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Characterization of expansin-like proteins from the plant pathogen Zymoseptoria tritici

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Biotechnology

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Master thesis

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Abstract

The demand to increase food production for a growing human population in a sustainable manner while handling issues such as food waste and poverty remains a significant global challenge. Climate change has shown to further complicate these issues. A specific example of this being that it has shown to lead to the development and spread of plant diseases, which can affect important crops, such as wheat.

The fungal pathogen *Zymoseptoria tritici*, which was previously known as *Mycosphaerella graminicola*, poses a significant threat to wheat crops by displaying host specificity and resistance to fungicides. As a result, it has come under recent scientific scrutiny. A better understanding of the plant cell walls dynamics is crucial for the defense against such pathogens.

Expansins, which are non-enzymatic proteins present in the plants cell wall, have been identified in fungi and microbial species. The fungi and microbe expansins have been found to engage in pathogenic and mutualistic patterns. Plant cell wall expansins are responsible for the relaxation and loosening of the cell wall, which is a detrimental process for crops considering it can affect different properties of the plant. In this thesis, four uncharacterized expansin-like proteins from Z. tritici were examined to determine if they also possess similar abilities to plant cell wall expansins.

In this study, select polysaccharide substrates such as celluloses, hemicelluloses and others that are present in the plant cell wall were treated with the expansin-like protein produced at the laboratory. This was done to determine if they can loosen the polysaccharides present in the cell wall through protein-substrate interaction. Later, it was examined whether they can be quantified by means of HPAEC-PAD and MALDI-TOF. Optimization of various parameters such as pH, temperature and enzyme concentration and the various polysaccharide substrates utilized are important in the characterization of the expansin-like proteins.

To get a better comprehension of the mature proteins, potential post-translation modifications (PTMs) such as N-glycosylation were investigated by employing the N-deglycosylation enzyme EfEndo18A.

In this study, the four expansin-like protein – F9, F11, F12 and F14 – were cultivated and purified. Protein F9 produced the most promising results in regard to N-deglycosylation. The F11 protein appeared to have properties that made it challenging to purify, while F14 was

shown to have a molecular weight almost double the expected size. Analysis of the expansin-like proteins' effect on polysaccharide substrates did not reveal any distinctive activity on the substrates in the current study, but this remains a large avenue that is still in its infancy and with the potential to be applied in various fields.

Sammendrag

Kravet om å øke matproduksjonen for en voksende menneskelig befolkning på en bærekraftig måte samtidig som man håndterer problemer som matavfall og fattigdom forblir en betydelig global utfordring. Klimaendringer har vist seg å ytterligere komplisere disse problemene. Et spesifikt eksempel på dette er at det har vist seg å føre til utvikling og spredning av plantesykdommer, som kan påvirke viktige avlinger, som hvete.

Den soppsykdomsfremkallende *Zymoseptoria tritici*, som tidligere var kjent som Mycosphaerella graminicola, utgjør en betydelig trussel mot hveteavlinger ved å vise verts spesifisitet og resistens mot soppmidler. Som et resultat har det kommet under nylig vitenskapelig granskning. En bedre forståelse av dynamikken til plantecellevegger er avgjørende for forsvaret mot slike patogener.

Expansiner, som er ikke-enzymatiske proteiner til stede i plantens cellevegg, er identifisert i sopp- og mikrobielle arter. Sopp- og mikrobeekspansiner har blitt funnet å delta i patogene og gjensidige mønstre. Plantecellevegg ekspansiner er ansvarlige for avslapning og løsning av celleveggen, noe som er en skadelig prosess for avlinger med tanke på at det kan påvirke ulike egenskaper ved planten. I denne avhandlingen ble fire ukarakteriserte expansin-like proteiner fra Z. tritici undersøkt for å avgjøre om de også har lignende evner til plantecellevegg expansiner.

I denne studien ble utvalgte polysakkaridsubstrater som cellulose, hemicellulose og andre som er til stede i plantecelleveggen, behandlet med ekspansin-lignende proteiner produsert på laboratoriet. Dette ble gjort for å avgjøre om de kan løsne polysakkaridene som er til stede i celleveggen gjennom protein-substratinteraksjon. Senere ble det undersøkt om de kunne kvantifiseres ved hjelp av HPAEC-PAD og MALDI-TOF. Optimalisering av ulike parametere som pH, temperatur og enzymkonsentrasjon og de ulike polysakkaridsubstratene som ble brukt, er viktige i karakteriseringen av expansin-lignende proteiner.

