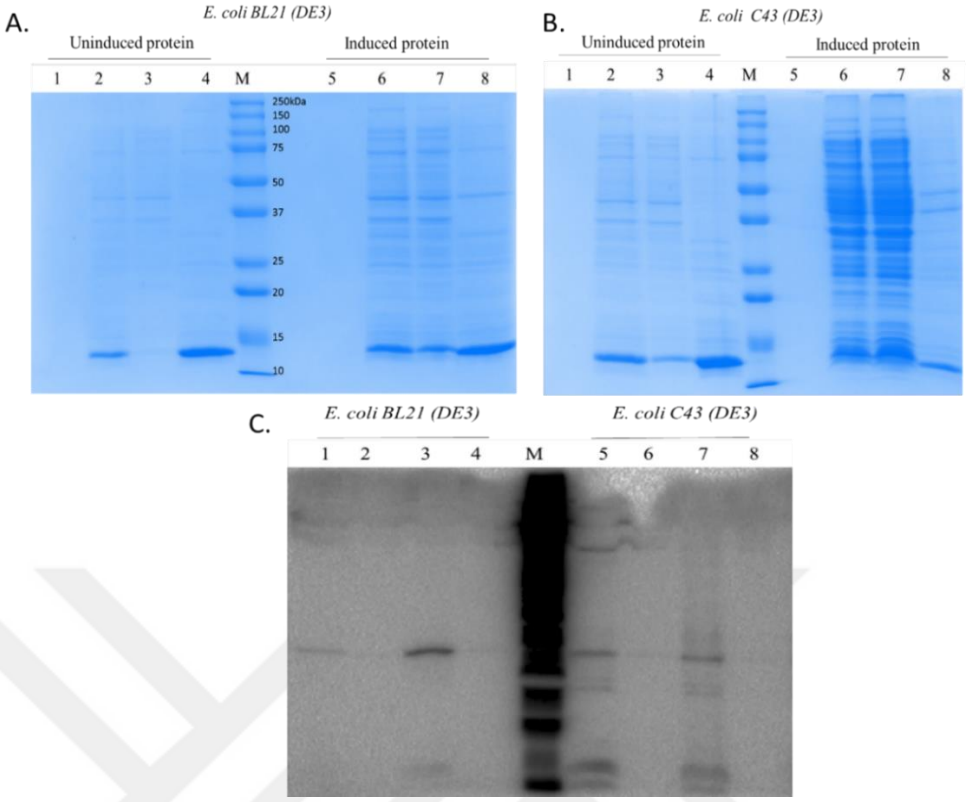

Figure 4.9. SDS PAGE and western blot results of IPTG induction at 37°C for 4 hours with C-His OipA expression in *E. coli BL21 (DE3)* and *C43 (DE3)*.

A is SDS PAGE result of *E. coli BL21 (DE3)* group; lanes 1, 2, 3, and 4 are induced supernatant, total protein, soluble protein, and insoluble protein, respectively. M is a pre-stained protein marker (Bio-Rad). Lane 5, 6,7, and 8 are induced supernatant, total protein, soluble protein, and insoluble protein, respectively. **B** is the SDS PAGE result of the *E. coli C43 (DE3)* group. The sample order is the same as A. **C** is the western blot results of two groups. Lane 1, 2, 3 and 4 of *E. coli BL21 (DE3)* are uninduced total protein and soluble protein, induced total protein and soluble protein, respectively Lane 5, 6, 7 and 8 of *E. coli C43 (DE3)* order is same as *E. coli BL21 (DE3)* group.

When results obtained from SDS PAGE and western blot data were investigated and the induced protein samples stronger protein profile than uninduced protein samples is noticed. The supernatant was not given protein band in gel and insoluble protein was not observed overexpression of C-His OipA. However, expression of C-His OipA protein in the each of total protein and soluble protein around the 32kDa band was observed. So, total protein, soluble induced and uninduced protein samples were analyzed in western blot to check SDS PAGE results. The desired protein band size (32kDa) was shown that C-His OipA protein was detected with rabbit His tag antibody. When E. *coli BL21 (DE3)* and *C43 (DE3)* were compared, samples of *E. coli*

C43 (DE3) were given better protein profile and seen more expression of C-His OipA protein in desired protein band size according to SDS PAGE results. However, total protein sample of E. *coli BL21 (DE3)* was given a stronger band in the expression of C-His OipA protein according to western blot results. It was decided to use total protein for subsequent induction studies.

In the third setup, recombinant C-His OipA protein was expressed in both *E. coli BL21 (DE3)* and *C43 (DE3)* strains by adding 1 mM final concentration of IPTG, at different temperatures and times which was at 37 ˚C for 4 hours and 6 hours, at 30 ˚C for 6 hours, at 25 ˚C for 8 hours, 22 ˚C for 16 hours and at 20 ˚C for 18 hours. Before adding IPTG, 10 ml of uninduced samples were taken from both bacteria cultures. After induction, 10 ml of induced samples were taken from each culture. Total protein was extracted by following the total protein extraction protocol (second setup). Total protein samples were performed on western blot. (Figure 4.10)

