

T.C.

ÇANAKKALE ONSEKİZ MART UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MASTER OF SCIENCE THESIS

RECOMBINANT PRODUCTION OF A GLYCOSIDASE BY USING AN *IN-VIVO* CLONING SYSTEM AND ITS IMMOBILIZATION VIA DIFFERENT STRATEGIES

Burcu PEKDEMİR

Biomolecular Sciences

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The study entitled "**Recombinant Production of a Glycosidase by Using an *In-vivo* Cloning System and Its Immobilization via Different Strategies**", submitted by Burcu PEKDEMİR under the supervision of Assoc. Prof. Dr. Sercan KARAV was defended on **01/31/2020**. This thesis has been approved unanimously as **Master of Science** in **Biomolecular Sciences** of Çanakkale Onsekiz Mart University, Graduate School of Natural and Applied Sciences by the jury.

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PLAGIARISM DECLARATION PAGE

I declare that all the information and results presented in visual, audio and written form are obtained by myself observing the academic and ethical rules and, all other results and information indicated in the thesis but not specific to this study are cited.

Burcu PEKDEMİR

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Burcu PEKDEMİR

Çanakkale, January 2020

LIST OF ABBREVIATIONS AND SYMBOLS

[E]	Enzyme concentration
[S]	Substrate concentration
2-AA	2-Amino benzoic acid
Asn	Asparagine
bp	Base pair
$C_2H_3NaO_2$	Sodium acetate
dH_2O	Distilled water
DHB matrix	2-5 dihydroxy benzoic acid/2-hydroxy-5-methoxy benzoic acid
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
e.g.	Exempli Gratia
EndoBI-1	Endo-*β*-*N*-acetylglucosaminidase
et al.	Friends
G	Relative centrifugal force
GlcNAc	*N*-acetylgalactosamine
HCl	Hydrochloric acid
HexNAc	*N*-acetylglucosamine
HF	Hydrogen fluoride
HMO	Human milk oligosaccharide
Ig	Immunoglobulin
*K*m	Michaelis-Menten constant
LB	Luria-Bertani
LPO	Lactoperoxidase
MALDI	Matrix-Assisted Laser Desorption Ionization
MES	2-(*N*-morpholino) ethane sulfonic acid
$MgCl_2$	Magnesium chloride
MRS	De Man-Ragosa-Sharpe
MS	Mass Spectrometry
N-glycan	*N*-linked glycan
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NaH_2PO_4	Monosodium phosphate
Ni	Nickel
O.D	Optical density
O-glycan	*O*-linked glycan
PCR	Polimerase Chain Reaction
pH	Power of Hydrogen
Pic-BH_3	2-Picoline borane
PNGase F	Peptide-*N*-Glycosidase F
rpm	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulfate Poliacrylamide Gel Electrophoresis
Ser	Serine
subsp.	Subspecies
TBE	Tris-Borik acid-EDTA
TFMS	Trifluoromethanesulfonic acid
The	Threonine
TOF	Time of Flight
V_0	Initial reaction rate
V_{max}	Maximum reaction rate
WP	Whey protein powder
WG	Whey protein
g	Gram
M	Molar
L	Liter
kg	Kilogram
mg	Miligram
mm	Millimeter
mL	Milliliter
µg	Microgram
µm	Micrometer
µM	Micromolar
µL	Microliter
ng	Nanogram
kDa	Kilodalton

kV	Kilovolt
min	Minute
v	Volume
w	Weight
U	Unit
%	Per cent
°C	Degrees Celsius

ABSTRACT

RECOMBINANT PRODUCTION OF A GLYCOSIDASE BY USING AN *IN-VIVO* CLONING SYSTEM AND ITS IMMOBILIZATION VIA DIFFERENT STRATEGIES

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Master of Science Thesis in Biomolecular Science

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Human milk, except water, contains 54% lactose, 32% lipids, 8% free oligosaccharides (free glycans) and 6% protein. Although, the concentration of free oligosaccharides in human milk is very high, they are not digestible by infants. A study have demonstrated that these compounds stimulate the growth of beneficial bacteria, so they are used to promote the immune system of infants (Lo Cascio et al., 2007). After this critical role of free oligosaccharides has been discovered, the interest on these compounds has greatly increased. Because human milk can not be produced at industrial scale and the concentration of free glycans in other mammalian milks is too low (bovine milk 0.1%), the delivery of these compounds to the infants, which they have not access to breast milk is very limited. To solve this problem, scientists have found that other compounds that are structurally similar to free glycans are attached to proteins via *N*- or *O*- glycosidicially (glycoproteins). Glycans are important for cell-to-cell interactions, proper protein folding and stabilization as well as their prebiotic activity. However, the mechanism of these biological roles has not been elucidated well due the lack of isolation of glycans from glycoproteins. The current chemically and enzymatically isolation methods used for deglycosylation are not able to cleave all glycans and they require high heat and detergent use that might denature the structure of glycan and remaining polypeptide. To overcome this deficiency, a new enzyme, endo-*β*-*N*-acetylglucosaminidase was first isolated from *Bifidobacterium longum* subsp. *infantis* ATCC 15697 in the 2015 (Karav et al., 2015a). This enzyme does not require any heat denaturation of the substrate or detergent use for its activity that enables to produce the glycans without affecting their biological roles.

Therefore, this enzyme might be the only strategy to isolate these glycans in large scale and investigate their biological functions. In this thesis, firstly, endo-*β*-*N*-acetlyglucosaminidase which is not commercially available was produced recombinantly. A novel technique for molecular cloning of this enzyme encoding gene was used *in-vivo* cloning technique and the enzyme was immobilized by various strategies (adsorption, entrapment and covalent binding) to achieve the re-use of the enzyme. The activities of the resulting immobilized enzymes were tested on model therapeutic proteins and it was seen that the immobilized enzyme form by the adsorbtion strategy has the highest activity after the free enzyme form. Then resulting glycans were characterized using advanced mass spectrometry. As a result of long-term incubation, it has been shown that enzymes immobilized with different strategies can cleave all *N*-glycan structures.

Keywords: Endo-*β*-*N*-acetlyglucosaminidase, Immobilization, Glycans, Kinetic Characterization

ÖZET

HÜCRE İÇİ KLONLAMA SİSTEMİ KULLANARAK REKOMBİNANT GLİKOZİDAZ ÜRETİMİ VE İMMOBİLİZASYONU

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İnsan sütü su hariç %54 laktoz, %32 yağ, %8 serbest oligosakkaritler (serbest glikanlar) ve %6 protein içermektedir. Serbest glikanlar insan sütünde yüksek miktarda bulunmasına rağmen, bu glikanların bebekler tarafından sindirilmesi mümkün değildir. Bir araştırmada, bu bileşiklerin aslında faydalı bakterilerin gelişiminde ve dolayısıyla bebeklerin bağışıklık sistemini güçlendirmede kullanıldığı gösterilmiştir (Lo Cascio ve diğerleri, 2007). Serbest glikanların çok değerli bir prebiyotik kaynağı olduğunun anlaşılmasından sonra, bu bileşiklere olan ilgi hızla artmıştır ve önemli bir araştırma konusu haline gelmiştir. Anne sütünün endüstriyel anlamda üretilme olanağının çok sınırlı olması ve serbest glikanların diğer sütlerdeki oranının (inek sütü % 0.1) oldukça düşük olması nedeniyle, anne sütüne erişim olanağı olmayan bebeklere, serbest glikanların temini konusunda çok ciddi bir açık bulunmaktadır. Bu eksikliği gidermek amacı ile araştırmacılar kimyasal yapıca glikan özelliği gösterebilecek başka bileşiklerin keşfine yönelmiştir ve serbest glikanlara oldukça benzer başka bileşiklerin proteinlere *N*- ve *O*-glikosidik bağlarla bağlı şekilde (glikoproteinler) bulunduğunu göstermişlerdir. Glikanlar, prebiyotik etkileri yanında, hücreler arası etkileşimde, proteinlerin düzgün bir şekilde katlanmasında ve stabilizasyonunda da önemlidir. Fakat glikanların bu biyolojik ve fiziko-kimyasal özellikleri, glikoproteinlerden ayrılmaları için kullanılan yöntemlerin yetersizliği yüzünden tam olarak anlaşılamamıştır. Günümüzde kullanılan kimyasal ve enzimatik yöntemler, bütün glikanları glikoproteinlerden ayıramamakta ya da bu yöntemler güçlü kimyasal ve ısı kullanımı gerektirdiğinden, glikanların ve geride kalan protein kısmının yapısını bozmaktadır. Bu eksikliği gidermek için yeni bir enzim olan endo-*β*-*N*-asetilglukozaminidaz, *Bifidobacterium longum* subsp. *infantis* ATCC 15697'dan ilk 2015

yılında izole edilmistir (Karav ve diğerleri, 2015a). Bu enzim herhangi bir ısıl işleme ve kimyasal kullanımına gereksinim duymadan, glikanların biyolojik etkinliği kaybolmadan izole edilmesine olanak sağlamaktadır. Bu yüzden günümüzde glikanların yüksek ölçüde izole edilip biyolojik görevlerinin araştırılmasına olanak sağlayan tek strateji olarak bu enzimin kullanımı kabul edilmektedir. Bu tez çalışmasında ilk olarak, ticari olarak temini mümkün olmayan endo-*β*-*N*-asetilglukozaminidaz enziminin rekombinant olarak üretilmesi hedeflenmiştir. Bu enzimi kodlayan genin moleküler klonlaması için yeni bir teknik olan *in-vivo* klonlama tekniği kullanılmıştır ve farklı immobilizasyon stratejileri (adsorpsiyon, hapsedilme ve kovalent bağlama) denenerek enzimin geri kazanımının sağlanması hedeflenmiştir. Elde edilen immobilize enzimlerin aktiviteleri model terapötik proteinler üzerinde denenmiş ve serbest enzimden sonra en yüksek aktivite gösteren enzim formunun adsorbsiyon stratejisi ile immobilize edilen enzim olduğu görülmüştür. Daha sonra elde edilen glikanlar ileri kütle spektrometresi kullanılarak karakterize edilmiştir. Uzun süreli inkübasyon sonucunda, farklı stratejiler ile immobilize edilen enzimlerin bütün *N*-bağlı glikanları kesebildiği gösterilmiştir.

Anahtar sözcükler: Endo-*β*-*N*-asetilglukozaminidaz, İmmobilizasyon, Glikanlar, Kinetik Karakterizasyon

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CHAPTER 1
INTRODUCTION

Glycans are present in nature free or attached to proteins by *N*- or *O*- glycosidic bonds. The free glycans contains lactose core and this core is extended with *N*-acetylglucosamine (HexNAc), sialic acid and fucoses. If a glycan is linked to serine or threonine residues of protein with HexNAc, it is called *O*-linked glycans (*O*-glycans), whereas *N*-linked glycans (*N*-glycans) are linked to the asparagine residue of protein with HexNAc. *O*-glycan structures contain eight different cores, while *N*-glycans contain only one core consisting of two HexNAc and three mannose monomers (Figure 1). Different glycans are synthesized by adding monosaccharides such as galactose, fucose or sialic acid to the core by glycosyltransferase and glycosidase enzymes (Ohtsubo and Marth 2006). According to this modification of the core, *N*-glycans are classified as high mannose, complex or hybrid glycans.

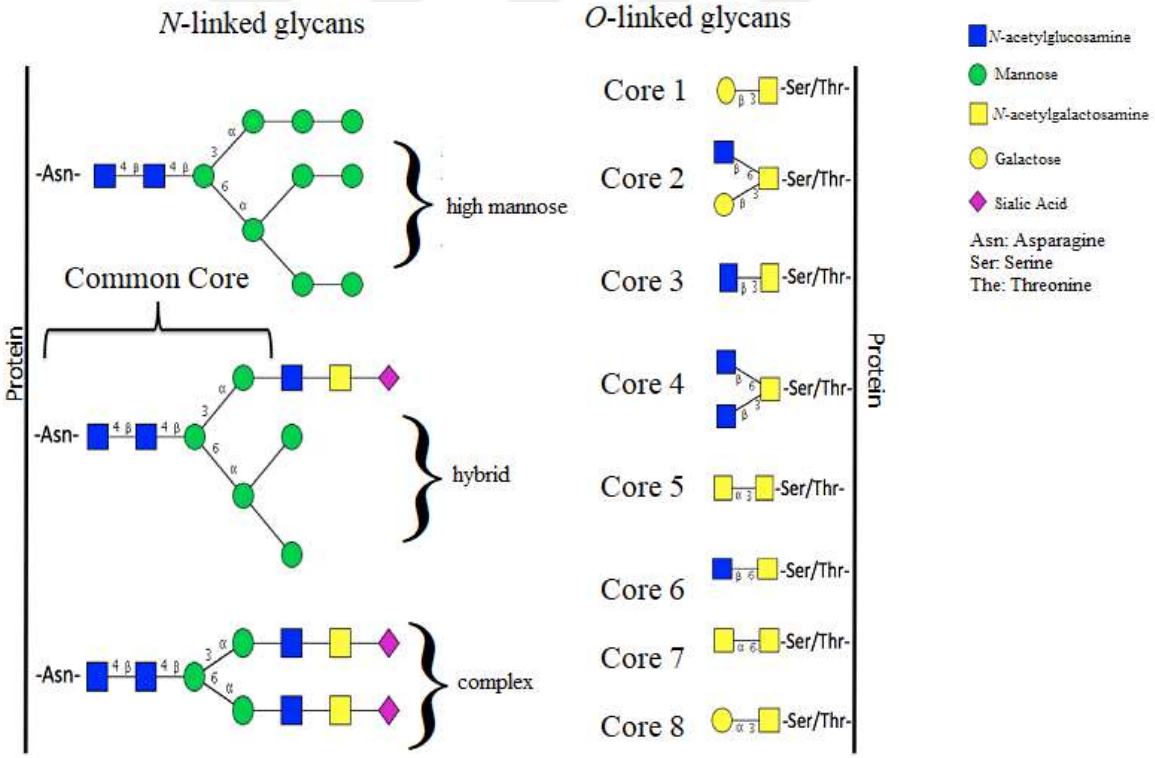


Figure 1. The cores of *N*- and *O*-glycans

1.1. Roles of Glycans

The interest in glycans has increased rapidly due to its physical and biological properties. Glycans have roles in intercellular interaction and cell-microorganism interaction, protein stabilization and structural support (Varki and Lowe 2009). In addition to these general properties of glycans, free glycans which are found in human milk block the pathogen attachment to the intestine epithelial cells, also help to growing beneficial microorganisms in the intestines of infants (Gopal and Gill 2000; Tao et al., 2008; Donovan et al., 2012; Ruhaak and Lebrilla 2012). In particular, it has been shown that sialic acid in free glycans, prevent diarrhea-causing rotavirus infection in infants and decrease the number of leukocytes bound to endothelial cells (Bode 2009). In addition, sialic acid is very important monosaccharide for development of brain and learning abilities of infants (Morgan and Winick 1980). Despite the known useful properties of free glycans, the industrial production of human milk is very limited, and it makes difficult to isolate and exploit these bioactive molecules on a high scale. In this study, it is aimed to isolate attached glycans from different glycoproteins that have similar chemical structure with them. These glycans are much more complex due to glycosyl-transferase and glycosidase enzymes, have high sialic acid content and are also more widely available in nature.

1.2. Source of Glycans: Glycoproteins

Glycoproteins commonly found in milk are known as immunoglobulin, lactoferrin, α-lactoalbumin, transferrin, osteopontin and glycol macropeptide (Walstra et al., 2005; Horowitz 2012). Especially whey, considered waste or by-product, is very rich in these glycoproteins (Mollea et al., 2013). Whey proteins are a good source of glycan due to their high concentration and high levels of glycosylated proteins.

Although the concentrations of glycoproteins in other animal milk vary, their aminoacid sequences of glycoproteins are highly similar. However, glycan structures which conjugated to these glycoproteins have very different properties. For example, Parc et al. (2000) examined the lactoferrin *N*-glycan types isolated from cow, human and goat milk and it was shown that only 13 glycan types were common among the 65 detected glycan structures.

Table 1. Various glycoproteins and their properties

Protein	Molecular Weight (kDa)	Concentration (g/kg)	Glycan Type
Glycomacropeptide	7	1.5 g (in a Liter), (Manso and Lopez 2004)	*O*-
Laktoferrin	86	0.1 (Recio et al., 2009)	*N*-
Transferrin	76	0.01-0.03 (Walstra et al., 2005)	*N*-
IgG*	150	1.8 (O'Riordan et al., 2014)	*N*-
IgA*	385	0.4 (Fuguay et al., 2011)	*N*-
IgM*	900	0.2 (Fuguay et al., 2011)	*N*-
Osteopontin	60	0.018 g (in a Liter), (Schack et al., 2009)	*O*-
α-lactalbumin	14.2	1.2 (Hart et al., 1979)	*N*-

*Ig: immunoglobulin

1.3. Deglycosylation Techniques

For deglycosylation of glycoproteins, many enzymatic and chemical methods are used. Chemical methods are widely preferred because of their easy applicability, low costs and being active on many substrates (Sojar and Bahl 1987). β-elimination and hydrazination methods are commonly used in chemical deglycosylation. The β-elimination is carried out under the alkaline condition to separate the glycans from glycoprotein (Dwek et al., 1993). Although reducing agents such as sodium borohydrate are used to protect the chemical structure of the separated glycans, the glycan structure and the remaining polypeptide structure can be disrupted by reactions known as peeling action during β-elimination (Carlson et al., 1968; Turyan et al., 2014). In addition, loss of glycans is

observed during the removal of highly amount of used salt in the *β*-elimination method (Turyan et al., 2014). The hydrazination technique is a method by which cleave of *N*- and *O*- glycan can be controlled by changing the reaction conditions. Kisiel et al. (2008) showed that hydrazination technique is a much more effective technique than *β*-elimination technique and that hydrazination technique is more efficient in terms of types of separated glycans. Although these two methods have recently been used and avoided disadvantages, they affect the chemical structure of both the remaining polypeptidee and deglycosylated glycans during deglycosylation. Furthermore, high amount of salt remaining within the separated glycans makes it difficult to analyze these glycans in the mass spectrometer.