For å få en bedre forståelse av de modne proteinene, ble potensielle post-translasjoner modifikasjoner (PTMer) som N-glykosylering undersøkt ved hjelp av N-deglykosyleringsenzymet EfEndo18A.

I denne studien ble de fire ekspansin-lignende proteinene - F9, F11, F12 og F14 - dyrket og renset. Protein F9 ga de mest lovende resultatene med hensyn til N-deglykosylering. Protein F11 syntes å ha egenskaper som gjorde det utfordrende å rense, mens F14 viste seg å ha en

molekylvekt nesten dobbelt så stor som forventet størrelse. Analyse av expansin-like proteiners effekt på polysakkaridsubstrater avslørte ingen distinkt aktivitet på substratene i den nåværende studien, men dette forblir en stor vei som fortsatt er i sin begynnelse og med potensiale til å bli brukt i ulike felt.

Abbreviations

BMGY	Buffered glycerol-complex medium with yeast extract
BMMY	Buffered methanol-complex medium with yeast extract
CBM	Carbohydrate-binding module
CPN	Cerato-platanin
CV	Column volume
E. coli	*Escherichia coli*
EXLA	Expansin-like A
EXLB	Expansin-like B
EXPA	Alpha-expansin
EXPB	Beta-expansin
HCP	Host cell proteins
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
ICS	Ion chromatography system
KGM	Konjac glucomannan
PASC	Phosphoric acid-swollen cellulose
P. pastoris	*Pichia pastoris*
SEC	Size exclusion chromatography
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TXG	Tamarind xyloglucan
YNB	Yeast nitrogen base
Z. tritici	*Zymoseptoria tritici*

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1. Introduction

1.1 Food production and importance of plant pathogens in food spoillage

One of the major challenges facing the world today is providing enough food of high quality to sustain the global population. In 2020, the United Nations declared that there must be a 60% increase in food production by 2050 to necessarily feed the projected 10 billion people on Earth. Not only does the increasing population play a crucial role, but environmental degradation, food waste, and poverty also add to the complexity of the issue. An increase of food production entails larger food loss. It has already been estimated that the wheat loss is approximately 21.5% due to crop pests and food diseases on the crop.

Pests and pathogens are abundant in crops and climate change makes them more susceptible to diseases and so on. Plant diseases are the main cause of loss of food that not only gives us less to eat, but also loss of species diversities and a lot of money is invested to prevent outbreaks in crops which down the line can affect the health of humans.

1.2 Fungi and their role in plant pathogenesis

Fungi are members of the eukaryotic kingdom and have the important role of breaking down organic compounds in dead plant and animal matter. They secrete enzymes such as cellulase, ligninase, protease to name a few, that secrete into the compounds and decompose them and it's a vital task for keeping the cycle of the ecosystem. Decomposing the organic matter release carbon, nitrogen, and phosphorus back to the soil that can be utilized by other plants. Some fungi create symbiotic relationships where the fungi and the plant that both benefit from such as enhancing nutrient uptake and plant well-being.

These types of fungi are referred to as saprophytic and are considered non-pathogenic.

It's important to distinguish however the difference between non-pathogenic and pathogenic fungi because pathogenic fungi cause harm in other organisms instead of having the role of maintain the ecosystem.

1.3 Zymoseptoria tritici, a major plant pathogen

Zymoseptoria tritici is a fungus from the Dothideomycetes class and is a part of the fungal kingdom. It used to be known as Mycosphaerella graminicola until its renaming due to new taxonomic discoveries which prompted for a new distinct classification for the species in 2011.

Z. tritici is a fungal plant pathogen that has developed mechanism to have a host specificity towards wheat plants, infecting them and colonizing and it has become highly efficient at it (Fagundes et al., 2020). The organism is quite robust and can withstand several conditions such as changes in environment and host resistance (Chen et al., 2017). In addition to that they have developed quite the resistance towards fungicides (Bellah et al., 2023) which are meant to prevent the outbreak of the well-known Septoria tritici blotch.