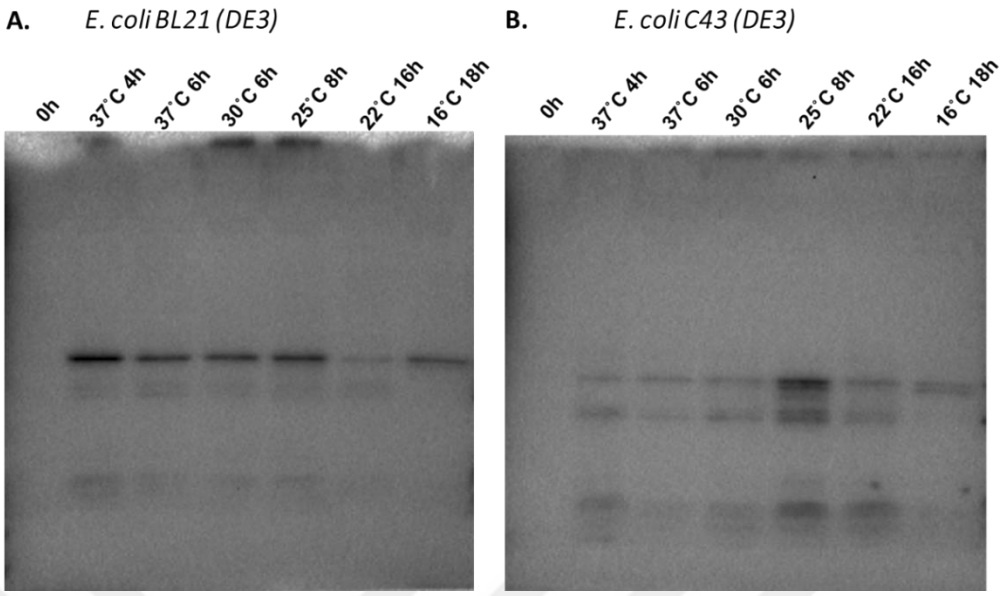

Figure 4.10. Western Blot results of total protein of IPTG induction at different temperatures and times with C-His OipA expression in E. *coli BL21 (DE3)* and *C43 (DE3)*.

A is *E. coli BL21 (DE3)* total protein samples, 0.h is an uninduced sample, 37˚C 4h is a total protein with induction at 37˚C for 4 hours, 37˚C 6h is total protein induction at 37˚C for 6 hours, 30˚C 6h is total protein induction at 30˚C for 6 hours, 25˚C 8h is total protein induction at 25˚C for 8 hours, 22˚C 16h is total protein induction at 22˚C for 16 hours, 16˚C 18h is total protein induction at 16˚C for 18 hours. **B** is *E. coli C43 (DE3)* total protein samples, the sample order is the same as A.

When the western blot results were examined, total protein samples obtained by induction from *E. coli BL21 (DE3)* culture at different temperatures and times were determined clearly result than that obtained from *E. coli C43 (DE3).* Total protein samples of *E. coli BL21 (DE3)* were seen more expression of C-His OipA protein in the 32kDa protein band. It has been shown that the best result for overexpression of C-His OipA protein was obtained with IPTG induction at 37˚C for 4 hours in *E. coli BL21 (DE3)* culture. The induction at 37˚C for 4 hours in *E. coli BL21 (DE3)* culture determined as an optimized condition for recombinant protein purification.

4.1.5 Increasing the solubility of the C-His OipA protein

The C-His OipA protein was overexpressed under the optimized induction conditions. The pellet containing induced C-His OipA protein was extracted with both B-PER™ and thiourea/urea solutions. Total protein extraction was started with B-PER™. For this, 50ml of *E. coli DH5α* culture pellet was dissolved with 2ml of B-PER™ and incubated on ice for 30 minutes. At the end of the incubation, observed that the viscosity of the mixture was very high. B-PER™ protocol has been revised as a result of research and optimization studies to reduce viscosity. By revision, final concentrations of 0.1mg/ml lysozyme, 1U DNase1, 0.1%, Tween20, 1X Protease inhibitor cocktail were added in 50 ml culture pellet which was resuspended in 2ml B-PER™ solution and incubated on ice for 30 min. After incubation the mixture was sonicated on ice and the total protein was stored at -20ºC.

Thiourea/urea solution was used as an alternative to B-PER™ to increase the solubility of the protein mixture containing C-His OipA, a membrane protein under induced conditions. The pellet obtained from the same culture and the same volume was used for comparison with B-PER™. For this, total protein extraction was made by using 2 ml Thiourea/urea for 50 ml of culture pellet and stored at -20ºC. Total protein samples obtained by two methods were analyzed by both SDS PAGE and western blot. In order to compare total protein band profile, 20 ul of protein in the same volume was loaded with 4X Laemmli buffer to each well in SDS PAGE.

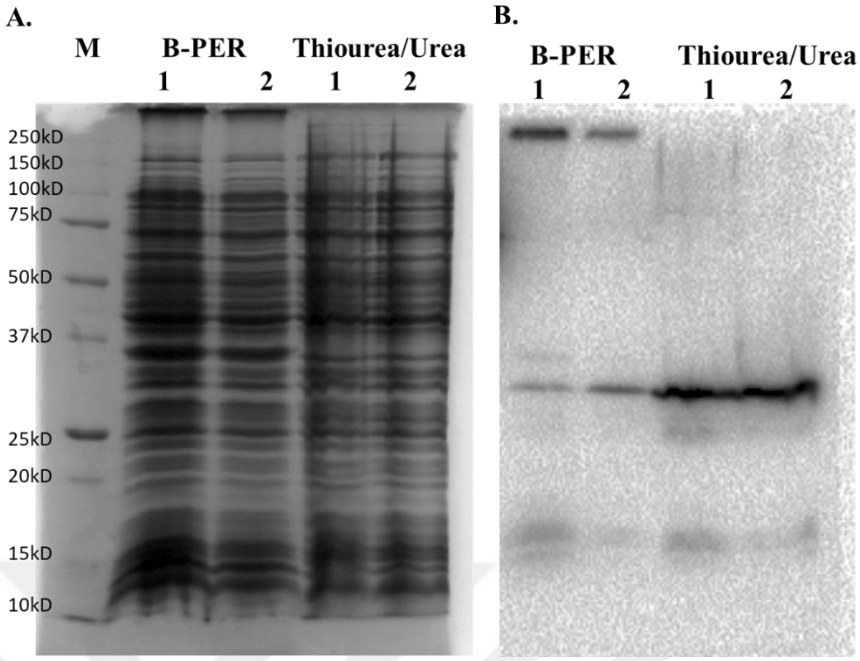

Figure 4.11. SDS PAGE (stained with CBB) and Western blot results of total protein extractions with different solutions.

A is SDS PAGE results of total protein extracted; M is pre staiend marker (Biorad). B-PER 1 is total protein extracted with B-PER. B-PER 2 is 1:2 dilition of B-PER 1. Thiourea/urea 1 is total protein extarcted with Thiourea/urea solution. Thiourea/urea 2 is 1:2 dilition of Thiourea/urea 1. **B** is western blot results of total protein extracted. The sample order is the same as A.