Peptidyl-*N* glycosidase F (PNGases F) enzymes are generally used during enzymatic cleave for glycans (Altmann et al., 1995). PNGases F separate glycans from proteins by hydrolyzing the bonds to which they are attached to asparagine residues (Takahashi 1977; Nuck et al., 1990). Although PNGases F can separate all glycans regardless of the size of the glycans, the activity of the PNGases F stops where a fucose conjugated to the *N*-acetylglucosamine by 1,3 bond in the glycan core (Tretter et al., 1991). Furthermore, for the activity of PNGases F, denaturation of glycoproteins with high temperature and detergent usage is required firstly. Under these conditions, the isolated glycans can be degraded and mass spectrometer analysis of the glycans is negatively affected by the detergent contamination. Novel enzymes called endoglycosidase F1, F2 and F3, which do not require substrate denaturation, have been discovered, but their usage has been very limited due to their activity on a small number of glycan types (Trimble and Tarentino 1991).The linkages of novel endo-*β*-*N*-acetylglucosaminidase enzyme to be used in this study and different enzymes are effective on the *N*-glycan core (Figure 2).

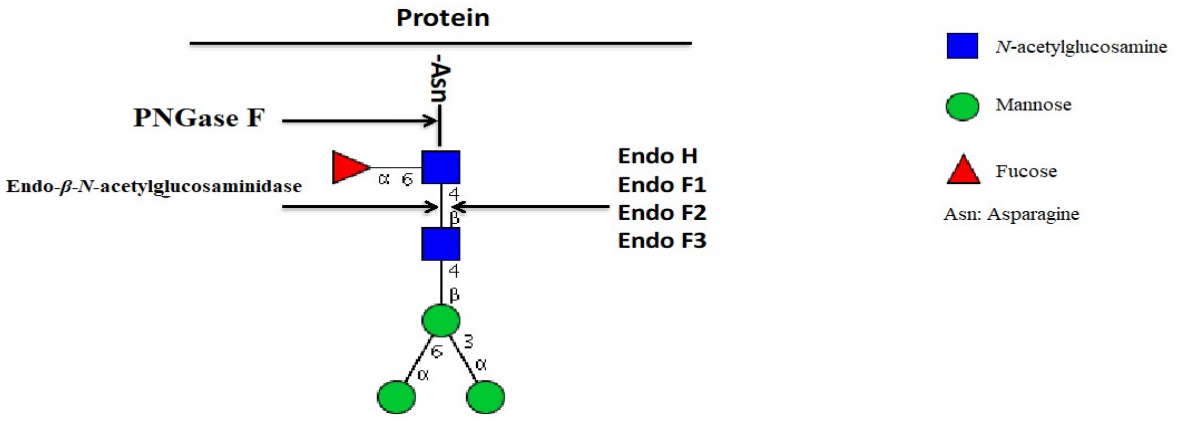


Figure 2. The activities of various glycosidases on *N*-glycan core

1.4. Can the New Glycosidases be the Only Way to Isolate High Quality Glycans?

EndoBI-1 is a novel enzyme and used during deglycosylation of glycans. This enzyme was isolated first time from *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (Karav et al., 2015a). This enzyme has many advantages over other chemical and enzymatic methods. Its activity is not affected by fucosylation of the core, and substrate denaturation is not necessary, also it is active on all glycan types (Karav et al., 2015a, 2015b). Another important advantage is that the separated glycan group (acidic or neutral) can be manipulated by changing the reaction conditions (Parc et al., 2015). For example, if the reaction was carried out at pH 7, 65 °C for 130 minutes and 1/1500 enzyme / protein, neutral glycans are predominantly isolated, but if the reaction was performed at pH 5.3, 40 °C for 360 minutes and 1/500 enzyme / protein, acidic glycans isolation can be possible. Considering all these properties and advantages, endo-*β*-*N*-acetylglucosaminidase stands out as the only strategy that allows the investigation of biological roles by separating all glycan types from glycoproteins without degradation their chemical structure. A detailed comparison of endo-*β*-*N*-acetylglucosaminidase and other enzymes active on glycans is shown in Table 2.

Table 2. Comparison of the effects of endo-*β*-*N*-acetylglucosaminidase and other glycosidases on different glycan types (+: high activity, -: inactive, +/-: les activity)

Enzyme	Natural Proteins (Not Denatured)	Core Fucosylation	High-Mannose	Hybrid	Two-Branches	Three-Branches	Four-Branches
PNGase F	-	-	+	+	+	+	+
Endo-*β*-*N*-acetylglucosaminidase	+	+	+	+	+	+	+
Endo H	+/-	+	+	+	-	-	-
Endo F1	+/-	+	+	+/-	-	-	-
Endo F2	+/-	+	+	-	+	-	-
Endo F3	+/-	+	-	-	+	+	-

1.5. Why is Enzyme Immobilization Necessary?

Recombinantly produced enzymes have high costs as well as they are generally sensitive to experiment conditions such as changing temperature, pH and substrate concentration (Tischer and Kasche 1999; Mateo et al., 2007). These problems can be solved by enzyme immobilization (Sheldon 2007). Enzyme immobilization is defined as the adsorbtion / entrapment of enzymes to a support material where they can continue their activities despite the loss of mobility (Datta et al., 2013). Many immobilization strategies were developed after Nelson and Griffin immobilized the first invertase enzyme in 1916 (Nelson and Griffin 1916). Which immobilization technique to use is chosen according to physico-chemical characteristic of the enzyme, support material and substrate properties (Nisha et al., 2012).

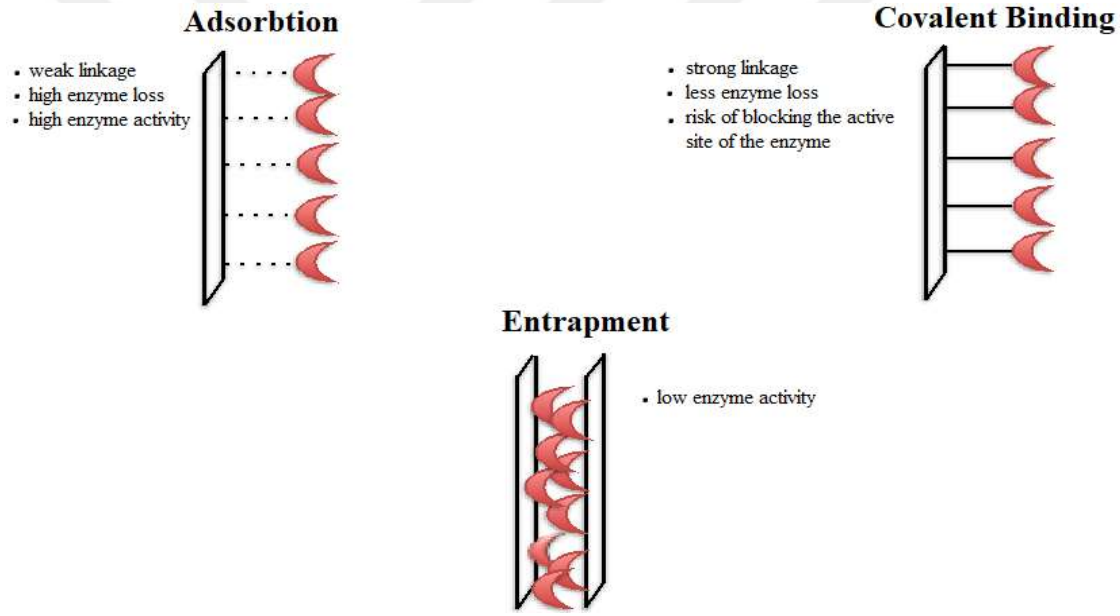


Figure 3. Different immobilization techniques

Enzyme immobilization techniques are divided into two classes as reversible (adsorption, ionic interaction) and irreversible (covalent binding and entrapment) (Figure 3). Reversible enzyme immobilization techniques generally form a weak interaction between the enzyme and the support material. Since this weak interaction does not block the enzyme active site, its mobility increases, and high enzyme activity is observed. However, due to this weak interaction, when the immobilized enzyme is re-used, the enzyme is separated from the support material and enzyme loss occurs (Sheldon 2007). Irreversible immobilization methods prevent the loss of enzyme over time by forming a

strong bond between the enzyme and the support material or by entrapping the enzyme between the two membranes (Karyakin et al., 2000; Schuster et al., 2001; Jal et al., 2004). However, as these methods involve the risk of blocking the active site of the enzyme, enzyme activity is likely to decrease (Datta et al., 2013; Kim and Herr 2013).

1.6. Objective of the Study

In this study, the following items were aimed to fill a large gap in the glycobiology literature: 1) Recombinantly producing the gene region encoding the endo-*β*-*N*-acetylglucosaminidase enzyme from *Bifidobacterium infantis* genomic DNA and using the new technique which is *in-vivo* cloning technique for molecular cloning. 2) To analyze the purity and activity of the produced enzyme. 3) Try different immobilization strategies (adsorption, entrapment and covalent binding) to ensure the recovery of the enzyme. 4) To make kinetic characterization of immobilized enzymes and compare with free form of the enzyme. 5) Deglycosylation of different glycoproteins with immobilized glycosidase enzyme

CHAPTER 2
PREVIOUS STUDIES

Glycans are present in nature as *N*-linked, *O*-linked or free glycans. The core structures of *N*- and *O*-glycans are produced by biosynthetic pathways. Functions or recognition properties are determined by subultimate and terminal sugars at "outer" positions of a glycan (Stanley and Cummings 2009). Glycans are built by glycosyl-transferase and glycosidase enzymes. Glycan chains are synthesized by glycosyltransferases which are anabolic component, but specific glycan linkages are hydrolyzed by glycosidases. Glycan structure is determined by both types of enzymes in glycan biosynthesis. (Ohtsubo and Marth 2006).

Biological roles of glycans can be determined by using the genetic modification of glycosylation in human diseases. Glycans also known as oligosaccharides play in major structural, physical and metabolic roles in biological systems (Varki and Lowe 2009).

Prebiotics are known as the non-digestible food components and they benefit to growth and activity of bacteria in the intestine that can protect the host health. (Gibson and Roberfroid, 1995). Human milk is rich in oligosaccharides (glycans) and is an important prebiotic source. In addition, human milk oligosaccharides (HMO) affect the microflora in the first weeks of an infant. (Coppa et al., 2004; Coppa et al., 2006). Also they have a role as micronutrients for infant developing and protection in the life of newborn (e.g. these oligosaccharides may have sialic acid which helps to nervous system development) (Morgan and Winick 1980).

Glycoprotein deglycosylation has been obtained in three different way; biosynthetically such as using tunicamycin (glycosylation inhibitor) (Le and Doyle 1986; Soderquist and Carpenter 1984), chemically (Kalyan and Bahl 1981; Bahl and Sojar 1987) and enzymatically (Koide and Muramatsu 1974; Umemto et al., 1977). In the deglycosylation of glycoproteins biosynthetically, the inhibitors of glycosylation have been employed as indirect method to obtain small quantities of a protein without the *N*-linked carbohydrate (Soderquist and Carpenter 1984). To determine the functional significance of carbohydrates in glycoproteins, this approach is useful but it is not suit as a preparative method. In the deglycosylation of glycoproteins chemically, $(HF)^3$ has been preferred (Mort and Lamport 1977). However, this method has some disadvantages. After process, poor yields are obtained also large amount of material is needed. Furthermore, the cleavage of *O*-linked carbohydrates is not generally complete under reaction conditions which do

not disrupt the dissociation of first and fourth structures of proteins. For chemical technique, trifluoromethanesulfonic acid (TFMS) mixture has been used but after chemical technique, deglycosylated proteins lose activity for a long time. Therefore, despite the fact that trifluoromethanesulfonic acid is highly efficient and reactive, it causes the degradation and inactivation of some proteins. (Kalyan and Bahl 1981; Edge et al., 1981). In the deglycosylation of glycoproteins enzymatically, in order of endoglycosydases H, F, and D from *Streptomyces plicatus* (Tarentino and Maley 1974), *Flavobacterium meningosepticum* (Elder and Alexander 1982) and *Diplococcus pneumoniae* (Kodie and Muramatsu 1974), have been employed (Fukuda 1985; Umemoto et al., 1977). However, these approaches have some disadvantages because of limitation of enzymes. The enzymes are costly and also have narrow substrate specificity with less-sensitive to glycoproteins. Consequently, this method is not suit for functional studies of carbohydrates in glycoproteins (Sojar and Bahl 1987).

Endoglycosidases are still widely used to characterize the purified glycoproteins as biochemical. Endo F enzyme from *F. meningosepticum* (Tarentino and Trimble 1991), was showed that is true endoglycosidase due to it cleaved the *β*1,4-linked di-*N*-acetylchitobiose core of *N*-linked glycans, like endo H from *Streptomyces plicatus*, oligosaccharides with a single reducing-end GlcNAc are cleaved except the core-proximal GlcNAc because it remains attached to the originally glycosylated asparagine. As a valuable reporter group, these GlcNAc residues can serve to determine the extent of site-specific glycosylation on defined glycoproteins (Reddy et al., 1988). Initial specificity studies suggested that, unlike endo H, endo F hydrolyzed biantennary oligosaccharides, although at a lower rate than high-mannose glycans (Tarentino and Trimble 1991).

Endo-*β*-*N*-acetylglucosaminidase (EndoBI-1) enzyme which was obtained from *B. longum* subsp. *Infantis* ATCC 15697 capable of cleavage of *N*-*N*-diacetyl chitobiose moiety found in the *N*-glycan core of high-mannose, hybrid and complex *N*-glycans. Fucosylation of the *N*-glycan core does not affect on EndoBI-1 activity and this enzyme have the heat resistance. So, it is useful for various industrial processes (Garrido et al., 2012).

Despite the wide potential use of the endo-*β*-*N*-acetylglucosaminidase enzyme, which allows for high isolation of glycans, no studies have been reported about production of this enzyme with *in-vivo* cloning strategy and kinetic characterization of this enzyme after different immobilization techniques.

CHAPTER 3
MATERIAL AND METHOD

3.1. Material

3.1.1. Substrates

In-vivo cloning and production of EndoBI-1 enzyme which has been immobilized in 4 different ways in this study were carried out with using *Bifidobacterium longum* subsp. *infantis*. For kinetic characterization of immobilized enzymes and characterization of released glycans, 0.1-0.8 mg/mL of whey protein was used.

3.1.2. Chemicals and Kits Used in Molecular Analysis

The SulfoLink immobilization kit (44995), The AminoLink immobilization kit (44890), Bolt 4-12% Bis-Tris Plus Mini precast gel (NW04120BOX), 20X Bolt MES SDS Running Buffer (B0002), 4X Bolt LDS Sample Buffer (B0007), 10X Bolt / NuPAGE ™ Reducing Agent (B0004), GeneJET Plasmid Miniprep kit (K0502), 2X PCR Master Mix (K0171), Ultra Pure Agarose (16500-500G), SeeBlue Pre-stained Protein Standard (LC5925), 6X DNA Loading Dye (R0611), HisPur Ni-NTA Resin (88222) were purchased from Thermo Fisher Scientific (Waltham, MA USA).

(*R*)-2-Amino-3-mercaptopropionic acid **(**L-Cysteine; C7352), Glycerol Solution ($C_3H_8O_3$; 15524), Sodium Chloride (NaCl; 31434), Calcium Chloride Dihydrate ($CaCl_2 \cdot 2H_2O$; 12022), Sodium Acetate Trihydrate ($C_2H_9NaO_5$; 25022), Sodium Azide (NaN_3; 71289), Potassium Phosphate Monobasic (KH_2PO_4; 04243), Imidazole ($C_3H_4N_2$; 56750), Sodium Phosphate Dibasic Dihydrate ($Na_2HPO_4 \cdot 2H_2O$; 04272), Agar (05039), Lactoperoxidase from bovine milk (L2006-5MG), Acetic Acid (CH_3COOH; 27225), Methanol (CH_3OH; 947.046.2500), Ethanol (C_2H_5OH; 920.027.2501)) were purchased from Sigma-Aldrich Chemical (St Louis, Missouri, USA).

Ethylene diamine tetra acetic acid disodium salt (EDTA; EDT001.500), 2- amino-2-hidroymethyl-propane-1,3-diol (Tris; TRS001.1), Boric Acid (H_3BO_3; BOR001.500) were purchased from BioShop Canada (Mainway, Burlington, Ontario).

De Man-Rogosa-Sharpe (MRS; 1.106.610.500), Anaerocult A (113829.0001), Anaerotest (115112.0001) were purchased from E.Merck (Darmstadt, Germany).

1 kb DNA Ladder (D010-500), Kanamycin A ($C_{18}H_{38}N_4O_{15}S$; K-120-5) were purchased from Gold Biotechnology (U.S.)

CoomasieBrillant Blue R-250 ($C_{45}H_{44}N_3NaO_7S_2$; 0472) was purchased from

Amresco Biochemicals (Parkway Cleveland, OH, ABD).

QuickClean II gel extraction kit (L00418) was purchased from GenScript (Piscataway, NJ, USA).

Expresso Rhamnose Cloning and Expression Kit (49011-1) was purchased from Lucigen (Middleton, WI, USA).

Qubit protein assay kit (Q33211) was purchased from Thermo Scientific (Life Technologies, Grand Island, NY).

RedSafe Nucleic Acid Staining (21141) was purchased from Intron (Burlington, MA, USA).

LB-Broth-Miller (Luria-Bertani; J106), Ampicillin (0339) were purchased from Amresco Inc. (West Chester, USA).