The fact that the fungus is so resilient and creates such a havoc on wheat is quite the problem considering that wheat is the most important source of food on earth (Igrejas et al., 2020). What the fungus does to wheat varies from isolate and wheat genotype but usually it causes necrosis and formation of pycnidia on the infected wheat leaves. For wheat to show symptoms can take anywhere between one to two weeks and its highly dependant on several factors. A significant amount of resources goes into fighting the Septoria blotch disease by developing resistant wheat variants, fungicides and educate farmers how to by implementing several strategies such as crop rotation, when it is the most optimal to plant the different wheat types and to be on the lookout for the weather considering the spores of *Z. tritici* thrive in humidity and moisture (Department of Jobs, Precincts and Regions, 2022) . Humidity makes leaves prone to germination. Moisture is what the optimal condition is for the dispersal of the spores.

1.4 Plant cell wall

The plant cell wall is a protective layer that encloses the cell membrane of plants. It provides structural integrity and protection to the cell as well as it maintains it shape and doesn’t burst due to osmotic shock. Additionally, the cell wall acts as a physical barrier against pathogens and pests. It can repel the attack from foreign bodies by recognizing pathogens with the use of special receptors and signals to the host that it has been compromised by producing antimicrobial compounds.

The plant cell is distributed with different layers such as primary and secondary cell walls, the warty layer, and the middle lamella. These layers consist most commonly of cellulose,

hemicellulose, and pectin. The composition will always depend on the species, what kind of tissue and the stage of growth and the amount depends on which layer it exists in.

The three components make up a large amount of the cell wall in addition to proteins and other molecules in a matrix in plants. Cellulose is a polysaccharide consisting of a linear chain of glucose unit and they form the primary structure of the cell wall. Its linear polymer structure makes it a prominent substrate for enzymatic degradation studies. Hemicellulose are heterogeneous polysaccharides that are more complex in comparison to cellulose. They also contribute to structural integrity of plant cell walls alongside cellulose. The different sugar units can be xylose, mannose, galactose to name a few.

In this study different types of carbohydrates and polysaccharides were utilized to determine if the cell wall was able to be loosened.

1.4.1 Whatman filter paper

Whatman filter paper is a filter paper that is made from cellulose fibers. It is generally utilized for filtration and separation processes in the laboratory. Since Whatman filter paper is manufactured it has a high purity and consistency. Considering it's made out of cellulose fibers that is from wood pulp its worth exploring whether it's possible to loosen its cell wall with the expansins.

1.4.2 PASC

Phosphoric acid swollen cellulose is cellulose treated with phosphoric acid. This occurs by protonation of the phosphoric acid when it's dissolved in water which makes it release H^+ protons. These protons interact with the hydroxyl group from the cellulose and create positively charged hydroxyl groups. The positive charge disrupts the hydrogen bonding present on the cellulose which makes it so that water is more easily able to penetrate. By the water uptake the cellulose structure becomes swollen which in turn makes it more porous.

1.4.3 Aspergillus Niger biomass

Aspergillius Niger is a filamentous fungus with a complex structure. The cell wall of the fungi contains several types of polysaccharides such as glucans, chitins, galactomannans, α-glucans, and several other components such as proteins and lipids.

1.4.4 Tamarind xyloglucan

Tamarind xyloglucan is a polysaccharide from the seeds of tamarind tree. As the name implies, it is a type of xyloglucan which is a hemicellulose present in the cell wall of several plant species. Just as xyloglucan, tamarind xyloglucan consists of glucose units that are held together by β-1→4 glycosidic bonds which gives the typical linear backbone structure.

Xyloglucan and TGX have side chains that contains different types of monosaccharides such as xylose, glucose, and galactose to name a few. They can be modified in form of acetylation, methylation, and phosphorylation.

1.4.5 β-Glucan

β-Glucan is a type of polysaccharide that is present in the cell walls of organisms such as fungi, bacteria, algae, and some grains. It consists of glucose units held together by beta-glycosidic bonds that form either linear or branched chains. β-Glucan can be linked in β-1→4, β-1→3 and occasionally β-1→6 depending on the source.

This type of polysaccharide is usually soluble in water, though its solubility is heavily dependent of factors such as molecular weight, degree of branching and where it comes from.

β-Glucan has several applications in today's technology such as cosmetics, food, beverage, and dietary supplements.

1.4.6 Beechwood Xylan

Beechwood Xylan is a hemicellulose polysaccharide that is present in the cell walls of beechwood. It is made up from a backbone of xylose with is held together by β-1→4 glycosidic bonds which makes it a variant of xylan.

Beechwood xylan have side chains that contains different types of monosaccharides such as arabinose and glucuronic acid. An important aspect of xylan is that it can be hydrolysed into the simple monomer xylose or converted into xylooligosaccharides through the usage of enzymes or chemicals.