4.1.6 Recombinant protein purification

4.1.6.1 Recombinant protein purification with affinity chromatography method

Purification of recombinant C-His OipA protein was tried with affinity chromatography containing nickel column. Insoluble protein mixture as a mobile phase in the nickel column was prepared with a solubilization protocol (M&M at 3.2.1.10 Recombinant protein purification). Then purification experiments were carried out in affinity chromatography following HisTrap Hp column (GE Healthcare)

instructions. Optimization studies were the concentration of imidazole in the elution buffer was tried as 1M and 750mM; buffer flow rates from the column were changed as 0.3ml / min for refolding buffer, 0.1ml / min for elution buffer, 0.5 ml/min for other buffers and all buffer were prepared again without BME. During the preparation of the insoluble protein mixture, the filtering process was tested with 0.22 μm and 0.45 μm filters and loaded onto the column with and without filters. The optimal conditions obtained after the optimization trials and the experimental results were displayed using the western blot. After the optimization studies, 1M imidazole elution buffer was adjusted so that the solution flow rate passing through the column decided to be 0.3 ml/min, BME was removed from all buffers and the soluble protein mixture was loaded onto the column without filtering. The sample was collected from each step to analyze. When the optimal conditions obtained from optimization studies were tested in this process, the collected samples were analyzed in western blot (Figure 4.12). The results of western blot analysis was shown that the C-His OipA protein could not be completely purified in the experiment using HisTrap Hp column; during the purification process, the obtained C-His OipA protein could not be fully bound to the column so the yield percentage of the experiment was low when the band densities at about 32 kDa were compared.

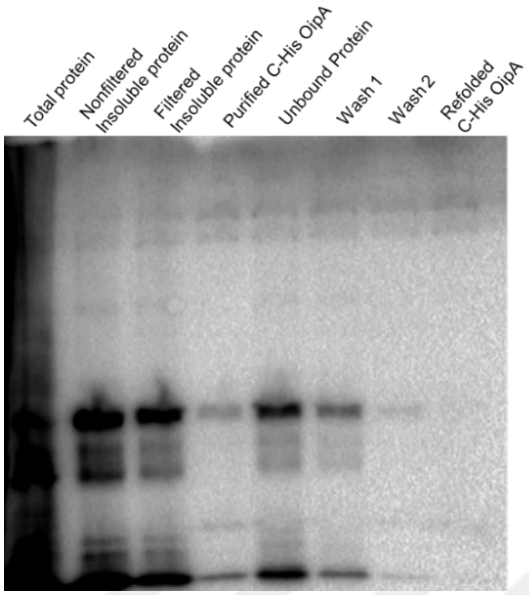

Figure 4.12. Result of western blot of affinity chromatography to purified C-His OipA protein.

The order of proteins is as follows, total protein; nonfiltered insoluble protein is insoluble protein dissolved without filtering; filtered insoluble protein is insoluble protein mixture dissolved with filtering, purified C-His OipA is obtained from purification under the optimal conditions; unbound protein is collected after loading the dissolved protein mixture into the column; wash 1 is collected at first washing step; wash 2 is protein sample collected at second washing step, refolded C-His OipA is collected at refolding step.

4.1.6.2 Recombinant protein purification with magnetic bead method

As a first try, two pellets were obtained from *E. coli BL21 (DE3)* culture in 100 ml LB media under the optimal IPTG induction conditions that were at 37˚C for 4 hours. The pellets were prepared to extract protein with three different methods for purification. Lysate 1 as a total protein was extracted from pellet 1 with lysis solution with 8M urea and 1X protease inhibitor cocktail (brand). Lysate 2 was extracted from pellet 2 with B-PER™ containing 0.1% Tween 20, 1X protease inhibitor cocktail (brand). Lysate 3 was obtained from lysate 2 with centrifugation. Lysate 1 was a soluble mixture where lysate 2 and 3 were highly viscous. All lysates were used to purify in a magnetic bead assay. While Lysate 2 and 3 were used in the purification process, one hundred percent efficiency was not achieved in adhesion of the beads to

the magnetic stand due to their viscosity structure Lysate 1 did not encounter such a problem. As loading the gel, Lysate 2 and 3 were hardly loaded due to its viscous structure. The samples obtained from purification were analyzed with SDS PAGE and western blot. (Figure 4.13)

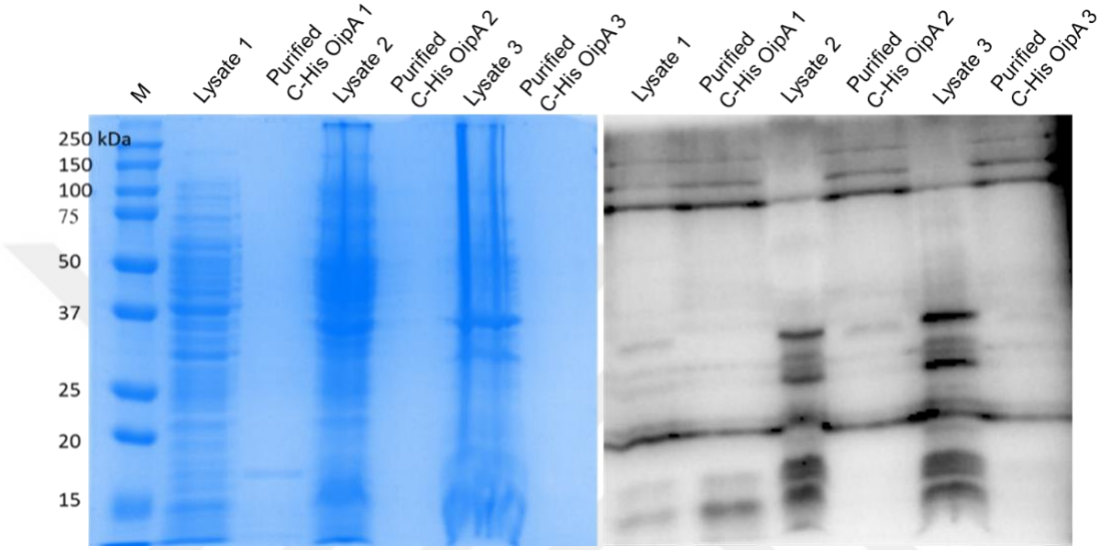

Figure 4.13. Results of SDS PAGE staining with CBB and western blot of magnetic bead purification of C-His OipA protein.