3.1.3. Instruments Used in Molecular Analysis

In this study, autoclave (Nüve), incubator (IndemNüve EN 400), -80°C freezer (Operon), Thermal Cycler (Bio-Rad), Millipore pure water system, Gel Imaging System (VilberLourmat, France), horizontal electrophoresis and power supply (Thermo), sensitive scales (Shimadzu), NanoQuant DNA concentration meter (Tecan Infinite® M200 PRO), i-control™ microplate reader software (Tecan Infinite® M200 PRO) , -20 °C freezer (Bosch), refrigerator (Bosch), cooling centrifuge (Hettich), heated stirrer (Wisd), mini centrifuge (CF-5 Wisd), Vibra Cell/Sonicator (Sonics), Shaker (Benchmark), vortex (Vortex Genıe 2), dry block heater (BSH1002 Benchmark), Centrifuge (Beckman Allegra X-15R), Vertical Electrophoresis System (Thermo), Shaker (Benchmark-Incu Shaker 10LR), Shaker (Major Science Waver Shakermicrowave (Bosch), pH meter (IsoLab), fume cupboard (Setra), BrukerrapifleX Tissue typer MALDI mass spectrometer (Germany, Bremen), lyophilizer (Christ), ice machine (izmak) were used.

3.1.4. Nutrient Media Used for Growth

Solid Nutrient Media; LB (Luria-Bertani; Amresco) + Agar (Sigma-Aldrich), and liquid media LB (Luria-Bertani; Amresco) broth media were used for growth of recombinant *E. coli* 10G cells. After surface sterilization, recombinant *E. coli* 10G cells, which contain the pRham Vector, were taken to culture in LB solid and liquid media contain the 30 µg/mL kanamycin. They were kept at 37°C. Recombinant *E. coli* 10G cells, which contain the pUC19 vector were taken to culture in LB solid media contain the 100 µg/mL ampicillin. They were kept at 37 °C. White colonies were observed when

recombinant *E. coli* 10G cells were grew in solid media.

MRS (Merck Milipore) broth + L-cys (0.05%) (Sigma-Aldrich) liquid medium was used as culture media for *B.infantis*. The cells were growth at 37 °C for 72 hours anaerobic conditions provided.

3.2. Method

3.2.1. Preparation of Whey Proteins for Deglycosylation

After the commercial purchase of whey protein powder, potential impurities and fat must be separated. For this purpose, 10 g of whey protein powder was dissolved in 40 mL of water with a vortex. After separating the foam formed by vortexing, it was centrifuged for 20 minutes at 4000 rpm to remove fat layer. The samples were re-centrifuged by adding 1:4 cold ethanol and the proteins were precipitated to remove the supernatant containing all other compounds. The resulting pellet was dried by lyophilizator and homogenized in mortar and stored at -20 °C until deglycosylation step.

3.2.2. Anaerobic Growth Condition of *Bifidobacterium longum* subsp. *infantis*

The modified MRS (Merck Milipore) medium added with L-cys (0.05%) (Sigma-Aldrich) was used as the medium. L-cysteine was added for lower the redox potential and provided better anaerobic media for the growth of bifidobacteria. Also, L-cysteine is an essential nitrogen source for these bacteria (Anonymous, 2003). In order to create anaerobic conditions, 2.5 liters of anaerobic jar (Merck), Anerocult A (Merck) - to keep the internal oxygen - and Anerotest (Merck) - to show that internal oxygen is depleted - were used (Figure 4).



Figure 4**.** Anaerobic jar used to create the anerobic environment, Anaerotest and Anaerocult A

Preperation of Modified MRS Media: 5.2 g MRS broth was dissolved in 100 mL of distilled water. After autoclave (at 121 °C for 15 minutes), 0.05 g L-cysteine was dissolved in 1 mL distilled water and added to MRS broth with 0.2 µm sterile syringe filter (Corning).

Cells were cultured in anaerobic media at 37 °C for 72 hours. 0.5 mL pre-sterilized (at 121 °C for 15 minutes) 50% glycerol was added with 1.5 mL growing culture into cryotubes. 20% glycerol stocks were kept at -80 °C until use (Figure 5).



Figure 5. Anaerobic system used for *B. infantis* development

Molecular Cloning of *Blon_2468* Gene Encoding Endo-*β*-*N*-Acetylglucosaminidase Enzyme

Expresso Rhamnose Cloning and Expression System (Lucigen) kit was preferred for molecular cloning (Figure 6). This kit provides faster and more reliable application than other conventional cloning systems. In this system, also known as *in-vivo* cloning system, the PCR product can be cloned by mixing with the vector and competent cell, which were provided by the kit, without any other enzymatic application. In this system, the vector contains 18 nucleotides at each sticky ends. The primers required for gene amplification were designed to suit these ends. Thus, strong binding can be achieved. After cloning, protein production was induced by rhamnose and purification was carried out by binding to nickel column with 6xHistidine tag which was found in vector.

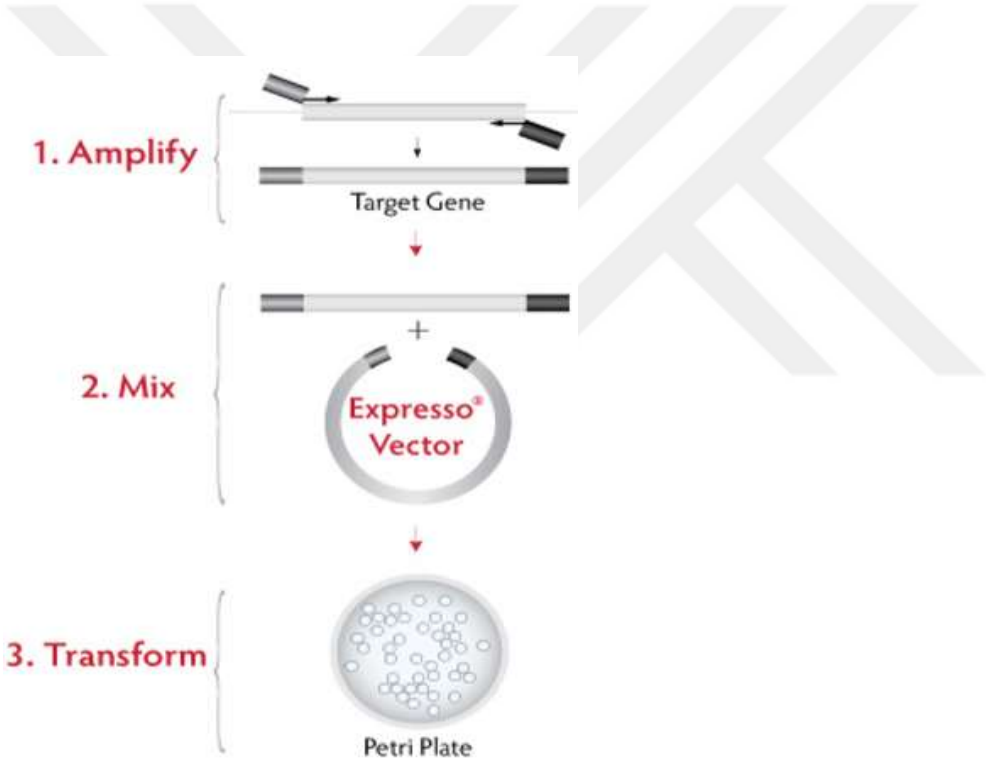


Figure 6. Expresso Rhamnose Cloning System

3.2.3. Primer Designing for PCR Amplification of *Blon_2468* Gene of Interest

For the primer designing, the amino acid and nucleotide sequences of *Blon_2468* gene were first obtained from the img.jgi.doe.gov database. Amino acid sequence is given below, and red sequences are known as transmembrane domain, which provides to enzyme to remain attached at the outer side of the cell. In order to produce this enzyme as recombinant in *Escherichia coli*, this region was not replicated, only black region was replicated.

Amino acid sequence:

MTFIKQMMPRYVASMTAGIVAAAMAATCAFAPVANADAVSPTQETIQSTGRHFM
VYYRAWRDVTMKGVNTDLPDDNWISMYDIPYGVDVVNIFSYVPSGQEEQAQPFY
DKLKSDYAPYLHSRGIKLVRGIDYTGVAVNGFRTFMKEQNKTESEATEADYDAY
AKQVIDKYMISVGLDGLDIDMEAHPNDADVKISDNVIRALSKHIGPKSAKPDTTMF
LYDTNGSYLNPFKNVAECFDYVAYQQYGSSSDRTARAAADYQPYIGNEFVPGLTF
PEEGDMNNRWYDATEPYEESHFYQVASYVREHNLGGMFVYALDRDGRNYDEDL
RRIVPSNLLWTKTAIAESEGMALDTAKTAANHYLDRMSLRQVIDDNAASADKAR
DMVGKAANLYETNKAVLGGDYGEGFSNTYDPTLEAGLLGIDISVLQQQIDKSSEII
GADTAESDAKTALRMARDAAIDGLTGKIYTADQVSAWSQALKAALDATVPVPTP
DSTDQNGNRDKVTNHKVQGQPKQLSATGISTDIIVAVGVTLAIAGVALSLSRKLS

Nucleotide sequence:

ATGACTTTCATCAAACAGATGATGCCGCGTTATGTGGCGTCTATGACCGCAGG
CATCGTAGCGGCAGCGATGGCCGCAACATGCGCGTTTGCACCGGTAGCGAATG
CGGACGCCGTTTCTCCGACACAGGAAACCATACAATCGACCGGTCGCCATTTC
ATGGTGTACTACCGCGCGTGGCGTGATGTCACGATGAAGGGTGTCAACACCGA
CCTTCCAGACGATAACTGGATTTCGATGTACGATATTCCGTACGGCGTCGACGT
GGTCAATATATTCAGCTATGTTCCATCGGGACAGGAAGAACAGGCGCAACCGT
TCTACGATAAGCTCAAATCTGATTATGCACCGTACTTGCATTCGCGTGGCATCA
AGCTGGTTCGGGGCATTGATTACACCGGCGTGGCGGTCAATGGTTTTCGTACTT
TCATGAAAGAGCAGAACAAAACCGAGAGCGAGGCGACCGAAGCTGATTATGA
TGCCTATGCTAAGCAAGTAATCGATAAATACATGATCTCCGTTGGCTTGGATG
GTCTGGATATCGACATGGAGGCGCACCCGAATGATGCCGATGTGAAGATCTCC
GACAATGTGATTCGTGCACTGTCCAAACACATCGGTCCCAAATCCGCCAAGCC
GGATACCACGATGTTCCTTTATGACACGAATGGATCTTATCTCAATCCGTTCAA
GAATGTGGCGGAATGCTTTGACTACGTGGCATACCAGCAGTACGGTTCTTCCT
CCGATCGCACCGCTAGGGCCGCCGCCGATTATCAGCCTTATATCGGCAACGAG
TTTGTGCCGGGCCTGACGTTCCCCGAAGAAGGGGACATGAACAATCGCTGGTA
TGATGCCACCGAGCCGTATGAAGAAAGCCATTTCTATCAAGTGGCGTCATATG
TGCGTGAGCATAATCTTGGCGGCATGTTCGTTTACGCGCTCGACCGAGACGGT
CGCAACTATGATGAAGACCTGCGTCGGATTGTACCGTCCAACTTGCTGTGGAC
CAAGACCGCCATTGCGGAGAGCGAGGGCATGGCGTTGGATACGGCAAAGACT
GCAGCGAACCATTACCTTGATCGCATGTCGTTGCGTCAGGTGATTGACGACAA

CGCCGCATCCGCGGATAAGGCGCGGGATATGGTGGGGAAGGCCGCTAACCTA
TACGAGACAAATAAAGCAGTCCTTGGTGGTGACTACGGAGAAGGTTTCTCCAA
CACATATGATCCGACATTGGAAGCTGGATTATTAGGGATTGACATCTCCGTGTT
GCAACAGCAGATTGATAAATCCAGTGAGATCATTGGTGCCGATACGGCGGAAT
CGGATGCAAAAACTGCACTCCGTATGGCACGTGATGCCGCCATCGATGGTTTG
ACCGGCAAGATCTACACAGCGGACCAAGTATCAGCGTGGTCGCAGGCATTGA
AGGCGGCGCTTGATGCCACCGTGCCGGTTCCGACGCCAGATTCCACCGATCAG
AATGGCAACCGTGACAAGGTTACCAATCACAAAGTGCAAGGCCAACCGAAGC
AACTGAGTGCGACCGGCATCTCGACGGATATTATCGTTGCCGTAGGCGTGACT
CTTGCCATCGCAGGGGTGGCTCTGTCTCTATCACGTAAGCTCTCCTGA

The primary design was performed considering all primary design rules. The blue and green sequences were added for binding to vector and the black sequence was added for necessary sequence to gene amplification (Table 3).

Table 3. Designed primers in order to molecular cloning

Primer	Base Sequence (5'-3')
Blon_2468F	CATCATCACCACCATCACAATGCGGACGCCGTTTCTCCG
Blon_2468R	GTGGCGGCCGCTCTATTAGCCGGTCGCACTCAGTTGCTT

An initiation codon was not added to forward primer. Because pRham N-His Vector has the ATG start codon near the 6xHis codon.

Blunt PCR product fuses with the vector in the host cell during recombination. The sticky ends of pre-linearized vector are not compatible each other, so there is no self-ligation risk in this method and obtained directional cloning (Figure 7).

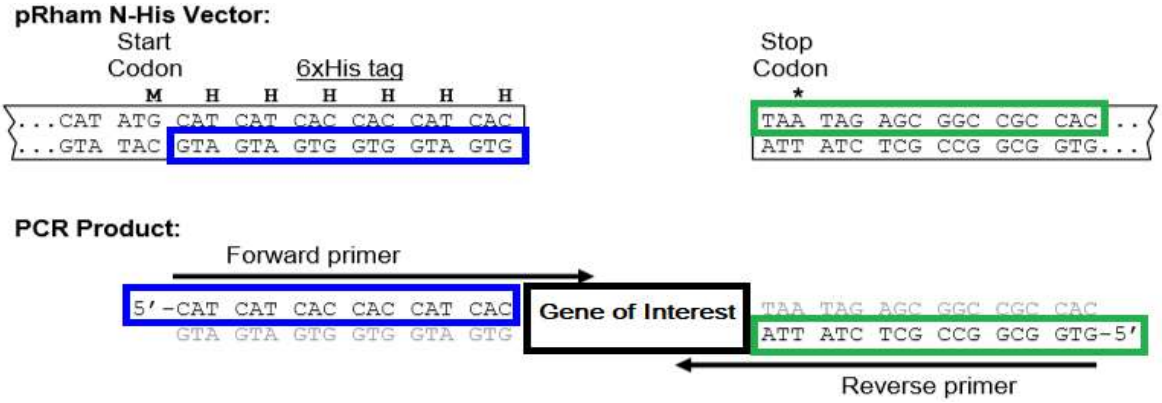


Figure 7. The sticky ends of PCR product and pre-linearized vector

3.2.4. PCR Amplification of *Blon_2468* Gene of Interest from *B.infantis* genomic DNA

The amplification of the gene of interest was performed as shown in a previous study (Karav et al., 2015).

Table 4. PCR mixture

Components	Volume	Stock Concentration	Final Concentration
Water	21 µL	-	-
Forward Primer	1 µL	10 µM	0.2 µM
Reverse Primer	1 µL	10 µM	0.2 µM
Master Mix	25 µL	2X	1X
DNA	2 µL	-	-
Total Volume	**50 µL**		

PCR mixture protocol was shown in Table 4. Amplification was carried out in 50 µL containing 2 µL DNA, forward and reverse primers at 0.2 µM and PCR Master Mix (2X) (Thermo Scientific) containing reaction buffer, 0.05 U/µL Taq DNA polymerase, 0.4 mM of each dNTPs and 4 mM $MgCl_2$. After the mixture was prepared, the PCR tubes were transferred to the Bio-Rad thermal cycler. Process was carried out as shown below:

First denaturation

1 cycle: (95 °C) 3 min (to release genomic DNA by breaking down cells) first denaturation

PCR cycle

35 cycles: (95 °C) 30 sec (Denaturation)

(60 °C) 30 sec (Annealing)

(72 °C) 1 min (Extension)

Final Extension

1 cycle: (72 °C) 10 min

3.2.5. Agarose Gel Electrophoresis

The electrophoresis assay was carried out in 1% agarose gel which was stained using 5 µL/100 mL of Red Safe Nucleic Acid Staining (Intron) dye, for 70 minutes at 100 volts with 1X TBE buffer. PCR products were visualized under gel imaging system ST4 1100 (VilberLourmat, France).

3.2.6. DNA Purification from Agarose Gel

700 mg gel containing the amplified gene region was removed and purified with GenScript QuickClean II gelextraction kit (GenScript, Piscataway, NJ, USA), (Figure 8). The concentration of the obtained DNA and the 260/280 ratio were then measured by using the Infinite 200 PRO NanoQuant Spectrophotometer. DNA concentration was determined as 25 ng/µL. Since this value was above the minimum concentration required for cloning, the cloning step was performed at this concentration. Purified PCR product was stored at -20 °C.

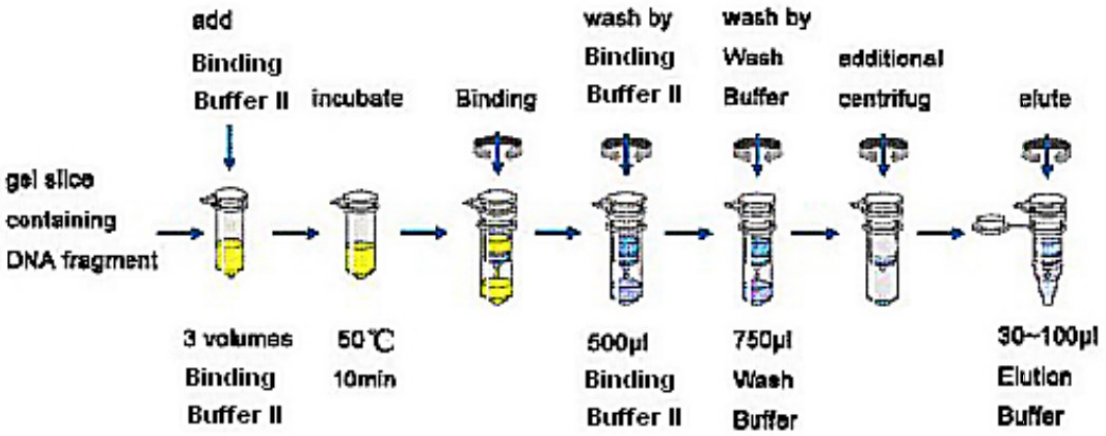


Figure 8. DNA agarose gel purification procedure

3.2.7. Molecular Cloning

For molecular cloning, pRham™ N-His cloning kit (Lucigen, Middleton, WI, USA) was used. The transmembrane domain regions were not amplified to facilitate protein expression and purification. This vector contains the kanamycin resistance gene in order to separate the positive transformants and 6xHistidine chain in the N-terminal region to perform purification (Figure 9).