1.4.7 Konjac Glucomannan

Konjac Glucomannan is polysaccharide from the konjac plants roots. It consists mainly of the monomers glucose and mannose which forms the glucomannan backbone and are linked together by β-1→4 glycosidic bonds, and occasionally β-1→3 which occurs near branching

points or where there is higher degree of polymerization. KGM can have side chains that acetyl groups that are connected to glucomannan.

1.5 Expansins

As described earlier, the plant cell wall consists of several layers of polysaccharides that are crosslinked together. The two main polysaccharides cellulose and hemicellulose present in the cell wall create a network which makes the primary cell wall more rigid.

The plant needs to be able to modify itself and grow, and it achieves such purpose by secreting expansins into the cell wall space. Expansins are non-enzymatic proteins that in theory have the ability to loosen the cell wall in addition to reduce its rigidity in a pH-dependant way (Cosgrove, 2017). It is believed that expansins can have an impact on a lot of development stages of the plants, such as germination, fruit ripening and softening, leaf initiation and leaf growth and crop yield for instance (Marowa et al., 2016).

The currently accepted hypothesis is that the expansins disrupt non-covalent bonds between cellulose microfibrils and hemicellulose which in turn enhances the cell wall extensibility. Cellulose microfibrils consist of a linear chain of glucose monomers that are held together by hydrogen bonds (Cosgrove, 2023). Expansins have domains that can bind and disrupt the hydrogen bonds which facilitate sliding and the separation of the strong cellulose microfibrils (McQueen & Cosgrove, 1994). Expansins also interact with hemicellulose that is interwoven with the cellulose microfibrils, and a slippage is induced between the two of them which further loosens the cell wall (Cosgrove, 2023).

These types of proteins are categorized into the four subfamilies alpha-expansins (EXPA), beta-expansins (EXPB), expansins-like A (EXLA) and expansins-like B (EXLB) (Cosgrove, 2015). Alpha- and beta-expansins possess the ability to perform the sliding and slippage albeit they work a little differently. EXLA and EXLB secrete into the cell wall and their structure is supposed to be similar to other expansins although their current biological function is unknown at the moment. These types of subfamilies are for expansins and expansins-like found in plants (Cosgrove, 2015).

While expansins were first discovered in plants, they are also present in fungi and bacteria. There are several theories on how exactly they are present in fungi and bacteria too. One plausible explanation is horizontal gene transfer which essentially means that the common ancestor of the expansin-like protein in other organisms is plants (Cosgrove, 2017). It could

also be due to convergent evolution where the fungi and bacteria had to develop similar proteins that could perform similar functions. Just like the plant expansins, the expansins from fungi and bacteria have no enzymatic activity either (Cosgrove, 2017).

Fungal expansins occasionally have domains or motifs that are very similar to plant expansins (Cosgrove, 2017). Loosenins, EXPNs, ceratoplatanins which are expansins-like protein that exists in fungi resemble the N-terminal domain of plant expansins. According to studies, these expansin-like protein promote plant colonization or pathogen virulence. The interaction between the proteins and the cell wall can cause modification of the plant cell wall which promotes fungal penetration which has been reported for several crops. A clear example is the swollenin which has CBM1 and an expansins-like region. which breaks cotton fiber structure. Unlike expansins, swollenin does not cause cell wall loosening nor expansion which could be due to the expansins-like not having the ability to promote cell wall extension or the CBM1 binds to locations too distant from biomechanical hot spots. This reveals that although swollenin is related to expansins it acts and functions differently from them while also being different structurally.

In this study, four uncharacterized expansin-like proteins from Jean-Guy that originate from *Z. tritici* were cloned into Pichia Pastoris strain X33 at previously at INRAE, within the FunTime project funded by the French Research National Agency (ANR-14-CE06-0020, https://mycor.nancy.inra.fr/IAM/?p=3931).

1.5.1 F9

This protein has a molecular weight of 24,892 kDa, a molar extinction coefficient of 51825, and a pI of 6.24. This protein has an EXPN-CBM63 domain complex. The presence of EXPN signifies that the protein has similar structure or function as plant expansins. It might not act the same way as expansins do, but they share a similar ability to possibly interact and modify the cell wall. It also has the Carbohydrate-Binding Module 63, which is a protein domain that is able to potentially bind to carbohydrates and polysaccharides such as cellulose and hemicellulose for instance.