A is representative SDS PAGE result. The order of proteins is as follows; M is pre-stained protein marker (Bio-Rad), lysate 1, purified C-His OipA protein 1 is obtained from lysate 1, lysate 2, purified C-His OipA protein 2 is obtained from lysate 2, lysate 3 and purified C-His OipA protein 3 is obtained from lysate 3. **B** is a representative western blot result. The same order is as 'A'.

According to the results of SDS PAGE and western blot, the first try of purifying C-His OipA protein was not successful. When total protein band profiles were compared, it was observed that lysate 2 and 3 had 37 kDa protein band denser than lysate 1. According to the SDS PAGE results, lysate 2 and 3 viscose forms were caused to irregular running in the gel. A second experiment was designed to get rid of this viscous form.

In the second experiment, a total protein obtained with B-PER™ was used as lysate. Two pellets were obtained from 100 ml of bacterial cultures grown under optimized IPTG induction conditions. One of the pellets was used to extract total protein and the other was used to obtain insoluble total protein. To get rid of the viscous form for both lysate, Lysozyme, Tween20, and Dnase1 were added to B-PER™ in the lysis stage, kept on ice for 30 minutes, and sonicated on ice. After these processes, the viscous form was removed. During the magnetic bead purification process, it was noticed that the efficiency of magnetic beads adhesion to the stand increased, when non-viscous lysate forms were used. Western blot was performed to interpret the obtained results. (Figure 4.14)

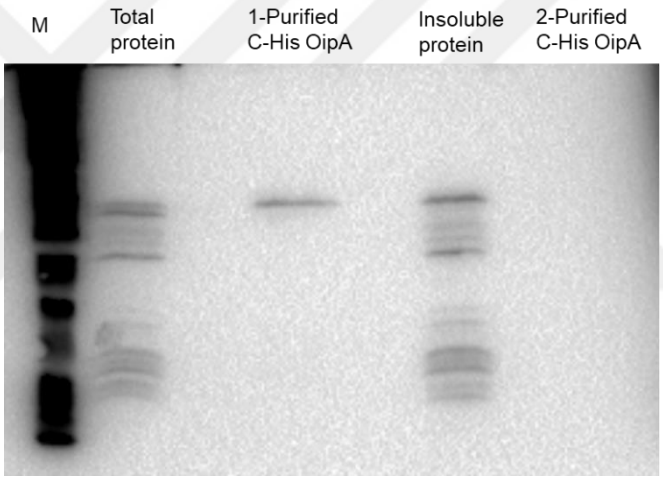

Figure 4.14. Result of western blot of magnetic bead purification to purified C-His OipA protein.

The order of proteins is as follows, M is a pre-stained protein marker (Bio-Rad), Total protein, 1-purified C-His OipA protein was obtained protein from total protein, insoluble protein and 2-purified C-His OipA protein was obtained from insoluble protein.

Purification of the C-His OipA protein has been successful in the second experiment When the total protein mixture viscosity was decreased, the yield of the purified protein was increased. was obtained from the experiment. In purification using total protein, the C-His OipA protein was purified. The purified protein quantification was made with Bradford assay and its concentration was calculated as 43 µg / ml. The

recombinant C-His OipA protein complex was in denature form as it was purified under ingdenaturing conditions. It must be refolding to be active form.

4.1.7 Increasing the purification efficiency of C-His OipA

Total protein was extracted from the pellet obtained from 50 ml of induced culture using 6 ml of thiourea/urea solution. Purification process was performed under denature conditions with magnetic bead. The protein samples were collected from each stage. Western blot results of the collected samples are shown in Figure 4.15. Based on the results, it was shown that there was a loss of the C-His OipA protein at each step. Three protein bands, at 100 kDa, 37-25 kda and 25 kDa seen in the western blot results and they were cut from SDS PAGE and sent to LC-MS/MS analysis. The results of LC-MS/MS analysis was shown the band between 100 kDa and 37-25 kDa belongs to the C-His OipA protein. Since the C-His OipA protein is produced by subtracting the signal peptide sequence which weighs 32 kDa, the band between 37-25 kda was thought to be the monomer form of C-His OipA.

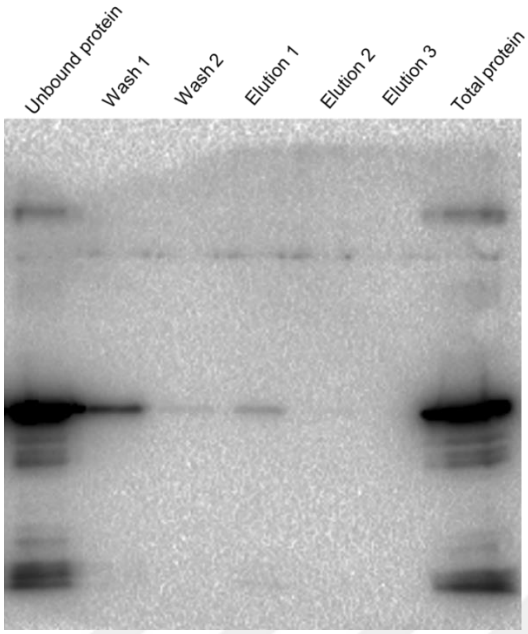

Figure 4.15. Western blot result of protein samples obtained from magnetic bead purificaiton.

The order of proteins is as follows, unbound protein; wash 1 is obtained from the first wash; wash 2 is obtained from the second wash; elution 1 is obtained from the first elution step ; elution 2 is obtained from the second elution step; elution 3 is obtained from the third elution step and total protein sample containing C-His OipA (9.second).