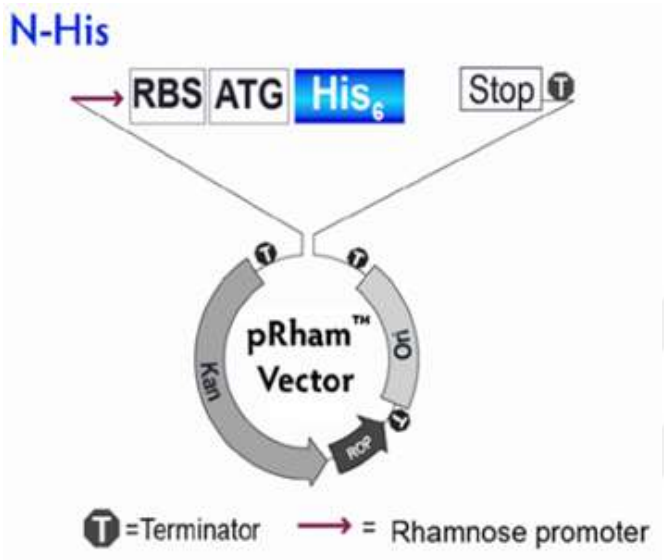


Figure 9. Vector used for cloning

Prior to the cloning process, 'the recovery medium' which was provided by the kit (the medium used for rapid recovery of cells after cloning) was taken from -80 °C to room temperature. Then, the *E. coli* 10G cloning cells were taken from -80 °C freezer and thawed completely on wet ice. 2 µL of (25 ng) pRham N-His Vector DNA and 2 µL of PCR product were added to 40 µL of cloning cells and stirred briefly with pipet tip. They have not been pipetted up and down for preventing the cells to warm and the air bubbles. This mixture was transferred to a 15 mL falcon tube and kept on ice for 30 minutes. Then thermal shock was carried out in 42 °C water bath for 45 seconds to insertion of the vector and PCR product into the cell. This mixture was then quickly contacted with ice for 2 minutes to allow cell pores to close. After transformation, 960 µL recovery medium was supplemented to cells for allowing rapid repair of the cells and were incubated at 37 °C for 1 h at 250 rpm. 100 µL of these transformed cells were taken to culture on LB-agar containing 30 µg/mL kanamycin plates. In this experiment, only *E. coli* 10G cells were taken to culture on LB-agar containing 30 µg/mL kanamycin plates as the negative control.

Since these cells do not have vector containing antibiotic resistance gene, no growth should be observed. In this way, the contamination and the stability of the antibiotic have also been tested. As a positive control, the pUC19 vector provided by the kit was screened on LB-agar plates with 100 µg/mL ampicillin. So, the accuracy of our transformation protocol was tested with this control group.

3.2.8. Confirmation of the Gene of Interest by Colony PCR

To confirm the presence of pRhamTM-N-His transformants carrying the *Blon_2468* genes, the colony PCR assay was carried out. Thirteen randomly selected colonies were amplified by two different primer sets (sequencing primers 'pRham forward and pETite reverse' provided by the kit and primers which was designed in order to gene of interest amplification at the first stage). Sequencing primers (Table 5) bind to the immediate outside of the cloning site (Figure 10), such that if the gene of interest was inserted successfully into cloning site, it can amplify this region and after the DNA gel electrophoresis, we can see a band of the size of the gene. In case of desired gene cannot be inserted successfully, since there is no region for amplification by these primers, there is no-band on the gel. In this way successful transformants can be separated. Likewise, our designed primers (Table 3) bind to the first and last 20 nucleotide sequences of the gene of interest. So, gel electrophoresis results in a band that is 50 bp less due to the region to which they bind, compared to the sequencing primers.

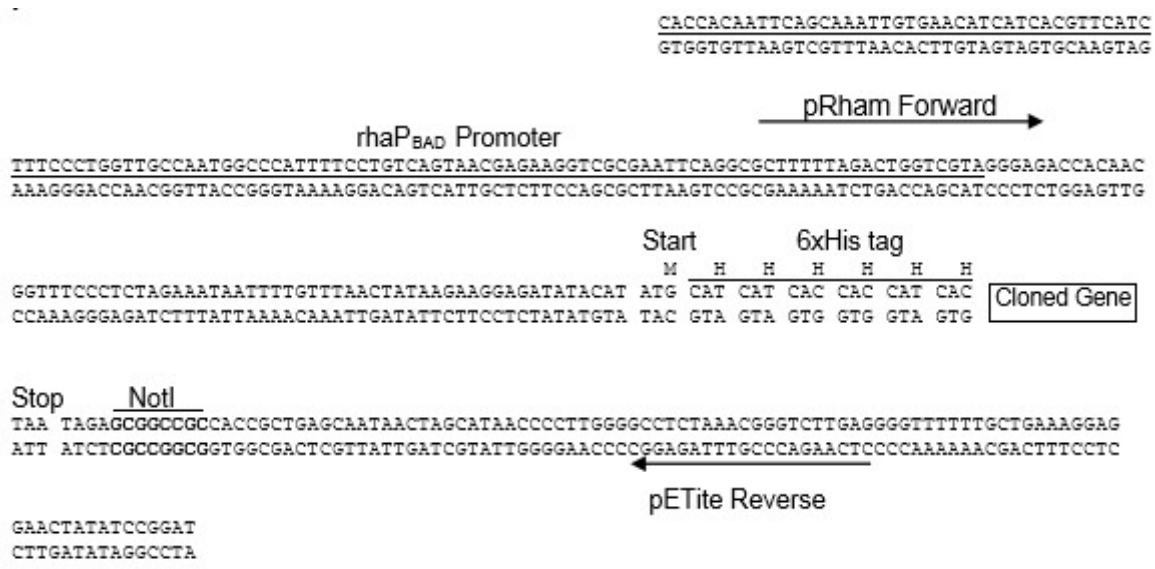


Figure 10. The sequence of pRham vector and sequencing primers strategy

At the beginning of this experiment, a part of each colony was taken into PCR tube to be used in PCR amplification and other part of each colony was inoculated to 5 mL LB + kanamycin medium in a 15 mL falcon tube to use in case of positive results. Amplification was carried out in 50 µL containing a part of colony (for each thirteen randomly selected colonies), forward and reverse primer sets at 0.2 µM and PCR Master Mix (2X) (Thermo Scientific) containing reaction buffer, 0.05 U/µL Taq DNA polymerase, 0.4 mM of each dNTPs and 4 mM $MgCl_2$. After the mixture was prepared, the PCR tubes were transferred to the Bio-Rad thermal cycler. Process was carried out as shown below:

First denaturation

1 cycle: (95 °C) 3 min (to release genomic DNA by breaking down cells) first denaturation

PCR cycle

35 cycles: (95 °C) 30 sec (Denaturation)

(60 °C) 30 sec (Annealing)

(72 °C) 1 min (Extension)

Final Extension

1 cycle: (72 °C) 10 min

Table 5. Sequence of sequencing primers

Primer Name	Base Sequence (5'-3')
pRham forward	GCTTTTTAGACTGGTCGTAGGGAG
pETite reverse	CTCAAGACCCGTTTAGAGGC

PCR products were run on 1% agarose gel stained with 5 µL/100 mL of RedSafe Nucleic Acid Staining (Intron) for 70 minutes at 100 volts for 70 minutes and screened with the gel imaging system ST4 1100 (VilberLourmat, France).

The plasmids of positive results were isolated with GeneJET Plasmid Miniprep kit (Thermo Scientific). DNA concentrations were determined at A260/A280 wavelength in NanoQuant instrument (Tecan Infinite M200 PRO). After isolation, obtained DNA for five positive samples (42.1, 55.5, 62.3 and 145 ng/µL) were sequenced in Genetic Analyzer (MedSanTek Lab, Istanbul) for final confirmation.

3.2.9. Protein Production and Purification

Recombinant protein production was carried out using the 6xHistidine tag that found in the vector used. In this system, recombinant proteins can be bound to nickel columns by the 6xHistidine tag, while the proteins produced by *E. coli* 10G cells were removed from the system. The five falcon tubes, each containing 30 mL LB + 30 µg/mL kanamycin medium were inoculated with 100 µL of recombinant *E. coli* 10G cells and they were growth for 6 hours at 200 rpm and 37 °C until the cell density reached approximately O.D = 0.6. Protein production was stimulated by addition of rhamnose (0.2% final concentration), and the cells were incubated overnight at 37 °C. Bacterial pellets were taken by centrifugation at 4,000 x G (Beckman Allegra X-15R; Falcon) and 4 °C for 15 minutes. All subsequent steps were performed at 4-8 °C. 5 mL of lysis buffer was mixed with the pellets and then mixture was placed on ice for 30 minutes. The lysed cells were centrifuged at 12,000 x G (Beckman Allegra X-15R; Falcon) for 10 minutes to remove cell debris. To ensure that all of the protein bound to the resin, protein was allowed to stand for 1 hour at 100 rpm after the addition of 1 mL Ni-NTA resin. The protein was purified using affinity chromatography with 15 mL pre-coated Ni-loaded columns (Bio-Rad, Hercules, CA) (Figure 11). The mixture was washed with 5 mL washing buffer to get rid of potential impurities. Then 5 mL elution buffer was used for elution of resin-attached protein. The purified protein was concentrated by using a 30-kDa-cut off centrifugal filter device (15 mL) (Millipore) and the EndoBI-1 purity was determined by SDS-PAGE. The concentration of protein was measured using Qubit (Life Technologies) and the purified enzyme was kept at −80 °C.



Figure 11. Nickel resin system for protein purification

SDS-PAGE assay was performed with concentrated EndoBI-1 enzyme to determine purity. To compare the molecular weight of proteins, SeeBlue™ Pre-stained Protein Standard (Invitrogen) was used. 2 µL, 5 µL, 10 µL, and 20 µL samples taken were incubated with Bolt™ / NuPAGE™ Reducing Agent (10X) (Novex) and gel-compatible Bolt™ LDS Sample Buffer (4X) (Novex) throughout 10 min at 70 ˚C. After incubation, samples were touched to ice for 2 minutes in order to prevent the continued high temperature and then 40 µL of each sample was taken and loaded to a 4-12% Bis-Tris (Invitrogen) precast gel. The samples were run at 200 V for 25 min with 20X MES SDS Running Buffer (Novex) (Thermo Scientific-EC 300 XL). Proteins separated according to their molecular weight were stained with Coomassie Brilliant Blue at 55 rpm for 1 day. To ensure clarity of the bands, destaining solution was applied for 3 hours and was changed at intervals. When the gel image was evaluated, it was seen that EndoBI-1 enzyme which was loaded at different concentrations, was obtained with a high purity.

3.2.10. Determination of Recombinant Enzyme Activity on the Model Protein Lactoperoxidase

The activity of the purity-verified EndoBI-1 enzyme was tested by the SDS-PAGE gel mobility assay on the model protein lactoperoxidase. The molecular weight of lactoperoxidase (78 kDa) shows a 10% molecular weight decrease after enzymatic

deglycosylation, and this difference can be detected on the SDS PAGE gel. Therefore, it is a common method used in the activity determination of glycosidases. To compare the molecular weight of proteins, SeeBlue™ Pre-stained Protein Standard (Invitrogen) was used. 13 µL of dH_2O and 3 µL of lactoperoxidase (2.4 mg/mL) were put into two eppendorfs (2.0 mL) and denatured at 95 ˚C throughout 5 min. After incubation period, the tubes were placed onto ice and allowed to cool and then 5 µL of sodium phosphate buffer (0.2 M, pH = 5) was added to both tubes. 5 µL of dH_2O was added into the first tube (control tube) and 5 µL of EndoBI-1 enzyme was added into the second tube these mixtures were incubated throughout 1 hour at 160 rpm and 37 ˚C. Following incubation, samples were re-incubated with Bolt™ / NuPAGE™ Reducing Agent (10X) (Novex) and gel-compatible Bolt ™ LDS Sample Buffer (4X) (Novex) for 10 minutes at 70 °C. Samples taken from the incubation were waited on ice for 2 minutes to prevent the high temperature from continuing and then 40 µL of each sample were loaded to a 4-12% Bis-Tris (Invitrogen) precast gel. They were run at 200 volts for 25 minutes with the 20X MES SDS Running Buffer (Novex) (Thermo Fisher -EC 300 XL). Proteins separated according to their MW stained with Coomassie Brilliant Blue at 55 rpm for 1 day. To ensure clarity of the bands, destaining solutionwas applied for 3 hours and was changed at intervals.

Immobilization of Recombinant Enzyme by Different Techniques

Enzyme immobilization is important for the recovery of the enzyme, the inhibition of enzyme contamination in the obtained product, and in some cases manipulating the activity of the enzyme. In addition, recombinant enzymes are generally unstable and sensitive to experiment conditions such as changing pH, substrate concentration and temperature. Enzyme immobilization can decrease or even eliminated limitations. Although very different immobilization methods have been developed, there are risks such as low enzyme activity and the separation of the enzyme from the bound matrix depending on the strategy used. Therefore, determine the optimum strategy by trying different immobilization methods were aimed in this study. The obtained recombinant enzyme was immobilized by four different techniques (alginate adsorbtion (1) and entrapment (2) sulfo-link (3) and amino-link (4) covalent bond) techniques (Figure 12).

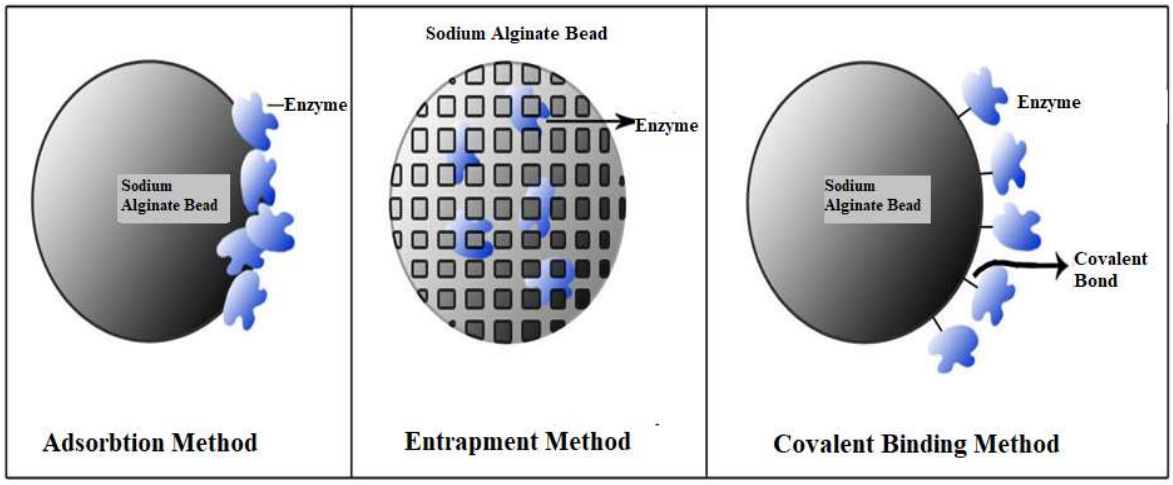


Figure 12. Strategies based on immobilization methods tested in the project; adsorbtion, entrapment and covalent binding

3.2.11. Enzyme Immobilization by Adsorbtion Method

The adsorbtion method is the adherence of the enzyme molecule to a matrix by non-covalent weak interactions. This process can be carried out in the polymer matrix or in the gel as well as in semi-permeable membranes or with microcapsules / micelles. The most important feature that differentiates this technique from other immobilization techniques is that the enzyme molecule is not bound to matrix with a physical or chemical force. Thus, the risk of blockage enzyme active site decreases and increases the chance of high activity. However, weak interaction causes easy separation of the enzyme from the matrix and therefore decreases the rate of re-use of the enzyme.

Sodium alginate was used as support material in this experiment. In addition, sodium acetate buffer solutions at different pH (6.34, 4.83 and 3.63) were used to determine which pH intervals were more efficient.

To prepare 0.1 M 100 mL $C_2H_3NaO_2$ buffer solution, 1.3608 g $C_2H_3NaO_2$ was dissolved with 100 mL of distilled water then was measured pH as 7.88. The pH of buffer solutions (6.34, 4.83, and 3.63) were adjusted with 50% HCl. For 2% sodium alginate solution preparation, 50 mL dH_2O was added to 1 g of sodium alginate and mixed. A 5% $CaCl_2$ buffer solution was prepared to form sodium alginate beads. 1.25 g of $CaCl_2$was dissolved in 25 mL of dH_2O in 50 mL beaker. 2% Sodium Alginate solution was slowly dropped with G22 syringe to the 5% $CaCl_2$ solution in a beaker. Approximately 7 mg and 1 mm beads were obtained, and 10 beads were made for each of three different pH values (Figure 13).