1.5.2 F11

A protein with the molecular weight of 14,768 kDa, a molar extinction coefficient of 15150, and a pI of 5,69. F11 has an EXPN-CPN domain complex, meaning that there is a cerato-platinin domain present. It has been studied that cerato-platanin is found in types of

filamentous fungi and are part of biological processes such as plant-fungal interactions, fungal adhesion and pathogenesis. Cerato-platanin are carbohydrate-binding proteins that can bind to chitin and N-acteylglucosamine oligosaccharides.

1.5.3 F12

The F12 protein has a molecular weight of 22,024 kDa, a molar extinction coefficient of 28460, and a pI of 6,75. It also has a EXPN-CBM63 domain complex like F9.

1.5.4 F14

Another protein that demonstrates a molecular weight of 28,878 kDa, a molar extinction coefficient of 38640, and a pI of 5,43. Unlike the two F9 and F12 it does not have the EXPN-CBM63 complex, only the EXPN domain.

1.6 Aim of the study

In this study the objective is to investigate if the four non-enzymatic expansin-like protein are able to impact the cell wall. As mentioned, expansins have in theory the ability to loosen the cell wall and reduce its rigidity and the job is to find what the preferable conditions are. There are several expansins and expansins-like that have yet to be characterized and studied. Learning more about these kinds of proteins and by characterizing them furthers the understanding of what the proteins do and what their potential applications in the future can be.

The expansin-like protein will be tested on different kind of substrates to be able to see if there is substrate specificity towards either celluloses, hemicelluloses or other type of polysaccharides that exist on the plant cell wall based on the results.

In addition to that also being able to identify potential PTMs during the folding of the protein. Characterization of the proteins furthers the understanding of them and what the potential applications can be.

The applications of the expansin-like proteins for cell wall component could lead to a further understanding of how the cell wall functions and it could be incorporated as a defence mechanism against pathogens and pests as well as other environmental stressors such as climate variation which leads to wheat plants being more susceptible to infections.

2. Materials

2.1 Equipment and materials

Table 2.1.1 Summary of the equipment and materials used in this study.

Equipment	Manufacturer
ÄKTA pure chromatography system	Cytiva
Amicon Ultra Centrifugal Filter, 3 kDa MWCO	Sigma Aldrich
BD Emerald Sterile 2mL Syringe	BD
Beakers 100, 250, 500, and 1000 mL	VWR International
BioPhotometer	Eppendorf
BioFrac Fraction Collector	BIO-RAD
Cellstar Centrifuge tubes 15 mL and 50 mL	Greiner bio-one
Cultivation flasks 100 mL, 1000 mL, 5000 mL	Duran Group
Cuvettes (disposable)	BRAND
Cuvettes (glass)	Eppendorf
Eppendorf tubes 1.5 mL	Eppendorf
Eppendorf tubes 2.0 mL	Eppendorf
Gel Doc™ Ez Imager	BIO-RAD
Glassware 1 L beaker	Duran Group
HisTrap™ HP	Cytiva
ICS-5000	Thermo Fisher Scientific
Inoculation loops	Sarstedt
MASTERFLEX Easy-Load	Cole-Parmer
Measuring cylinders 10, 25, 50, 100, 250, 500, 1000 mL	Duran Group
Mini-PROTEAN Tetra Vertical Electrophoresis Cell	BIO-RAD
Mini-PROTEAN® TGX™ Precast Gels Stain-Free Gels (10 and 15	BIO-RAD

well)	
Multifuge X1R Refrigerated Centrifuge	Thermo Fisher Scientific
NGC chromatography system	BIO-RAD
Parafilm	Bemis
Petri dishes 90 mm	Heger
Pipette tips	Sarstedt
Pipettes	Thermo Fisher Scientific
Power Supply, PowerPac 300	BIO-RAD
ProteoSEC Dynamic 3-70 kDa HR resin	Protein Ark
Serological pipettes	Sarstedt
Stain-Free Sample Tray	BIO-RAD
Syringe 10 mL (12 mL) Henke-Ject	Henke Sass Wolf
TX-400 Rotor with Buckets, 15ml and 50ml Adapters	Thermo Fisher Scientific
Vivaflow 200 Reusable cassette	Sartorius
Filter Upper Cup 250 mL bottle top filter 0.22 µm PES membrane	VWR
250ml Millipore Stericup Filter Unit 0.22 µm PES Membrane	Sigma Aldrich

2.2 Chemicals

Table 2.2.1 Chemicals used in this study.