Eluted C-His OipA obtained in magnetic bead purification has a lower protein band density in the range of 37-25 kda compared to proteins obtained at other stages in western blot analysis. Passive elution process was performed to recover the C-His OipA in the protein collected from the magnetic bead purification stages. 20 µl of proteins obtained from the wash and elution steps were loaded onto the 12% polyacrylamide gel and performed by SDS PAGE. The protein band in the range of 37-25 kda were cut from the gel according to the previous western blot results. This process was done from five gels. Each piece of gel cut from polyacrylamide gel was treated with 600 µl passive elution buffer. Approximately 600 µl of protein samples in 5 tubes obtained at the end of the experiment and they were combined. For this mixture, BCA was performed with BSA standards prepared using passive elution buffer. The concentration of C-His OipA protein obtained by passive elution was measured as 367.16 µg/ml. The C-His OipA protein was shown by Western blot (Figure 4.16).

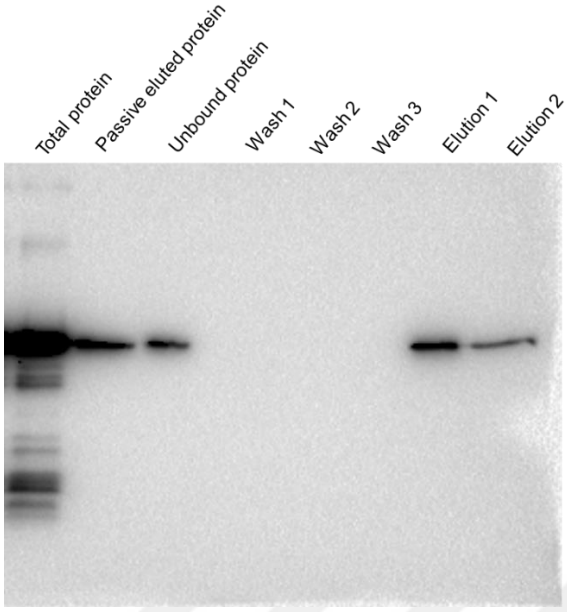

Figure 4.16. Western blot result of protein samples obtained from magnetic bead purification after passive elution assay.

The order of proteins is as follows, total protein containing C-His OipA protein; passive eluted protein is obtained from passive elution assay; unbound protein is obtained from binding step; wash 1 is obtained from the first wash; wash 2 is obtained from the second wash; elution 1 is obtained from the first elution step ; elution 2 is obtained from the second elution step in magnetic bead protein purification.

4.2 The Obtaining of Human Gastric Cancer Cell Membrane Proteins

AGS cell line was grown in petri dish under cell culture conditions. AGS cells were counted using Thoma cell counting chamber. Then, the membrane proteins were extracted from $5x10^6$ AGS cells using MEM-PER kit. In the extraction of membrane proteins, the incubation time at 37°C was changed from 10 minutes to 20 minutes to better see the phase partitioning. The obtained membrane proteins were in solution containing mild detergent mixes, so they had a viscous form. However, the membrane proteins in solution based on mild detergent was in nature conformations. For pull down assay, the obtained membrane proteins did not need to refold. The membrane proteins of AGS cells were performed by SDS PAGE and visualized using silver staining. According to SDS PAGE results, the membrane protein viscous form was

caused to irregular running in the polyacrylamide gel. So, to ensure the membrane proteins get a better result in the gel that detergents were removed with acetone precipitation. For this, 100 µl of membrane protein was used to acetone precipitation. In the end of acetone precipitation, obtained pellet was dissolved with 50 µl 4X Laemmli buffer containing BME and then analyzed with SDS PAGE.

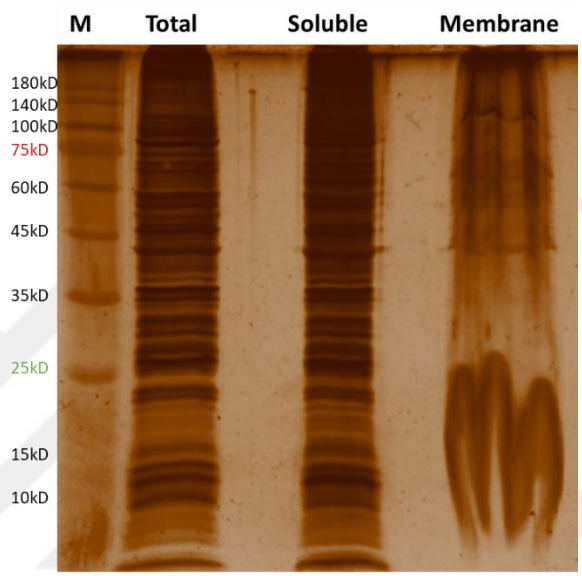

Figure 4.17. SDS PAGE stained with silver stained results of protein extractions from AGS cell line.

M is pre staiend marker (GeneMark). Total is total protein extracted from AGS cell line with RIPA buffer. Soluble is hydrofilic protein fraction from membrane protein extraction ;Membrane is hydrophobic protein fraction from membrane protein extraction using AGS cell line.

4.3 The Discovery of OipA Binding Partner

4.3.1 Pull down assay

Refolded C-His OipA protein as a bait protein and AGS membrane proteins as a prey protein were used to pull down assay. For first setup, the assay was performed using 50 µl nickel magnetic beads of bait and prey protein at a ratio of 1: 4. 100 µl of refolded C-His OipA protein at 10 ng/µl concentration was added to 50 µl nickel magnetic beads under nature conditions ann incubated at room temperature for 30 min. After incubation, 400 µl AGS membrane proteins were mixed with immobilized refolded C-His OipA protein into nickel magnetic beads and incubated at room temperature for 30 min. Then, the assay was continued to magnetic bead purification protocol. 125 µl wash buffer was used in each wash steps. 50 µl elution buffer was used in each elution step. The protein samples were collected from each steps. The obtained protein samples were analyzed with native PAGE, western blot and, LC-MS/MS.