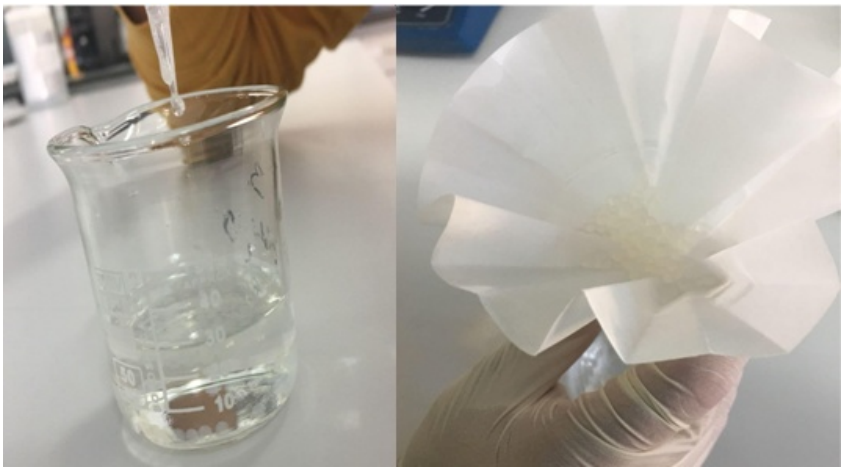


Figure 13. Preparation stage of alginate beads used for immobilization

30 beads were stirred in 5% calcium chloride buffer solution with the magnetic stir bars about 5 minutes. The beads were filtered through filter paper and washed with distilled water. After 1 hour, the 3.4 mL of $C_2H_3NaO_2$ buffer solution at different pH (6.34, 4.83 and 3.63) with 1 mL of endo-*β*-*N*-acetylglucosaminidase enzyme (1,88 mg/mL) were stirred with the magnetic stir bars in beakers. While the mixing process was in progress, 10 beads were put in the solutions of each beaker and continued the mixing for 60-90 minutes. At the end of process, the concentration of the unbound enzyme was measured with Qubit (Life Technologies). The amount of bound enzyme was determined by subtracting the unbound enzyme concentration from the concentration of initial enzyme.

3.2.12. Enzyme Immobilization by Entrapment Method

In this method, as in the previous method, sodium alginate support material was used. However, the strategy in this method is not based on ionic interaction of the enzyme to the matrix surface as in the adsorbtion method. It is based on the entrapping of the enzyme into beads during the formation of alginate beads. The most important criterion in this method is the concentration of prepared alginate beads. Sodium alginate solutions with a concentration of 2%, 3% and 4% were used to compare which sodium alginate concentration is more efficient. Firstly, 5% calcium chloride buffer solution was prepared to make sodium alginate beads. 3.75 g of calcium chloride was dissolved with 75 mL of dH_2O and 25 of 75 mL were transferred into three different beakers. 0.33 g of sodium alginate was first added to 8 mL of dH_2O and mixed to prepare sodium alginate solution. Thus, 4% solution was prepared kind of stock solution. Then, 2% and 3% solutions were prepared from this solution. 2.5 mL from produced enzyme as recombinantly (1.88

mg/mL) was added to the three different beakers which included sodium alginate solution at three different concentrations. Then, these solutions (final volume = 5 mL for each) were slowly dropped (with a syringe G22) to 5% calcium chloride solution which was stirred with the magnetic stir bars. Thus, when the beads were formed in the calcium chloride buffer solution, they were entrapped the enzyme. This system was stirred for 10 minutes and then Qubit (Life Technologies) was used to measure concentration of non-entrapped enzyme. The amount of enzyme entrapped was determined by subtracting the non-entrapped enzyme concentration from the initial enzyme concentration.

3.2.13. Enzyme Immobilization by SulfoLink Covalent Binding

The SulfoLink immobilization kit (Thermo Scientific) contains all necessary components for covalent binding of sulfhydryl sites present in the protein to the agarose support material (Figure 14).



Figure 14. The SulfoLink immobilization kit components

The agarose resins provided with the kit contain iodoacetyl groups which can react specifically with free sulfudryl at pH 7.5-9. With the 12-atom spacer arm, it aimed to solve the problem of low activity which is the biggest disadvantage of covalent binding of enzymes (Figure 15).

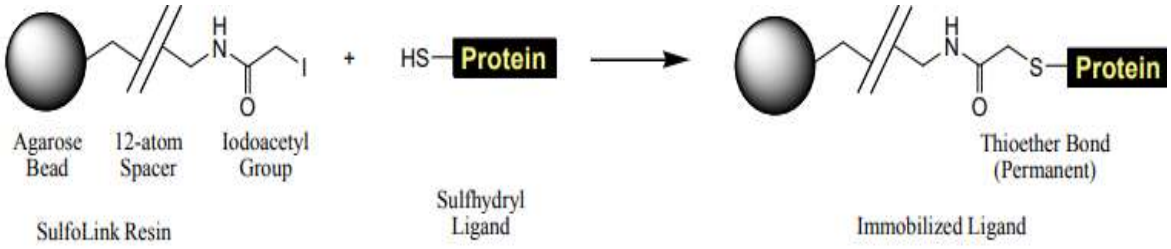


Figure 15. Principle of SulfoLink immobilization

For this technique, 900 µL of the recombinant enzyme solution (1.88 mg/mL) was first mixed with 1 mL sample preparation buffer. The prepared sample was transferred to a tube containing 6 mg of 2-MEA (50 mM) provided with the kit and incubated throughout 1.5 hoursat 37 °C. Then, 2 mL of coupling buffer solution was added to the desalting column in a 15 mL falcon tube and centrifuged at 1000 rpm and 25 °C throughout 1 min. This process was performed once more. When the enzyme sample achieved thermal equilibrium with room temperature, it was injected by pipette to the center of the walls of the column and transferred to the resin bed slowly. Then 0.1 mL coupling buffer solution was supplemented onto sample and centrifuged for 1 minute at 1000 rpm and 25 °C. After the centrifugation, 2 mL of coupling buffer solution was supplemented to supernatant. 0.1 mL of this reduced protein was stored for use in measurements of the protein immobilization efficiency. The enzyme obtained in this step was ready for the binding step to the SulfoLink Column.

SulfoLink column was mixed slowly by end-over-end, the top cap and the bottom tab were removed sequentially to avoid drawing air into the column. It was flowed through to a 15 mL falcon tube and centrifuged at 250 rpm and 25 °C throughout 1 minute. Thus, the storage buffer contained in the column was removed from the column. 2 mL of coupling buffer was addedinto throughout 1 minute at 1000 rpm and 25 °C. After this process was repeated one more time with 2 mL coupling buffer, the bottom tab of the column was closed, and 3 mL of the reduced enzyme sample prepared in the first step was added. The column was stirred throughout 15 minutes by end-over-end at room temperature. Then it was kept upright and incubated for 30 minutes at room temperature. After 30 minutes, the top and bottom column caps were taken out sequentially and the column was placed in a new 15 mL falcon tube. It was centrifuged throughout 1 minute at 1000 rpm and 25 °C. 0.1 mL of supernatant at the end of the centrifugation was stored for protein measurement and comparison (the supernatant contained that the amount of unbound enzyme. So, the amount of unbound enzyme was subtracted from the initial enzyme concentration. And the

amount of enzyme bound to column was calculated). The SulfoLink column was washed using 2 mL of washing solution and then centrifuged for 1 min at 25 °C and 1000 rpm. This step was carried out thrice again. Then, the column was washed with 2 mL of coupling buffer solution, after washing centrifugedfor 1 minute at 25 °C and 1000 rpm. 2 mL of degassed buffer was added to column to equilibrate the column for storage. This solution was prepared by mixing the chemical in BupH™ Phosphate Buffered Saline Pack provided by the kit, 500 mL of distilled water and 250 mg of sodium azide. The column was again centrifuged throughout 1 minute at 1000 rpm and 25 °C. This process was carried out thrice again. The bottom tab of column was closed, and 2 mL of degassed buffer solution was added. Finally, top cap was closed and stored at +4 °C.

3.2.14. Enzyme Immobilization by AminoLink Covalent Binding

The AminoLink immobilization kit (Thermo Scientific) was used and this immobilization method is based on covalent bonding of aldehyde functional groups in the support material and free amine groups in proteins used (Figure 16). In addition, a more stable immobilization is performed by Scihiff Base additional reaction (Figure 17).



Figure 16. The Amino Link immobilization kit components

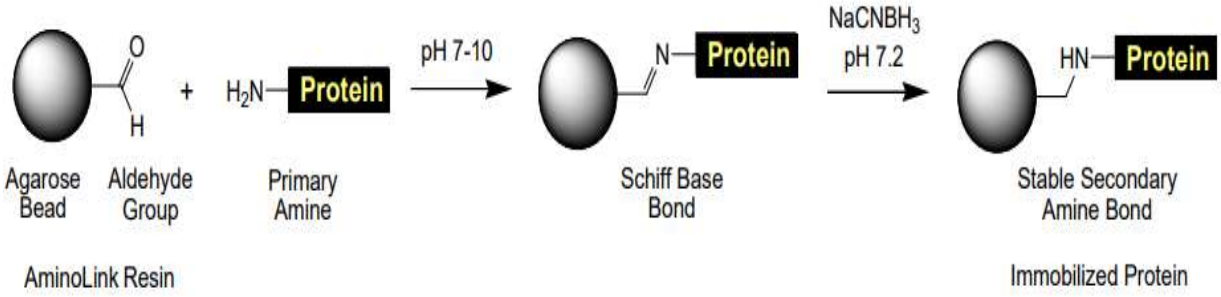


Figure 17. Principle of AminoLink immobilization

Before the immobilization assay, 250 mg of chemical (pH 10.0) in sodium azide and the BupH™ Phosphate Buffered Saline pack provided with the kit were dissolved with 500 mL distilled water. 4 mL of coupling buffer (pH 10.0) was used for 1 mL of enzyme solution.

The AminoLink Plus column was mixed slowly to provide intermal balance and homogen solution by end-over-end. The top cap and bottom tab were opened carefully. It was placed in a 15 mL falcon tube and centrifuged throughout 1 minuteat 240 rpm and 25 °C. Thus, the storage buffer contained in the column was removed from the column. 2 mL of coupling buffer was added to column then centrifuged throughout 1 minute at 1000 rpm and 25 °C. This process was repeated once more time and the bottom tab of the column was closed. 3 mL of the enzyme sample prepared in the first step was added to the column. 0.1 mL of this reduced protein was kept at +4 °C for use in measurements of the protein immobilization efficiency. Finally, the bottom tab of the column was closed, and the column was mixed for 2 hours. Then it was placed upright and incubated for 2 hours. At the end of 4 hours, the covers of the column were opened and put into a 15mL falcon tube, then centrifuged at 240 rpm and 25 °C for 1 minute. Thus, the unbound enzymes in the column were transferred to the falcon tube. 0.1 mL of sample was taken from the falcon and storedat +4 °C for comparison with the first sample. The column was washed using 2 mL of binding buffer solution (pH 7.2) and centrifuged for 1 min at 25 °C and 240 rpm. This process was carried out once again and bottom tab of the column was opened. In the fume hood, 2 mL of binding buffer solution (pH 7.2) was added to 40 µL of sodium cyanoborohydride in an eppendorf and mixed. This solution was transferred to column and top cap of column was closed. This column was incubated throughout 4 hours at room temperature by mixing. 2 mL of storage buffer was added and centrifuged for 1 minute at 25 °C and 240 rpm. This process was carried out thrice again. The bottom tab of the column was closed, and 2 mL of storage buffer solution was supplemented and finally, the

top cap was closed then kept at +4 °C.

Kinetic Characterization of Immobilized Enzymes

Kinetic characterization of EndoBI-1 enzyme immobilized by 4 different methods and characterization of separated glycans by mass spectrometry were performed. Whey protein powder containing high glycoprotein (such as lactoferrin and immunoglobulins) was used for kinetic studies.

3.2.15. Determination of Kinetic Parameters by Michaelis-Menten Model

The kinetic parameters of the produced EndoBI-1 enzyme on whey protein powder (WP) were determined according to Michaelis-Menten model. At the beginning of study, preliminary trials were conducted to determine the conditions that fulfill the [E] << [S] requirement. In these preliminary trials, the concentration of the product obtained using the constant enzyme concentration was observed (reaction curves) at different time intervals and at different substrate concentrations. For this purpose, 0.1-0.8 WG substrate glycans were cut with free-form enzyme were observed at 5 minute intervals for 50 minutes.

Lineweaver-Burk linear plotting technique was used for the calculation of kinetic parameters. This method is widely used because it minimizes error compared to non-linear graphs. In this method, 1/[S] vs. 1/V are used instead of [S] vs. V, and the known standard curve is linearized to give the slope *K*m/*V*max and the 1/*V*max point where the line intersects the y-axis (Figure 18).

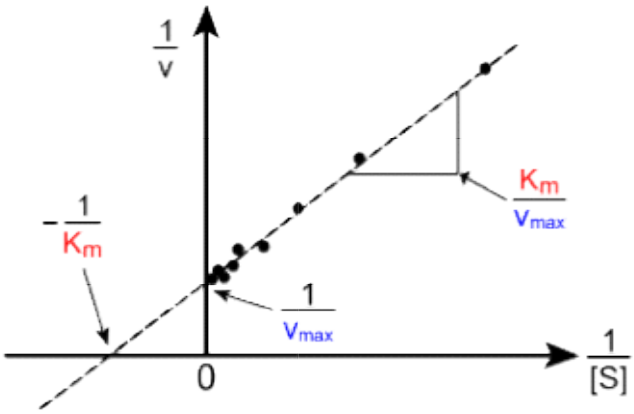


Figure 18. Lineweaver-Burk Plot

Lineweaver-Burke Equation;

$1/V_o = Km/V_{max} \times 1/[S] + 1/V_{max}$ (V_o; initial reaction rate, Km; Michaelis-Menten constant, V_{max}; maximum reaction rate, [S]; substrate concentration)

3.2.16. Characterization of Deglycosylated Glycans by Mass Spectrometry

Glycans cut from lactoperoxidase (LPO) and whey proteins (WP) by EndoBI-1 enzyme which was immobilized by different techniques were determined by MALDI-(TOF /) TOF-MS. 5 mg/mL substrate (total volume 200 µL) was incubated overnight at 37 °C for deglycosylation using free-form and immobilized forms enzymes (0.025 mg/mL).

3.2.17. Labeling of *N*-Glycans with 2-Amino Benzoic Acid (2-AA)

In order to obtain more effective results in mass spectrometry, the cut glycans were first labeled with 2-amino benzoic acid. Labeling was performed by modifying the method of Ruhaak et al. 50 µL of glycans (which were released by enzyme) solution was taken and 25 µL of 2-amino benzoic acid (48 mg/mL containing 15% (v/v) acetic acid and prepared in dimethylsulfoxide) and 25 µL of Pic-BH_3 (107 mg/mL prepared in dimethylsulfoxide) solutions were mixed by glycan solution. This solution was then incubated at 65 °C for 2 hours to complete labeling.

3.2.18. Purification of *N*-Glycans

A cotton-containing pipette tip was used for purification. For this purpose, cotton fibers were first placed on a 100 µL pipette tip. The ethyl esterified sample was then used as a loading solution in a separate eppendorf. The pre-prepared, cotton-containing pipette tip was first washed with 100 µL deionized water then with 100 µL 85% acetonitrile. The prepared loading solution was then passed through the cotton pipette tip at least 20 times. After this step, the pipette tip was washed 3 times with 85/14/1, v/v/v, acetonitrile/water/trifluoro acetic acid solution and 3 times with 85% acetonitrile to remove salt and other solvent residues. Elution was carried out with 10 µL of water.

3.2.19. MALDI-(TOF/) TOF-MS Analysis of *N*-Glycans (Ethyl-Esterified)

1 µL of the elution solution was dropped onto MTP 384 AnchorPlate and then dried. After this step, 1 µL of Super DHB matrix (w/w, 9/1, 2-5 dihydroxybenzoic acid/2-hydroxy-5-methoxy benzoic acid) solution (5 mg) which was prepared in 1/1, v/v, acetonitrile/1 mM NaOH was taken on this plate. After crystallization, the analysis was

carried out by MALDI mass spectrometer with BrukerrapifleX MALDI Tissuetyper (Germany, Bremen). Analyzes were performed by collecting at least 10000 laser shots in positive ionization in reflector mode. Before each analysis, BrukerrapifleX MALDI Tissuetyper^{TM} mass spectrometry was mass-calibrated with a peptide mixture. Mass spectra in the 1000-4000 mass range were obtained by applying an acceleration voltage of 25 kV. Analyzes of 2-amino benzoic acid-labeled *N*-glycan structures were performed using DHB (5 mg / mL, prepared in water) matrix and in the negative ionization mode using the method described above.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. PCR Product for Molecular Cloning by Designed Primers

PCR Amplification of *Blon_2468* from *Bifidobacterium longum* subsp. *infantis* genomic DNA resulted in a single band (1450 bp) in electrophoresis image (Figure 19), as expected.

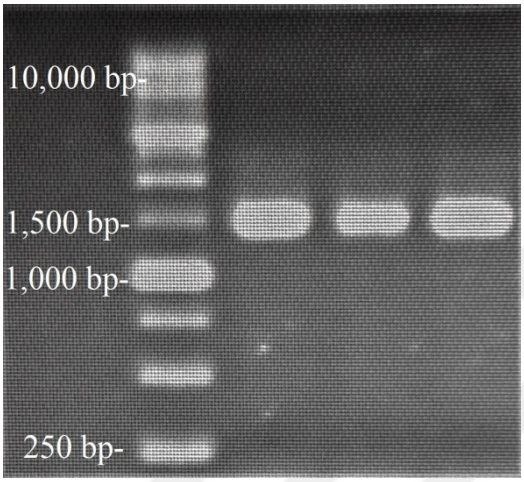


Figure 19. Gel image of PCR products with a gel imaging system

4.2. Transformation of Plasmid to *E. coli* 10G cells

After heat-shock transformation of plasmids (pRham vector) to *E.coli* 10G cells, they were taken to culture on plates overnight. 20-60 colonies were obtained after transformation (Figure 20). The growing of these cells on LB + agar + 30 µg/mL kanamycin media showed that the kanamycin-resistant vector was successfully transformed into the cell. However, it does not prove that the gene of interest was inserted into the vector successfully. The colony PCR assay was carried out for the next step to confirm insert gene in the vector. For this, the colony PCR assay was needed perform to confirm the gene of interest in pRham vector.