Chemical	**Manufacturer**	**CAS Number**
Acetic acid (glacial) (CH3COOH)	EMSURE	64-19-7
Agar powder (C14H24O9)	Sigma-Aldrich Corporation	9002-18-0
Bacto Yeast extract	BD emerald	Not Available
Ethanol absolute (C2 hours5OH)	VWR	64-17-5

Glycerol, 85% (C3H8O3)	Merck	56-81-5
Imidazole	PanReac AppliChem	288-32-4
Methanol	Merck KGaA	67-56-1
MilliQ water, filter Millipak Express 40, size 0.22 µm	Merck KGaA	Not Available
Meat peptone hygroskopisk	Thermo Fisher Scientific	73049-73-7
Potassium phosphate, monobasic	Sigma-Aldrich Corporation	7778-77-0
Sodium acetate (CH3COONa)	Sigma-Aldrich Corporation	127-09-3
Sodium acetate, ICS standard	Sigma-Aldrich Corporation	127-09-3
Sodium chloride (NaCl)	VWR	7647-14-5
Sodium hydroxide (NaOH)	EMSURE	1310-73-2
Sodium hydroxide, 50% solution for ion chromatography system (ICS) analysis	FlukaTM Honeywell International	1310-73-2
Tris Base (C4H11NO3)	Sigma-Aldrich Corporation	77-86-1
di-Potassium Hydrogen phosphate	VWR	7758-11-4

2.3 Substrates and standards

Table 2.3.1

Carbohydrates	**Manufacturer**
Aspergillus Niger Biomass	Made in house
Beechwood Xylan	Megazyme
Beta-glucan	Megazyme
Konjac Glucomannan	Megazyme
Native COS DP2-DP6	Made in house
Native XOS DP2-DP6	Made in house
PASC	Made in house
Tamarind Xyloglucan	Megazyme
Whatman filter paper	Cytiva

2.4 Standard solutions

Table 2.4.1. Standard solutions used in this study

Equipment	Use	Manufacturer
Benchmark protein ladder	SDS-PAGE	Invitrogen
Protein Assay Dye reagent concentrate	Bradford protein Assay	Bio-Rad
Reducing agent (10x)	SDS-PAGE	Invitrogen
Sample buffer (x4)	SDS-PAGE	Novex
1X Tris-Glycine-SDS buffer	SDS-PAGE	Bio Rad
20% ethanol	ÄKTA pure chromatography system and NGC chromatography system	VWR

2.5 Proteins

Table 2.5.1. Marked in purple is the signal peptide sequence, what is in orange is the KREAEA cleaving site and in red is the His-Tag on the protein.

Proteins
>Ztrit_110756_EXPN_CBM63 (F9) recombinant protein (UniprotID: F9XK90) **ε: 51825** MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTN NGLLFINTTIASIAAKEEGVSLEKREAEASVLPGITENKSHSVRRDLKTGLGTRYGKN CKEEDCWQSGACAFTNYKLPSTIDGSTCVSEAIWDSSAHCGGCIQVTYKGKTITIMV TNKTDGDANHLDMPPATWSKLTNGMTGGGVDGIEWDWVTCPITAPLTIHMHGGSSQ YWFAATVENATLRTTKMEVSTDNGKTWKGTERDINNFFVVDGVLPTTSAWVKVTSE SGSQVVVENVKLESGKSTVASENYAHHHHHH

>Zymo_39947_EXPN_CPN (F11) recombinant protein (UniprotID: F9X9Y5)

ε: 15150

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN STNNGLLFINTTIASIAAKEEGVSLEKREAEATTVSYDPGYDKANNPMTGVACSD GNNGLITRYGYQTYGSIPRFPYIGGSSDIAGYNSDQCGQCYSVSYNGGQPIYILAI DHTLEGLNISEEAMNALTGGQAEAVGRVDAQVTKVGVDMCGLAPRKRAVEFLA HHHHHH

>Ztritici_69140_EXPNCBM63 (F12) recombinant protein (UniprotID: F9X41)

ε: 28460

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN STNNGLLFINTTIASIAAKEEGVSLEKREAEAFTGRGTFYGGNVQGGMCSFASYT LPTGIDGTAISKLDWAGSGVCGACIKVTGLRGSTISMIVDQCPECPPHSLDLFQN SFGKIDDPQKGIIQLSWEFVDCPLNGLIYFRMKEGVSANWFSVQAVNASKRVKDI QVSTDHGATWQSGLTRMDYNFFQKSAGFGVDVVDLKVISIDGKERIAKNCQVIG GNTCNADGNFHHHHHH