For second setup, the assay was performed using 80 µl nickel magnetic beads of bait and prey protein at ratio of 1:4. 160 µl of refolded C-His OipA at 10 ng/µl concentration and 640 µl AGS membrane protein were used in second setup. As the parameter that changed from the first setup; AGS membrane proteins were mixed with immobilized refolded C-His OipA protein into nickel magnetic beads and incubated at 4°C for 2 hours. After incubation, second setup was completed with the magnetic purification protocol. 200 µl wash buffer was used in each wash steps. 80 µl elution buffer was used in each elution step. The obtained protein samples were analyzed with blue native PAGE, SDS PAGE, western blot and, LC-MS/MS.

4.3.2 Analysis of LC-MS/MS results

The obtained protein sample from pull down assay was carried out LC-MS/MS. Proteins with a unique peptide number higher than 2 were selected in the protein list obtained as a result of LC-MS / MS, and then 161 proteins were found. These 161 proteins were investigated whether they are cell membrane protein or cell membrane-associated protein by UniProt tool and literature review. After, 30 membrane and membrane associated proteins were determined. These determined proteins were networked with OipA protein using the Cytoscape program and shown in Figure 4.18.

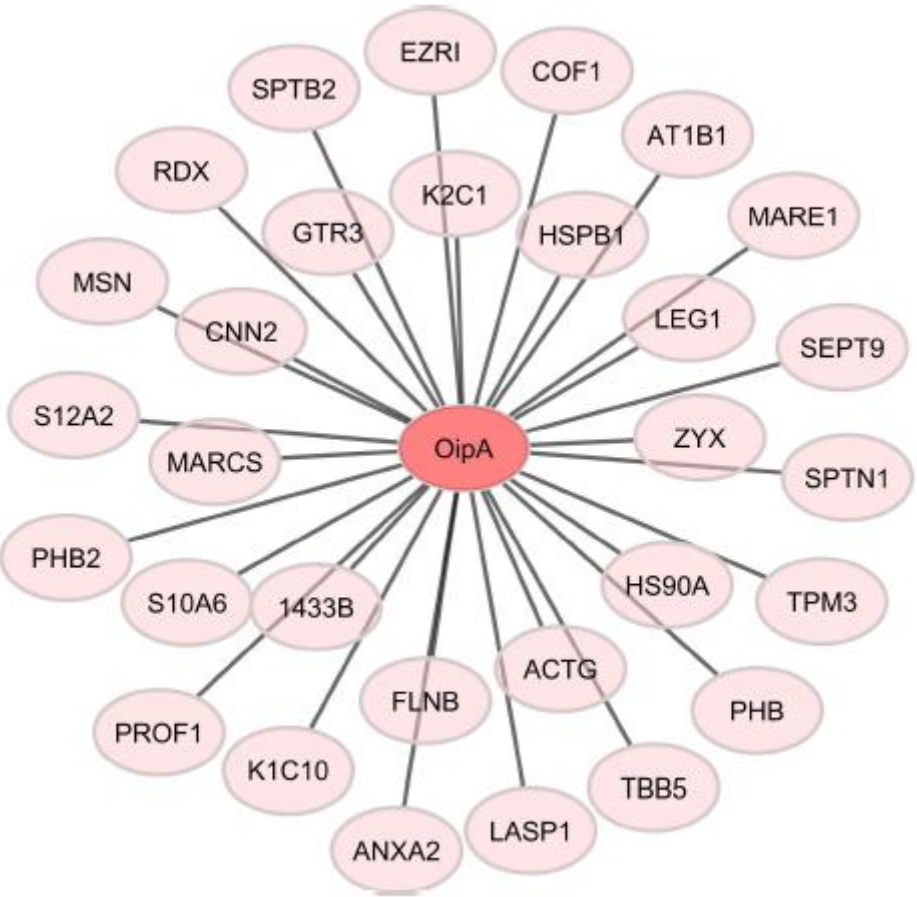

Figure 4.18. Network map between OipA and possible interaction partner.

A detailed literature study was conducted for proteins that can be associated with *H. pylori* infection and OipA using the 30 determined proteins. As a result of this study, 3 candidate interaction partners were determined. The candidate interaction partners are Prohibitin (PHB), Prohibitin-2 (PHB2) and Sodium/potassium-transporting ATPase subunit beta-1 (AT1B1). The relationship of candidate interaction partners to the OipA protein was visualized according to the unique peptide number and confidence score of candidate interaction partner.

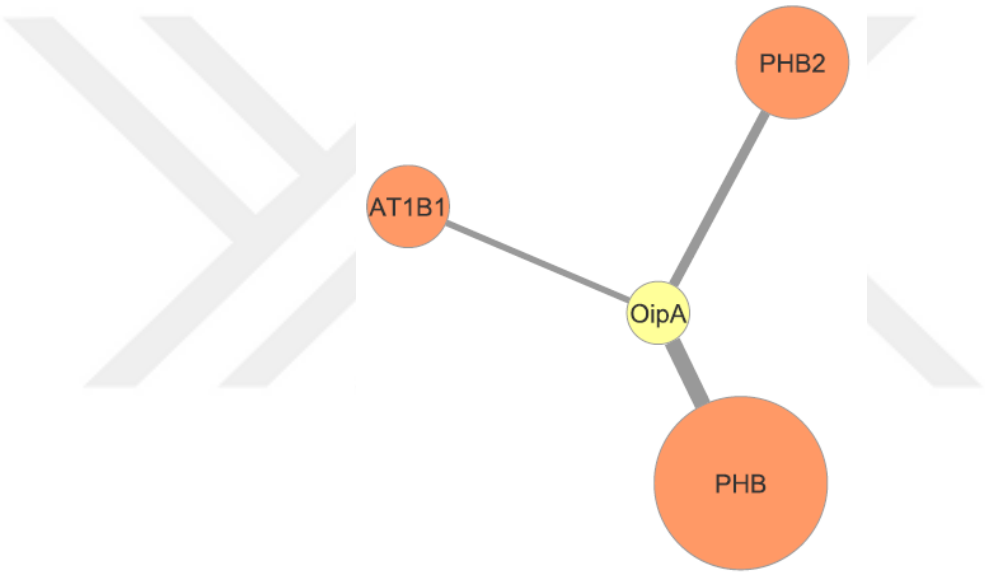

Figure 4.19. Network map between OipA and candidate interaction partners.