Figure 20. Obtained colonies after transformation

4.3. Results of Colony PCR for Gene of Interest in the pRham Vector

As a result of colony PCR using two primer sets showed that 5 colonies of 13 samples were succesful. As a result of the gel image, it was seen that the desired gene region was amplified from the samples taken from colonies 2, 3, 8, 12 and 13 (Figure 21). Thus successful transformants were distinguished from unsuccessful colonies. According to these results, 50% glycerol stocks were prepared from liquid cultures obtained from these colonies. The result of sequencing of plasmids confirmed our results. After all the controls of cloning, it was ready to move from the obtained recombinant cells to the enzyme production step.

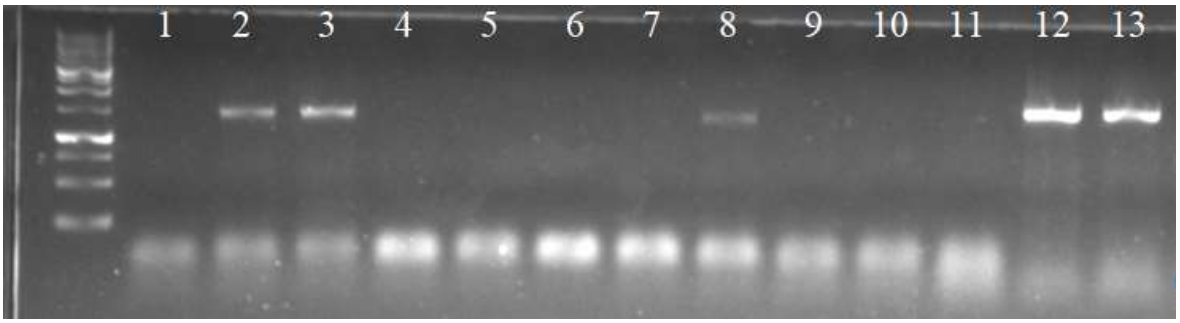


Figure 21. Gel image of Colony PCR with a gel imaging system

4.4. Control of the Enzyme Purity Obtained Recombinantly

After SDS-PAGE assay, when the gel image was controlled, it was seen that EndoBI-1 enzyme which was loaded at different concentrations, was obtained with a very high purity (Figure 22).

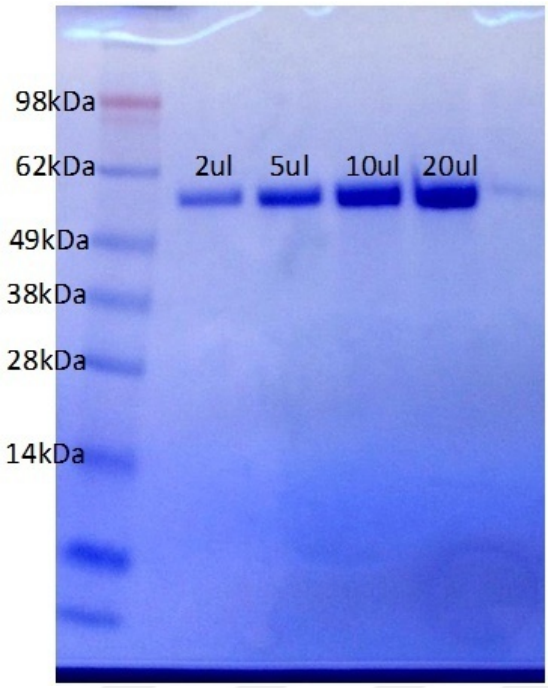


Figure 22. Control of the recombinant EndoBI-1 enzyme purity by SDS-PAGE assay

4.5. Confirmation of the Recombinant Enzyme Activity on Lactoperoxidase

Recombinant EndoBI-1 enzyme activity was tested on lactoperoxidase. It is a 78 kDa *N*-glycosylated protein. After SDS-PAGE method, it was showed EndoBI-1 activity on lactoperoxidase and was confirmed that lactoperoxidase glycans were released from the polypeptide chain (Figure 23).

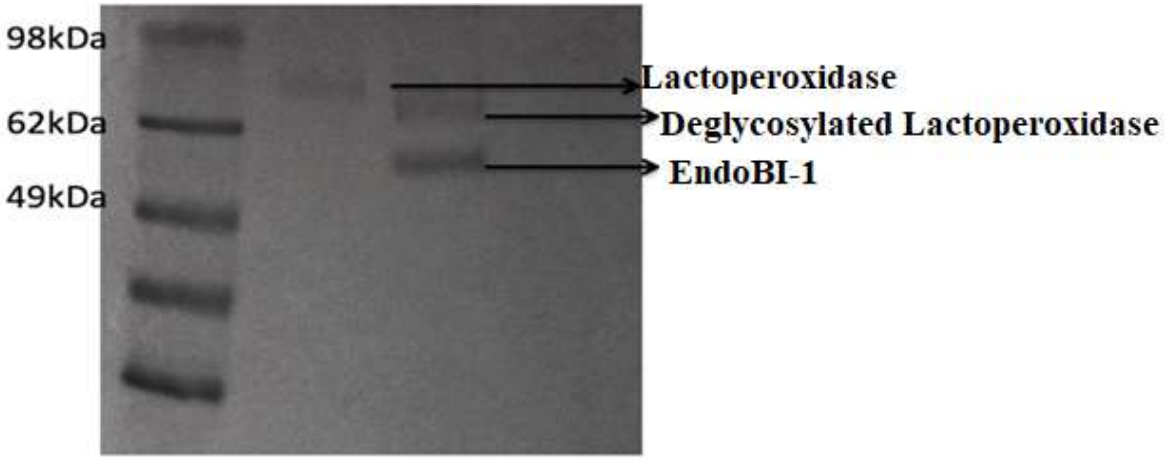


Figure 23. Control of the recombinant EndoBI-1 enzyme activity by SDS-PAGE assay

4.6. Immobilization Results

According to the adsorbtion immobilization technique results, adsorbtion performance increased at low pH values. Accordingly, 4.28% binding performance was obtained at 6.3 pH, 15.82% binding performance was obtained at 4.8 pH and 41.71% binding performance was obtained at 3.6 pH (Figure 24). Adsorbtion immobilization efficiencies were found to work with lower efficiency than other techniques. These results were consistent with the literature. When the immobilized enzyme is re-used, the enzyme is separated from the support material and enzyme loss occurs (Sheldon 2007).

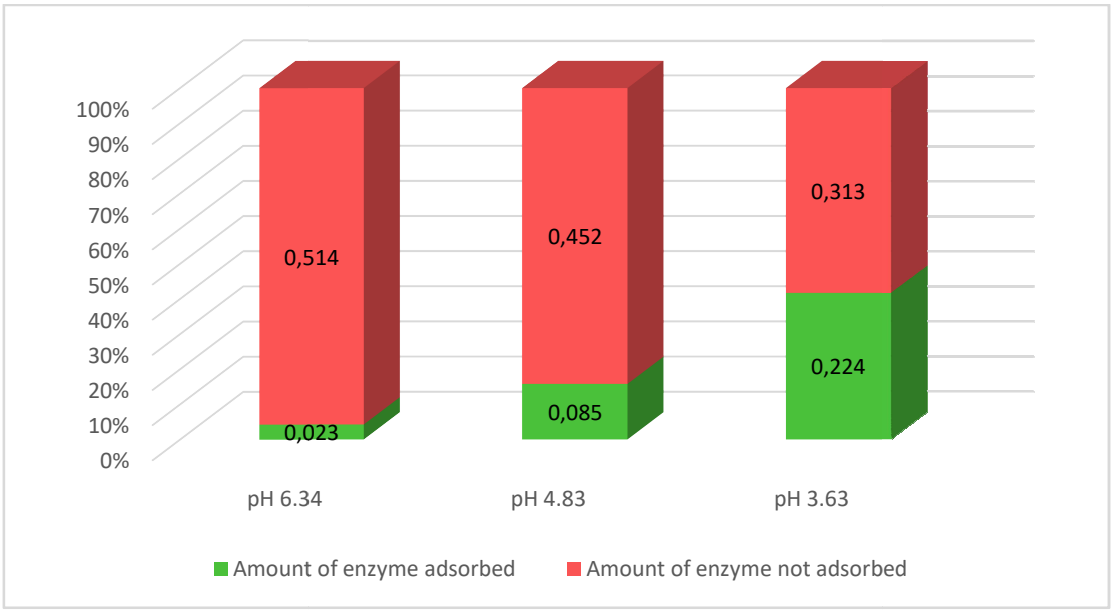


Figure 24. Adsorbtion immobilization performances in sodium acetate solution prepared in 3 different pH

According to the entrapment immobilization technique results, it was seen that high alginate concentration resulted high efficiency in immobilization by entrapment. While 2% alginate beads entrapped 15% of the amount of enzyme given, 3% alginate beads entrapped 63% and 4% alginate beads entrapped 79% of the amount of enzyme given (Figure 25). These values were quite high compared to the adsorbtion method. Because irriversible immobilization methods prevent the loss of enzyme over time by forming a strong bond (Karyakin et al., 2000; Schuster et al., 2001; Jal et al., 2004).

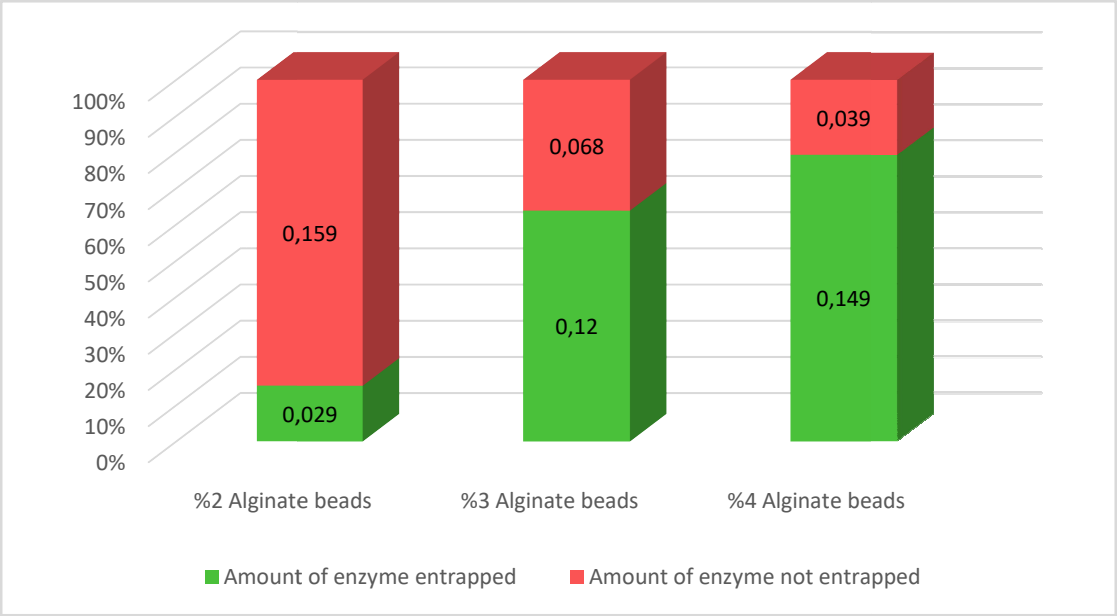


Figure 25. Entrapment immobilization performance for 3 different concentrations of alginate

According to the SulfoLink covalent binding immobilization technique results, 0.480 +/- 0.002 mg/mL enzyme was immobilized from 0.597mg/mL concentration given to the column. Thus, SulfoLink covalent binding yield was determined as %80.

According to the AminoLink covalent binding immobilization technique results, 0.479+/-0.009 mg/mL enzyme was immobilized from 0.548 mg/mL concentration given to the column. Thus, AminoLink covalent binding yield was determined as 87.4%.

4.7. Calculation of *K*m and *V*max value using Linearized Plotting

The kinetics of free-form EndoBI-1 on whey glycoproteins were calculated with each substrate (0.1-0.8) initial concentrations over 50 min for each reaction (Figure 26). The glycan release increased linearly for the first 20 minutes and all concentrations and the

reaction rate for each substrate were constant (slope of concentration / time was constant). Therefore, kinetic studies of enzymes in different forms were carried out under these conditions.

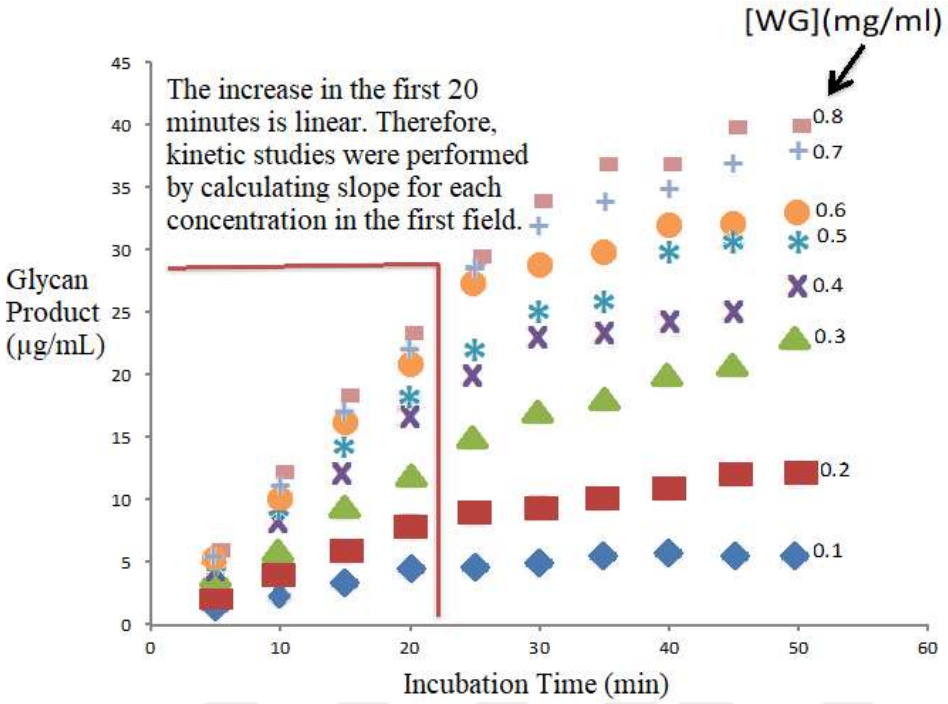


Figure 26. Glycan concentration (µg/mL) released from WG substrate ranging from 0.1-0.8 mg/mL using the EndoBI-1 enzyme in free form in 0.025 mg/mL. WG; whey protein

Lineweaver–Burk (1/[S] vs. 1/V) plotting technique was used for linearization method. Thus, kinetic parameters of each substrate were estimated. In the light of this method, the slopes obtained from the 20 minutesof our data were plotted together with the substrate concentration (Figure 27). E1, E2, E3, E4 and E5 represent respectively the free-enzyme, the enzyme adsorbed by alginate, the enzyme entrapped by alginate, the enzyme immobilized covalently by amino bond, and the enzyme immobilized covalently by sulfo bond.

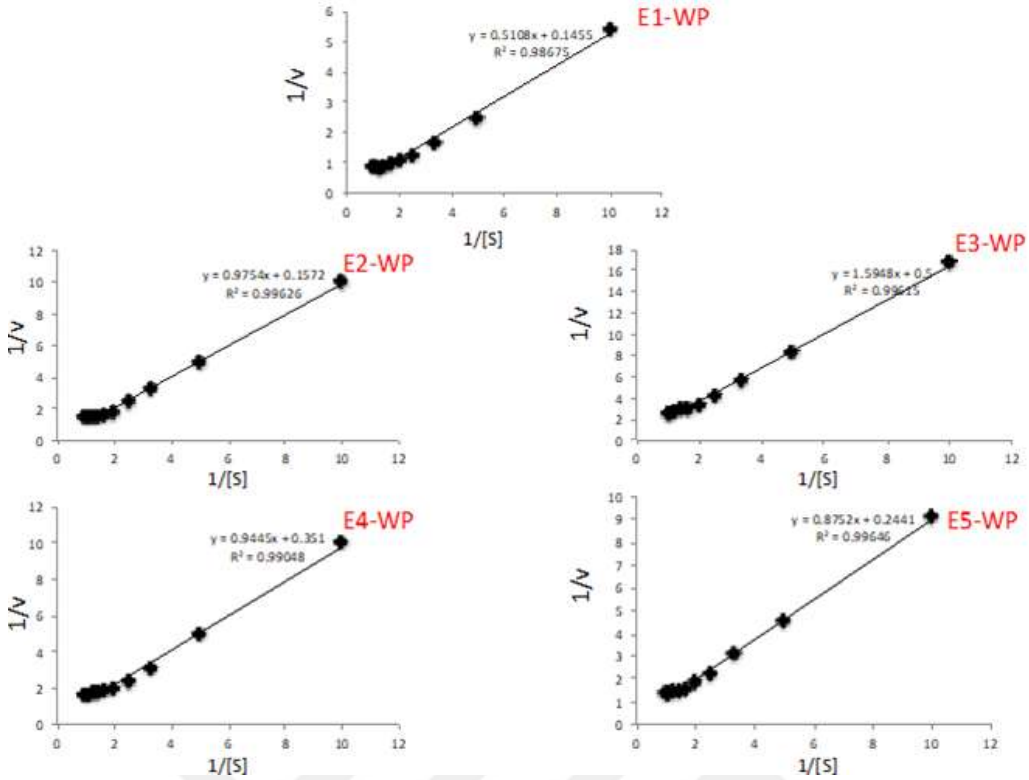


Figure 27. Lineweaver–Burke (1/[S] (mL/mg) vs. 1/V (mL × min/mg))

*V*max and *K*m values calculated according to the data obtained from these plots are given in Table 8. According to the results, the highest glycan yield was obtained from the free-form enzyme as expected. When the comparison between immobilization techniques was made, the highest activity was obtained by adsorption immobilization technique, while the lowest activity occurred after entrapment technique. Because irriversible immobilization methods involve the risk of blocking the active site of the enzyme so enzyme activity is likely to decrease (Datta et al., 2013; Kim and Herr 2013).