>Zymo_50652v2_EXPN (F14) recombinant protein (Uniprot: F9XNT0)

ε: 28460

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN STNNGLLFINTTIASIAAKEEGVSLEKREAEAVPKPELQHHHQHHPKRDIVWVTE YQEVVETIAVTKTVWVKPSQTQATPFSTQEVKQVQPAAYSPAPVAAPAYVAPAAQ SEPAAAPAYVAPVSSEPAAAYVAPVSSEAAAVPTSTYVPPPAPYVAPVASSSAAAAPA SSSAAAPSYNSPSDSGSSSGGQSYSGDFTWFDVGMGACGFTSTSDQDVVAVSHVL FDKVSTGNSNTNPLCKKIIYLIGADGNTYPGEIVDRCPSCAEGSLDLSESFFKKVT SNGDGRVLNMKWHMPLEQKLISEEDLNSAVDHHHHHH

2.6 Enzymes

Table 2.6.1

Enzyme	Use	Source
*Ef*Endo18A	N-deglycosylation	Made in house

2.7 Cultivation media for protein expression

2.7.1 BMGY- and BMMY media

The BMGY- and BMMY base are rich in nutrients such as vitamins, amino acids and nitrogen which later is infused with a carbon source and other ingredients to create BMGY and BMMY.

Materials

Yeast extract

Peptone

MilliQ water

Method

10g of yeast extract, 20g of peptone and 700 milliQ was placed within a 1 L beaker and around 500 mL of milliQ water was added and mixed with a magnetic stirrer. After it fully dissolves the content, it transferred over to a 1L bottle until it reaches around 700 mL, partially sealed with a blue cap and sterilized through autoclavation.

2.7.2 BMGY and BMMY

The four proteins were cultivated using a *P. Pastoris* expression kit from Invitrogen (page 55 and 58) using both BMGY and BMMY. The cultivation process is between 5-6 days were the first 3 days the the cells grow with the presence of carbon in the form of glycerol. When the cells reach a certain density, the feeding switches over from glycerol to methanol to promote secretion of protein rather than cell growth in the media by the host organism where they later on are extracted through purification.

Materials:

Potassium phosphate

YNB

Biotin

Glycerol (for BM**G**Y)

Methanol (for BM**M**Y)

BMGY-base

Method

100 mL 1M potassium phosphate buffer, 100 mL 10X YNB, 100 mL glycerol/methanol and 2 mL 500X biotin are placed within the 700 mL sterilized BMGY-base bottle and then carefully mixed under the extractor hood to create BMGY and BMMY depending on what carbon sourced utilized in the process.

2.8 Buffers

Several buffers were made for either maintaining the pH of liquids to provide a stable environment and ensuring the buffer capacity for the solutions.

2.8.1 Potassium phosphate buffer pH 6

1M Potassium phosphate buffer pH 6 was used for the cultivation process of *P. Pastoris* to make both BMGY and BMMY and the purpose it serves is to maintain a stable pH throughout the cultivation, provide phosphate source for the yeast cells and have a good buffer capacity.

Materials

1 M 132 mL of K2 hoursPO4

1 M 868 mL KH2PO4

Method

1M 132 mL of K2 hoursPO4 was combined with 1M 868 mL KH2PO4 to make a total volume of 1 L. The pH was adjusted to 6 using 4M KOH followed up by sterilizing it by the use of autoclavation and then stored at room temperature.

2.8.2 1M Tris-HCl buffer pH 8

Tris-HCl pH 8 was made as a base to create the three buffers; Buffer A, Buffer B and Washing buffer to ensure that the pH is maintained in the solution.

Materials:

121,14 g Tris-Base

MilliQ water

Method:

121,14g Tris-Base was placed within a 1 L beaker and around 800 mL of milliQ water was added and mixed with a magnetic stirrer. When the Tris-Base was fully dissolved 37% HCl was used to adjust the pH to 8. When the desired pH was reached the liquid was transferred to a 1L volumetric flask until the final volume of 1 L was reached, then filtered using a 0.22 µm filter and a membrane dry vacuum pump into a 1 L bottle with a blue cap, which was stored at room temperature.