Size of node is determined based on unique peptide number; size of edge is determined based on confidence score.

5. DISCUSSION AND CONCLUSION

In spite of gastric peristalsis, *H. pylori* demonstrates powerful interaction with gastric epithelial cells (17) and has developed some mechanisms to cause infection in the harsh acidic stomach environment. The pathogenesis of *H. pylori* infection and disease outcome are explained by the relationship between host genetics, environmental and bacterial virulence factors (84). The virulence factors take an important role for adherence, colonization, and activation of the host immune response. Especially, adherence of *H. pylori* to the mucus layer of the gastric epithelium has an significant role in the initial colonization (8). The interaction of bacterial outer membrane proteins with cellular receptors protects the bacteria from mechanisms like acidic pH, mucus, and exfoliation of stomach (40,105). The extraordinary large outer membrane protein (OMP) family of the bacterium includes proteins specifically involved in attachment, such as BabA, SabA, AlpA/B (105). The one of the important outer membrane protein is OipA that attach to gastric epithelial cells in bacterial pathogenesis. Thus, OipA has an affect in development of clinical outcomes such gastric cancer (84). The functional status of OipA are determined by by a slipped strand mispairing mechanism. The patients infected with *H. pylori* has a functional OipA have a higher risk of developing gastric cancer (11). OipA stimulates in vitro IL-8 secretion together with *cagA* and increases gastric inflammation in vivo (8). The protein is also involved in neutrophil infiltration, activation of focal adhesion kinase, cytoskeletal reorganization (77). Although the functions of the OipA protein are known with their general lines; the protein structure, interaction partner and its relationship are unknown (8,17).

The OipA protein, which is the focus of this thesis, is a highly hydrophobic and basic membrane protein. Therefore, the experimental protocols used in cloning, overexpression, isolation, purification and refolding experiments used in the thesis

differ from classical protocols by optimizing them. These developed protocols are also valuable in that they have the potential to be a resource for those who will work with membrane proteins.

In the LIC method, the vector and insert are annealed with specific nucleotide single-stranded overhangs. The sequence of the gene region to be cloned and vector are very important. In amplification step, Taq polymerase is not suitable for LIC method as it creates 3'A overhang of PCR product (106). In this thesis, PFU was used instead of Taq. As the PCR method; Nested PCR was used to amplify the target *oipA gene* region. For this, the 1093 bp *oipA* gene size was replicated with the external primer we designed and extracted with gel extraction kit. The obtained 1093 bp *oipA* was used as a template DNA in PCR using the primer suitable for the cloning strategy to be used. And this way non-specific band formation was prevented.

For cloning of the *oipA* gene, cloning studies were performed using two different vectors. At every stage of cloning strategy 1, especially T4 DNA polymerase. treatment and annenling steps optimization studies were performed. When colony PCR was made to the colonies obtained at the end of cloning, it was observed that there was no transformed colonies. Colony presence in negative control samples; It can be explained by the vector closing onto itself. In the realization of this situation, overhang structures at the 3' and 5' ends should not be formed, that is the T4 DNA polymerase should not worked. To solve this problem, the aLicator lic cloning kit was used to control the functioning of the T4 DNA polymerase enzyme in cloning strategy 2. There is a control PCR fragment in the kit that can control the T4 DNA polymerase work. Cloning experiments were performed simultaneously with the C-His *oipA* and the control PCR sequences. Colonies were seen in cloning experiments with the control PCR sequence, and when these colonies were made colony PCR, 898 bp DNA band was obtained. However, when colony PCR was performed on colonies seen in cloning

experiments for C-His *oipA*, 1154 bp DNA band could not be obtained. T4 DNA poly. enzyme did not work for cHis oipA sequence while it worked for control PCR fragment. A problem was thought to be caused by the C-His *oipA* sequence. An article encountered in a detailed literature study on this gave an idea that the problem experienced in the experimental procedure may arise from secondary structures that may occur in the gene sequence to be transferred (104). As stated in the article, secondary structures that may occur can affect the activity of T4 DNA polymerase and this problem can be solved by trying different T4 DNA polymerase activity temperatures. The conformation of the 3 'and 5' ends of the *oipA* gene sequence at different temperatures was examined using the UNAfold tool in silico environment. At high temperature, the conformation of the 3 'and 5' ends is more open and it has been found to be more suitable for the enzyme's work. In this study, it is predicted that cloning could not be completed due to the secondary folding of the single chain gene structures formed at the ends of the *oipA* gene during the T4 DNA polymerase activity because the exonuclease activity of the enzyme could not be completed or the binding did not occur. Upon this observation, experimental procedures were reviewed and T4 DNA polymerase. assays were optimized by trying different temperatures and times. For the C-His *oipA* gene sequence, the working conditions of T4 DNA polymerase were changed from 5 minutes at 25˚C to 30 seconds at 50˚C. With the optimized ligation-independent cloning method, cloning of an outer membrane protein with a high tendency to form a secondary structure was achieved.

The production of recombinant protein was initiated to perform protein level experiments using *oipA* cloned into pLATE11. In order that overexpression of C-His OipA protein, IPTG and lactose inducer concentrations and induction time trials were performed in induction studies. As another parameter, the strain of bacteria in which the protein will be expressed was changed. At the end of induction optimization studies, induction with 1 mM IPTG for 4 hours at 37ºC using *E.coli BL21 (DE3)* was found to be the optimal condition for overexpression of the C-His OipA protein.