Table 6. Kinetic parameters of different enzyme forms on whey proteins. *K*m: mg/mL, *V*max: µg/mL×min

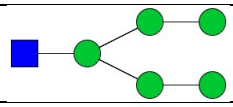
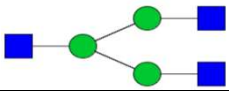
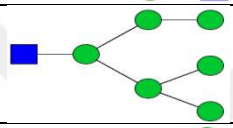
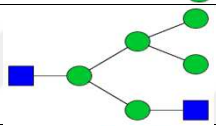
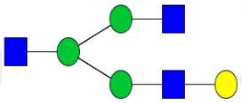
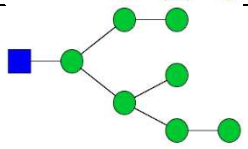
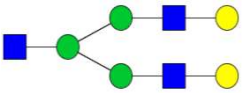
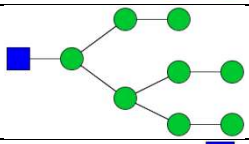
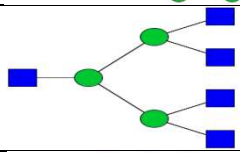
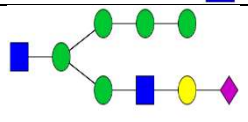
	E1	E2	E3	E4	E5
***K*m**	3.51	6.16	3.18	2.69	3.58
***V*max**	6.87	6.36	2.09	2.84	4.09

4.8. *N*-Glycan Structures

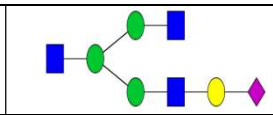
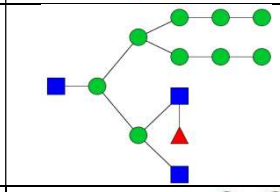
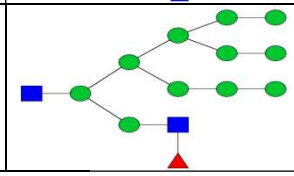
Glycan structures released from LPO and WP substrates by 5 different forms of enzyme are given in Table 9. According to the results, all immobilization techniques have

ability to separate the same glycans compared to the enzyme in free-form. Although these techniques show different levels of activity in the enzyme kinetics study, all forms of enzymes are capable of cutting all glycans when prolonged incubation is achieved. These results show that immobilization technique is not important in terms of the diversity of deglycosylated glycans.

Table 7. Glycan structures released from LPO and WG by EndoBI-1

Glycan	Glycan Structure	Substrate	Molecular Weight (m/z)
5Hex-1HexNac		LPO, WG	1151,29
3Hex-3HexNac		LPO, WG	1233,33
6Hex-1HexNac		LPO	1313,33
5Hex-1HexNac		WG	1356.57
4Hex-3HexNac		LPO, WG	1395,38
7Hex-1HexNac		LPO	1475,37
5Hex-3HexNac		LPO, WG	1557,42
8Hex-1HexNac		LPO, WG	1639,46
3Hex-5HexNac		WG	1640,61
6Hex-2HexNac-1NeuAc		WG	1807,50

Continuation of Table 7

4Hex-3HexNac-1NeuAc		LPO	1848,47
9Hex-3HexNac-1Fuc		WG	2352,48
11Hex-2HexNac-1Fuc		WG	2474,83

CHAPTER 5
CONCLUSION

Some bacteria consume released glycans from glycoproteins as a sole carbon source. These glycans, which are a source of nutrients to beneficial bacteria, provide the microflora in the human intestine to be shaped in a good direction (Gibson and Roberfroid, 1995). Human milk is known as rich glycans source, leading to the enrichment of infant intestinal microbiota by *B.infantis* (Lo Cascio et al.,2007; Marcobal et al., 2010). *B.infantis*, a key member of the microbiome, has been shown to consume *N*-glycan classes preferably (Karav et al., 2015). Glycans, which greatly promote infant development, are known to support the human immune system and defend against pathogens (Yolken et al., 1992; Choi et al., 2008). In addition, it has been shown that sialylated glycans protect against rota virus infection that causes infant diarrhea and are an important monosaccharide for infant development and learning (Yolken et al., 1992). As a result of this symbiotic relationship in the human intestine, interest in new prebiotics and symbiotic products has increased considerably. However, there is a need to obtain large amounts of pure glycans to elucidate the significant benefits of commercially unavailable glycans.

Whey, an abundant by-product of the cheese-making process, contains ~ 4.5 g glycoprotein per liter (de Wit et al., 1998). Glycans present in whey can be considered as source of bioactive glycans. *N*-glycans preferred by *B. infantis* present in significant amounts in whey glycoproteins (Karav et al., 2015). In this study, the variety of glycoproteins in whey which is a by-product of cheese making process was utilized. Therefore, whey protein was used as the substrate of the enzyme produced.

In addition to the need for glycans, the production of large-scale glycans is not commercially possible. *N*-glycans can be released from glycoprotiens by PNGaseF enzyme (commercial enzyme) produced from *Flavobacterium*. The activity of heat-sensitive *Flavobacterium* is limited due to core-fucosylation. A PNGase F (Peptide-*N*-Glycosidase F) hydrolyzes the amide side chain to cleavege asparagine-conjugated glycans, but this enzyme can not active on fucose α (1-3) bound (Takahashi 1977; O'Neill 1996). Commercially available PNGase F enzyme is expensive and denaturation is required for activity as well as being active at higher pH. These limitations adversely affect the use of *N*-glycan sources such as whey.

Endo-*β*-*N*-acetylglucosaminidase, a novel enzyme that separates glycans from glycoproteins, was recently isolated and the results of study were shown that this enzyme

is active under various combinations of temperature, pH and incubation time. Moreover, this enzyme has heat resistant structure. This feature of the enzyme EndoBI-1 allows the obtain glycan from the whey protein (which have densely *N*-glycan variety) and enables the study of biological properties and functions. Unlike PNGase F, EndoBI-1 separates the *N-N'*-diacetyl chitobioz fragment found in the *N*-glycan core, even if it is fucosylated (Karav et al., 2015a).

We started our study by cloning of EndoBI-1 enzyme by *in-vivo* cloning system. The transmembrane domains located in the *Blon_2468* gene region were not amplified to prevent binding of the enzyme to the cell wall during production. After amplification of the desired gene region, the PCR product was transformed into *E. coli* 10G cells by heat-shock. In this strategy, the tags added to the PCR product with the help of the primers were directly bound to the pRham linear vector in the cell. The ligation step was performed with the cell's own ligase without additional extra enzyme addition. In this way, no enzymatic step was required in the *in-vivo* cloning method. In the enzyme production step, the enzyme was collected from the nickel columns with the help of His tag. Since the tags found at the ends of the vectors are not compatible with each other, so we did not encounter the self-ligation risk. Since the cell used was suitable for recombination and production of the enzyme, these processes were performed in a single strain. After this method, the succes of the recombinant enzyme was tested by a number of processes. When the yield was evaluated and compared with other conventional cloning method, this method is more useful and faster. The activity of the EndoBI-1 enzyme was tested with lactoperoxidase to see differences of molecular weight. The molecular weight of lactoperoxidase (78 kDa) shows a 10% molecular weight decrease after enzymatic deglycosylation, and this difference can be detected on the SDS PAGE gel. Lactoperoxidase (LPO) enzyme, has high-mannose *N*-glycan chains, found in milk, tears and saliva have heme group also play in role antimicrobial defense system.

In this study, the produced enzyme was immobilized, and it was investigated which method would be useful for re-use. The recombinant enzymes are generally unstable and sensitive to changes of pH, temperature and substrate concentration. These disadvantages can be decreased or even eliminated by enzyme immobilization. Enzyme immobilization is important for the recovery of the enzyme, the inhibition of enzyme contamination in the obtained product, and in some cases manipulating the activity of the enzyme. Although very different immobilization methods have been developed, there are risks such as low enzyme activity and the separation of the enzyme from the bound matrix depending on the

strategy used. In this part of our study, the enzyme whose activity was confirmed was immobilized by 4 different techniques to determine the optimum strategy (alginate adsorbtion (1) and entrapment (2) sulfo-link (3) and amino-link (4) covalent bond). Enzyme active site does not blocked by adsorbtion strategy due to weak interaction between enzyme and support material. However, enzyme might be separated easily from the support material so enzyme loss can be occurred in this strategy (Sheldon 2007). Based on the results obtained from Qubit protein quantification measurements, covalent binding techniques and entrapment as immobilization efficiencies were found to work with higher efficiency than adsorption method. Weak interaction in the adsorption method is thought to lead to low yields. Although immobilization efficiency is very important, our priority in biological research is the high activity of the enzyme. The activities of immobilized enzymes were compared with free enzyme. For this purpose, kinetic characterization of EndoBI-1 enzyme was determined after immobilization. Whey protein powder and LPO containing high glycoprotein (such as lactoferrin and immunoglobulins) were used for our kinetic studies. According to the results, the highest glycan yield was obtained from the free form enzyme as expected. When the comparison between immobilization techniques was made, the highest activity was obtained by adsorption immobilization technique, while the lowest activity was observed after the entrapment technique. Analysis of the enzyme-cleaved glycans was carried out by MALDI- (TOF/) TOF-MS.

In the scope of this thesis, it was showed that when the glycan structures obtained are examined, all immobilization techniques have the ability to separate the same glycans compared to the free-form enzyme. Once the enzyme kinetics have been determined, although these immobilization techniques have shown that the enzyme exhibits a different level of activity, all enzyme forms are capable of cleavege all glycans when prolonged incubation is achieved.

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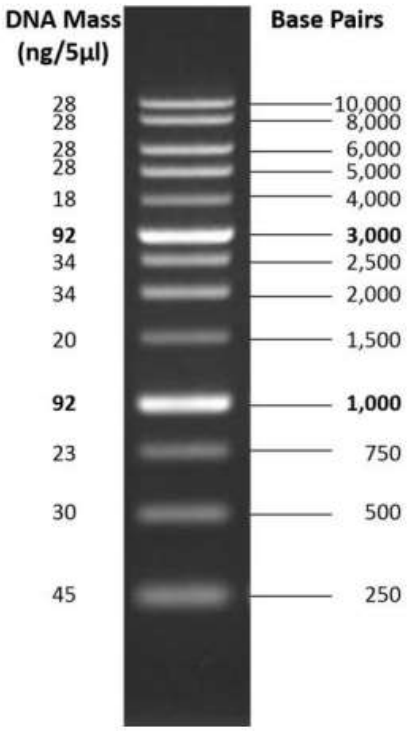
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APPENDICES

APPENDIX 1. DNA ladder used as marker in molecular analysis



Range: 250-10,000 bp

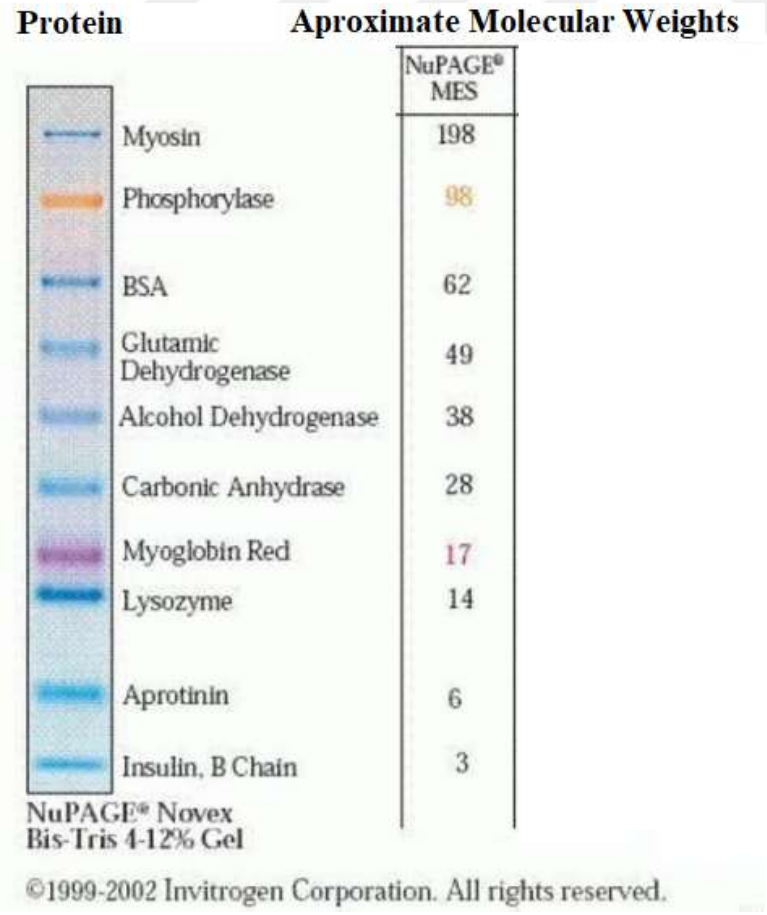
Number of bands: 13

Concentration: 100 µg/ml

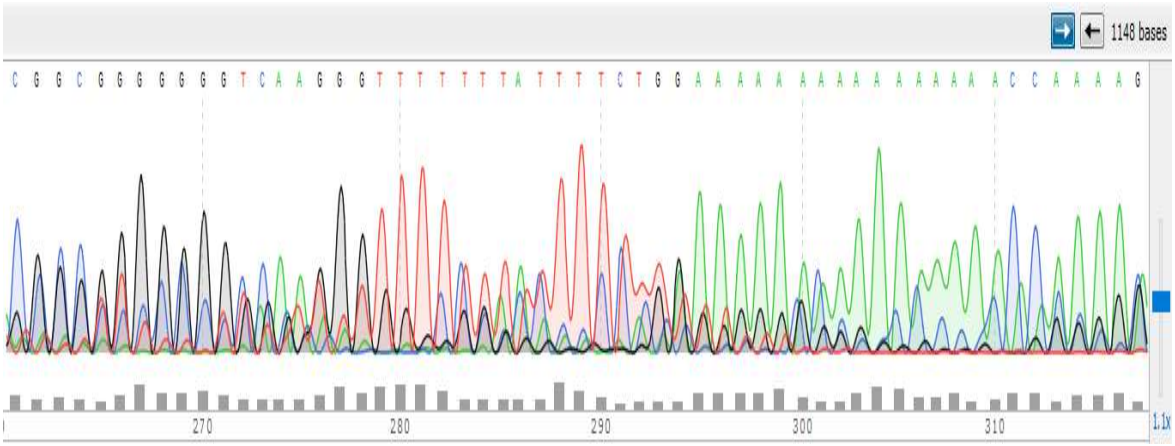
Recommended Load: 5 µl / well

Containing bromophenol blue & xylene cyanol FF as tracking dyes.

APPENDIX 2. Protein ladder used as marker in molecular analysis



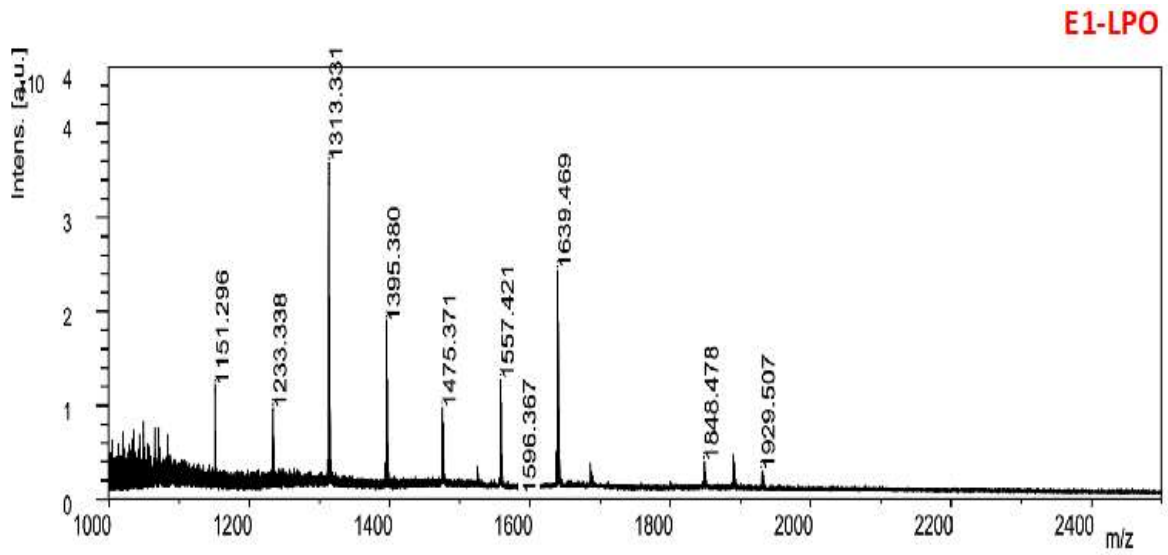
APPENDIX 3. A part for sequence reads for *Blon_2468* gene



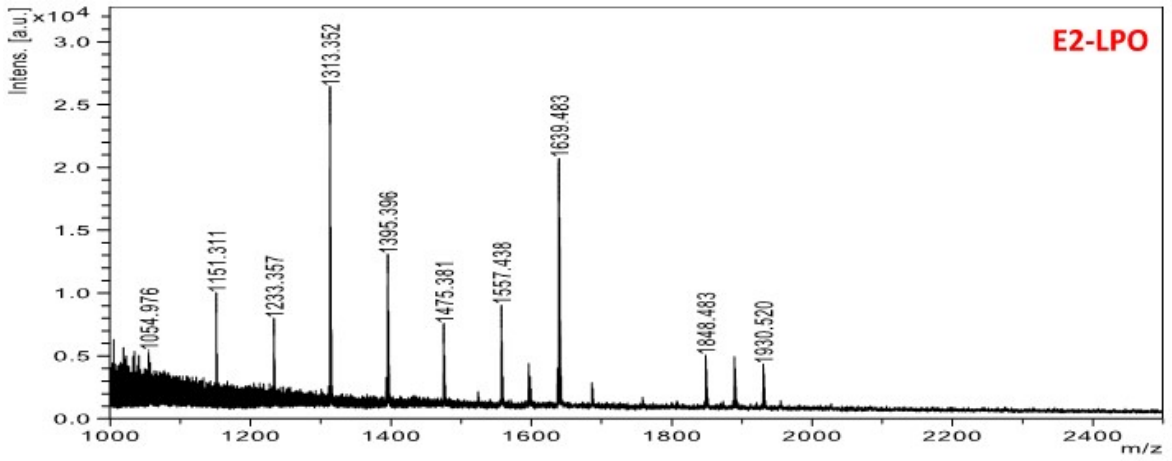
APPENDIX 4. A part from multiple sequence alignment performed for sequencing datas of *Blon_2468* gene in five recombinant samples.