Since it was not known whether the overexpressed OipA protein was released out of the cell or stored inside the cell, both the supernatant and the pellet obtained when the bacteria culture was centrifuged were used. Both soluble and debris (inclusion body) protein parts were obtained from the obtained pellet in protein isolation. When three different protein samples were examined, it was shown that the protein formed an inclusion body and was mostly located in the debris. Although it is not obvious, western blot results showed that C-His OipA protein is in the soluble part. Therefore, the total protein obtained from the pellet was used without splitting into two parts. Recombinant protein can form an inclusion body of the target protein in overexpression (107,108)

In order to increase solubilization of C-His OipA protein that was obtained as inclusion body, two different total protein isolation methods based on B-PER™ and Thiourea/urea buffer were used. When the two methods were compared, it was observed that the separation of proteins was better achieved in the isolation used with Thiourea/urea buffer. Protein aggregation was dissolved and inclusion body solubilization was increased. In the literature, it has been explained that urea and thiourea, which are chatrepoic agents, break down hydrogen bonds and turn the proteins from their natural form to their denatured form by weakening the hydrophobic effect (109). Thus, thioure/urea increases the solubilization of proteins, especially membrane proteins (110). In this study, the solubilization of the C-His OipA protein which is a membrane protein was achieved using thiourea/urea.

In order to purify labeled proteins, affinity chromatography methods that usually based on protein-protein interactions are used. In this thesis, a nickel-based column and nickel-charged magnetic bead affinity chromatography method were used to purify OipA protein produced with labelled 6XHis peptide on C terminal. In the purification experiment done by nickel-based column affinity chromatography (

HisTrap Hp column, GE Healthcare), sufficient purity and amount of C-His OipA could not be obtained. To solve this problem, optimization studies were done especially in the concentration of immidazole in the elution buffer. However, the desired result could not be achieved. The method used to obtain highly pure and amount of C-His OipA protein was changed and purification was planned using nickel-charged magnetic bead affinity chromatography method. Purification with the magnetic bead were carried out under denatured conditions to prevent protein accumulation. When the results of the purification with magnetic bead were examined, it was seen that more yield was obtained compared to the purification using the column. But the amount of pure C-His OipA was insufficient for further studies. In order to increase the amount of pure C-His OipA, it was planned to reduce the variety of proteins that bind non-specifically to magnetic bead in the purification. For this, passive elution was performed by cutting the band known to be between 35-25 kDa C-His OipA in the gel from the total protein extracted before purification. The protein mixture containing proteins between 35-25 kDa was used as lysate for purification. According to the purification results, the loss of C-His OipA protein in the samples collected during the purification step was minimized. The purification efficiency was shown to be increased by adding the passive elution step.

OipA is an important virulence factor as it provides a stronger effect by interfering with the signal pathways activated by CagA/T4SS. OipA mediates a tight attachment between bacteria and gastric epithelial cells through this strong effect that occurs either indirectly or by binding to the surface protein, in colonization (17). The functions and regulated signals of OipA in epithelial cells have been explained, but the surface protein with which OipA binding interacts in epithelial cells has not been identified. Thus, we performed this study to find the interaction partner of OipA, and identified proteins from AGS cell lines by pull down assay followed by LC-MS/MS analysis.

As a result of the analysis, it was noticed that there were proteins other than the cell membrane protein in the protein list that interacted with C-His OipA. The reason for this is thought to be that organelle membranes and membrane-associated proteins are extracted in membrane protein extraction. Since the protein we expect the OipA protein to bind to is the cell membrane protein, the list has been rearranged. As a result of a detailed literature review, 3 candidate interaction partners were found that could be associated with OipA and *H. pylori* infection which are Prohibitin (PHB), Prohibitin-2 (PHB2) and Sodium/potassium-transporting ATPase subunit beta-1 (AT1B1).

Prohibitin 1 ve Prohibitin 2 are pleiotropic protein in the cell. Prohibitin family members have been involved in the cellular proliferation, transcription, apoptosis, mitochondrial protein folding, and as a cell-surface receptor (111,112). Previous studies have confirmed that prohibitin is an anti-proliferation protein and can function as a tumor suppressor (113). Prohibitin's anti-proliferative function is down regulated by miR-27a what acts as an oncogene in gastric adenocarcinoma by targeting the protein (114). Also, PHB1 proteins localized in the cell membrane serve as viral or bacterial receptors to promote the entry of these microorganisms into host cells. Previous studies have also determined that PHB1 on the plasma membrane functions as a viral receptor protein. For examples, PHB1 interacts with chikungunya virus (115), PHB2 interacts with dengue virus (116) and PHB 1/2 interacts with severe acute respiratory syndrome coronavirus (117).

Previous studies have shown that PHB can be used as a biomarker in many types of cancer such as breast cancer, prostate cancer (118). KANG et al. have studied that PHB could be a potential biomarker in gastric cancer. Their studies have shown that PHB and mRNA levels were up-regulated in gastric cancer tissue compared to normal

gastric tissue. Based on this information in the literature, it is thought that PHB1 and PHB2 obtained as a result of LC-MS/MS analysis may be interaction partners of OipA.

Na^+/K^+ - ATPase is an integral membrane protein containing two subunits a large catalytic subunit (alpha) and a smaller glycoprotein subunit (beta) (119). Na^+/K^+-ATPase function in epithelial cells also plays a role in the formation of tight junctions through RhoA GTPase and stress fibers. The β1 subunit of Na^+/K^+-ATPase is responsible for epithelial cell polarization in cell-cell interaction (120). Marcus et al. showed that the expression of Na^+/K^+-ATPase is decreased in *H. pylori* infection. The decrease of this enzyme in the membrane leads to disruption of ion balance, nutrient intake and cell-cell interactions, causing stomach damage. In order for the colonization, *H. pylori* must attach to the epithelial cells with virulence factors. So epithelial cells are damaged with this attachment,. Since OipA also acts as an adhesin in attachment. It is thought that OipA may interact with the proteins involved in the ion transporter. Therefore, the interaction partner of OipA can be Na^+/K^+-ATPase; β1 subunit of Na^+/K^+-ATPase.

In conclusion, 3 candidate interaction partners of OipA were determined with this study. The binding of OipA protein and candidate interaction partner is an important step in the attachment of *H.pylori* to the gastric mucus layer. The interaction partners can be targeted to prevent this infection. The candidate interaction partners determined with this study are very valuable in terms of being the first in the literature. These proteins need to be supported by validation experiments for future studies. These candidate proteins can be used in vaccine studies as vaccine targets after validation experiments. The results would provide clues to understand the adhesion function of OipA and find targets of antiadhesion drugs against bacteria.

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7. CURRICULUM VITAE