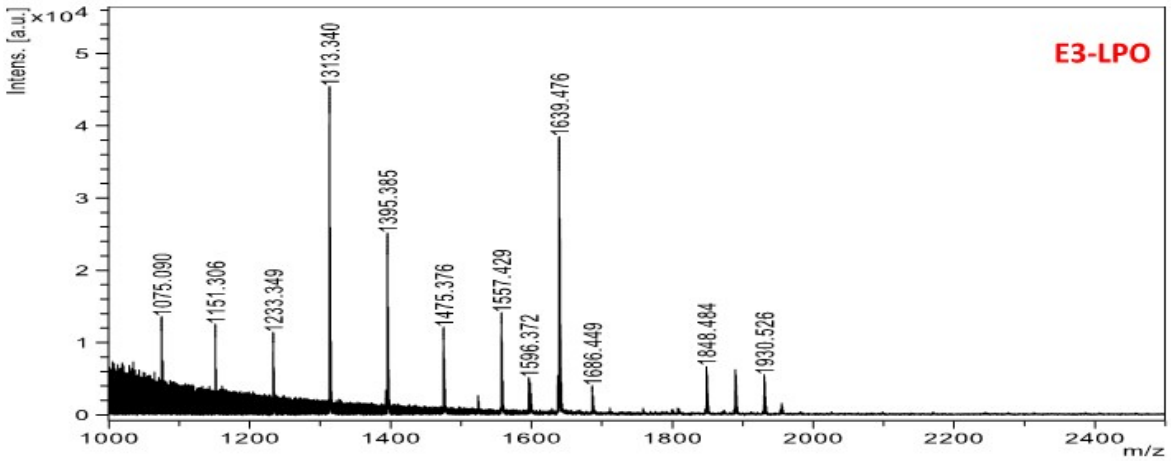
APPENDIX 5. MADI-(TOF/) TOF-MS spectra of the glycans deglycosylated by the immobilized enzyme EndoBI-1 from LPO and WG a) E1-LPO (free-ennzyme), b) E2-LPO (adsorbed), c) E3-LPO (entrapped), d) E4-LPO (amino-linked), e) E5-LPO (sulfo-linked), f) E1-WG (free-enzyme), g) E2-WG (adsorbed), h) E3-WG (entrapped), ı) E4-WG (amino-linked), i) E5-WG (sulfo-linked)



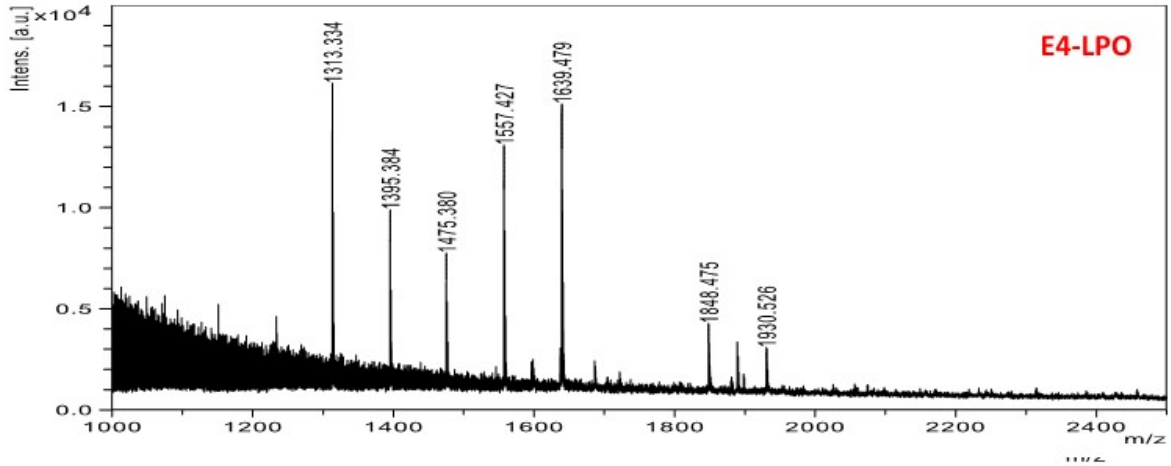
a) E1-LPO (free-ennzyme)



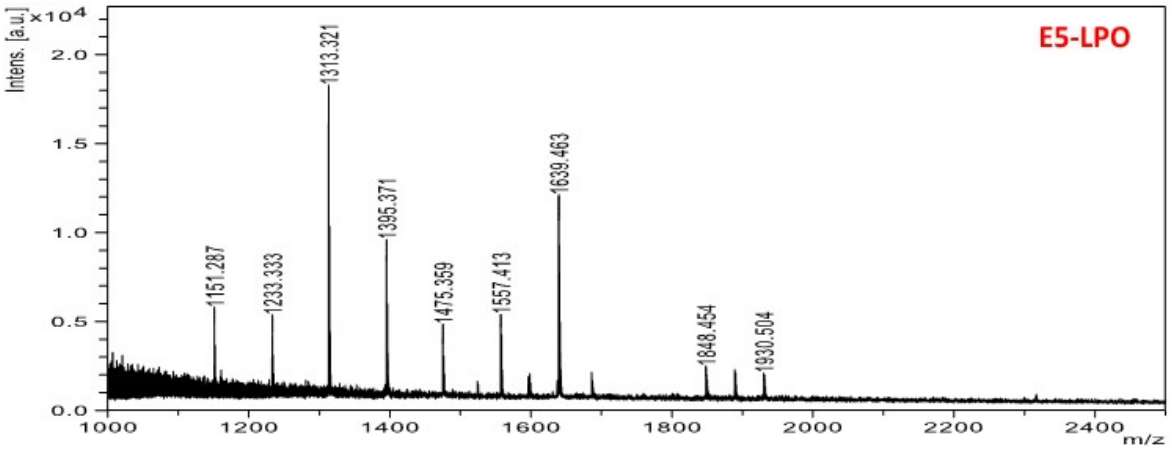
b) E2-LPO (adsorbed)



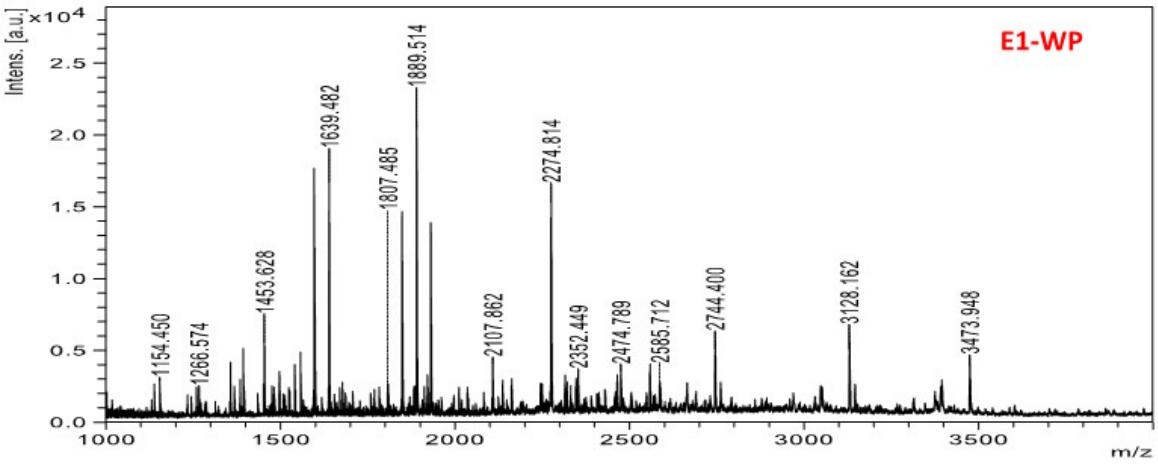
c) E3-LPO (entrapped)



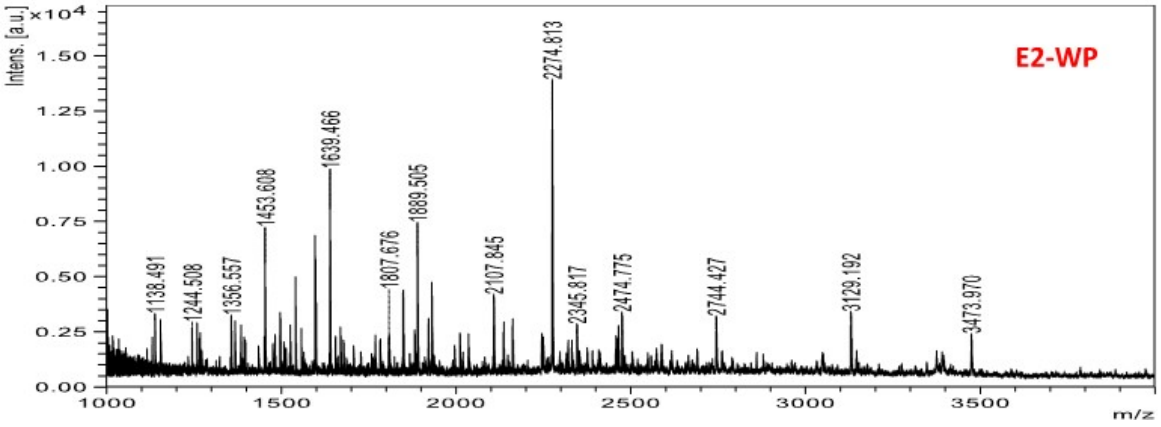
d) E4-LPO (amino-linked)



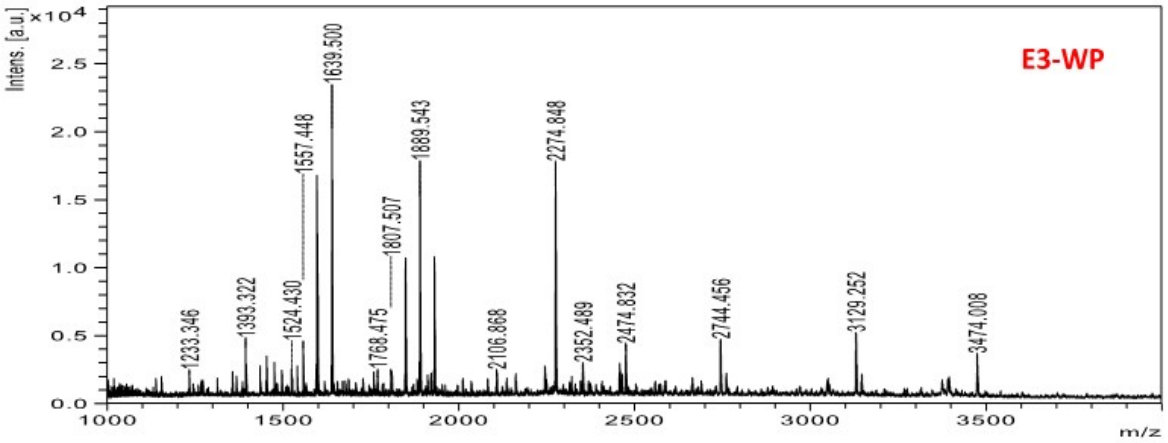
e) E5-LPO (sulfo-linked)



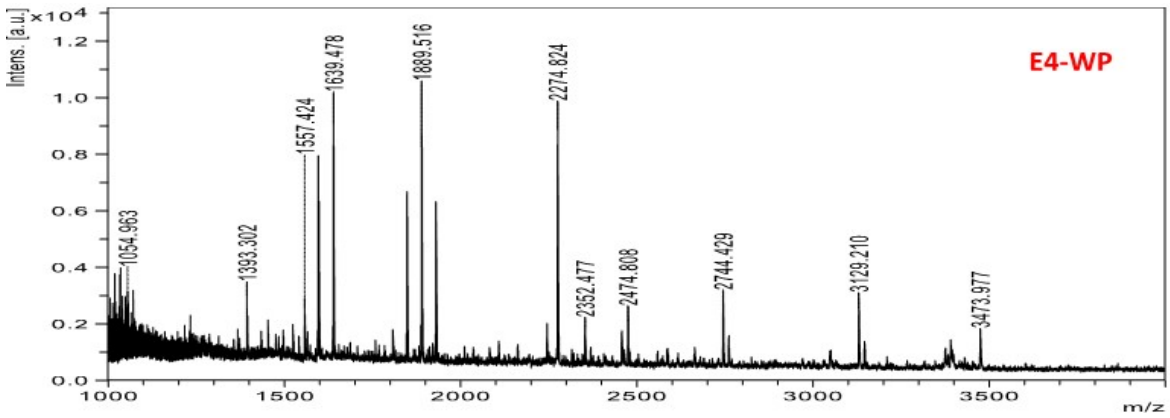
f) E1-WG (free-enzyme)



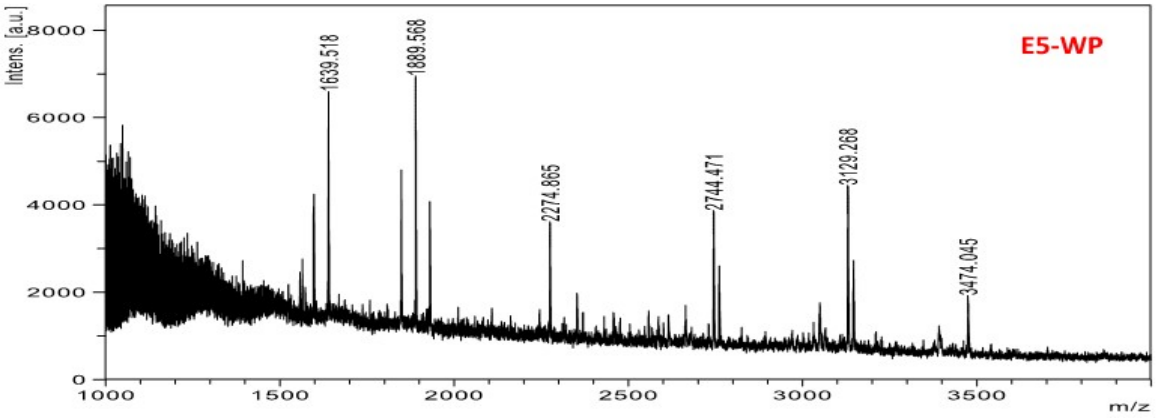
g) E2-WG (adsorbed)



h) E3-WG (entrapped)



ı) E4-WG (amino-linked)



i) E5-WG (sulfo-linked)

APPENDIX 6. Buffers and solutions used in study; a) 10X TBE electrophoresis buffer (1L) pH:8.3, b) lysis buffer (100 mL) pH: 8.0, c) elution buffer (100 mL) pH: 7.8, d) wash buffer (100 mL) pH: 8.0, e) destaining solution (100 mL)

a) 10X TBE electrophoresis buffer (1L) pH: 8.3

Component	**Number**
Tris base	54.0 g
Boric acid	27.5 g
EDTA	7.44 g
dH_2O	~1000 mL

b)lysis buffer (100 mL) pH: 8.0

Component	**Number**
50 mM NaH_2PO_4	0.6 g
300 mM NaCl	1.75 g
dH_2O	~100 mL

c)elution buffer (100 mL) pH: 7.8

Component	**Number**
50 mM Tris-HCl	0.6 g
400 mM NaCl	2.33 g
250 mM imidazole	1.70 g
dH_2O	~100 mL

d)wash buffer (100 mL) pH: 8.0

Component	**Number**
50 mM NaH_2PO_4	0.6 g
300 mM NaCl	1.75 g
20 mM imidazole	0.14 g
dH_2O	~100 mL

e) destaining solution (100 mL)

Component	**Number**
dH_2O (for 50%)	50 mL
Methanol (for 40%)	40 mL
Glacial Acetic Acid (for 10%)	10 mL

BIOGRAPHY

PERSONAL DETAILS

Name Surname : Burcu PEKDEMİR

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Date of Birth : 12.13.1995

EDUCATION STATUS

Bachelor's Degree : Çanakkale Onsekiz Mart University, Faculty of Science and Literature, Molecular Biology and Genetics, 2017

Master's Degree : Çanakkale Onsekiz Mart University, Graduate School of Natural and Applied Sciences, Department of Biomolecular Science, 2020

Foreign Language : English

SCIENTIFIC ACTIVITY

a) Publishing

1) SCI

Hilal OZKILINC, Zerrin KOZMA, Burcu PEKDEMİR, Birgul GULNAR., 2018. 'Characterization of Cryphonectria parasitica From Native Chestnut Trees in Kaz Mountain (Mount Ida)'. Journal of Agricultural Faculty of Gaziosmanpasa University http://ziraatdergi.gop.edu.tr/ JAFAG ISSN: 1300-2910 E-ISSN: 2147-8848 (2018) 35 (1), 38-45 doi:10.13002/jafag4357

b) Notifications

1) International

International Congress on Agriculture and Forestry Research (AGRIFOR), 04-09-2019 Pekdemir B. Karav S., 2019. 'Rapid *In-vivo* Cloning and Expression of Glycosly Hydrolases by pRham TM-N-His Vector'

c) Project

TÜBITAK 'Yeni Endo-*β*-*N*-asetilglukozaminidaz Enzimi Kullanarak Üretilen Ilk Seçici Prebiyotik Kaynağı; Glikanlar'

COMMUNICATION

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