2.8.3 Buffer A pH 8 (50 mM Tris-HCl, 150 mM NaCl and 10 mM imidazole)

Buffer A was used for both Vivaflow and His-Tag purification. Before His-Tag purification a buffer exchange was performed at the last step during the up concentration of the protein during Vivaflow to remove salts, detergents, and any other unwanted products in the sample. It also is used to set the pH of the sample to pH 8 to have optimal binding conditions during loading of the protein on the column. For the loading of the protein, it is recommended to use imidazole at a concentration between 20 and 40 mM, although the optimal imidazole concentration depends on the pH. The low concentration is to minimize the binding of the host cell protein and at the same time so that the His-Tagged proteins can bind to imidazole.

Sodium chloride is used to maintain the ionic strength of the buffer solution and Tris-HCl is used to maintain the pH of the solution.

Materials:

50 ml 1M Tris-HCl pH 8

8,766g NaCl

0,68 g Imidazole

Method:

8,766g NaCl and 0,68g imidazole was placed within a 1 L beaker and around 750 mL milliQ water was added in addition to 50 mL 1M Tris-HCl pH 8 which was then mixed with a magnetic stirrer. In case of needing to adjust the pH 37% HCl was used. When the desired pH was reached, the liquid was transferred to a 1L volumetric flask until the final volume of 1 L was reached, then filtered using a 0.22 µm filter and a membrane dry vacuum pump into a 1 L bottle with a blue cap, which was stored at room temperature.

2.8.4 Buffer B pH 8 (50 mM Tris-HCl, 150 mM NaCl and 500 mM imidazole)

Buffer B was the elution buffer for the His-Tag purification. The high concentration of imidazole enabled the elution of the proteins in the column. The proteins in the column were exposed of gradual increase of buffer B% with a gradient.

Sodium chloride is used to maintain the ionic strength of the buffer solution and Tris-HCl is used to maintain the pH of the solution.

Materials:

50 ml 1M His TrisHCl

8,766g NaCl

34,04g Imidazole

Method:

8,766g NaCl and 34,04g imidazole was placed within a 1 L beaker and around 750 mL milliQ water was added in addition to 50 mL 1M Tris-HCl pH 8 which was then mixed with a magnetic stirrer. In case of needing to adjust the pH 37% HCl was used. When the desired pH was reached, the liquid was transferred to a 1L volumetric flask until the final volume of 1 L was reached, then filtered using a 0.22 µm filter and a membrane dry vacuum pump into a 1 L bottle with a blue cap, which was stored at room temperature.

2.8.5 Washing buffer pH 8 (50 mM Tris-HCl, 150 mM NaCl and 25 mM imidazole)

The washing buffer was utilized after loading the protein on the column to remove any protein that might bind to the column non-spesifically in addition to remove any background noise in the sample.

Sodium chloride is used to maintain the ionic strength of the buffer solution and Tris-HCl is used to maintain the pH of the solution.

Materials:

50 ml 1M His TrisHCl

8,766g NaCl

1,702g Imidazole

Method:

8,766g NaCl and 1,702g imidazole was placed within a 1 L beaker and around 750 mL milliQ water was added in addition to 50 mL 1M Tris-HCl pH 8 which was then mixed with a magnetic stirrer. In case of needing to adjust the pH 37% HCl was used. When the desired pH was reached, the liquid was transferred to a 1L volumetric flask until the final volume of 1 L was reached, then filtered using a 0.22 µm filter and a membrane dry vacuum pump into a 1 L bottle with a blue cap, which was stored at room temperature.

2.8.6 SEC buffer pH 5 (150 mM NaCl and 20 mM NaAc)

After His-Tag purification the desired fractions were pooled together after detecting the proteins in the SDS-PAGE and up concentrated to 1-2 mL with SEC buffer twice to ensure that the protein is compatible with the mobile phase for SEC.

The buffer is also used during SEC to ensure that the SEC column maintains a stable pH and ionic strength in the SEC column. NaCl is used to maintain the ionic strength while NaAc is used to maintain the stable pH throughout the process. This is detrimental to ensure that the protein is stable in the mobile phase to prevent aggregation and/or precipitation.

Materials:

8,766 g NaCl

1,6406g NaAc

MilliQ water

Method:

8,766g NaCl and 1,6406g NaAc was placed within a 1 L beaker and around 800 mL of milliQ water was added and mixed with a magnetic stirrer. The pH was adjusted with 37% HCl. When pH 5 was reached the liquid was transferred to a 1L volumetric flask until the final volume of 1 L was reached, then filtered using a 0.22 µm filter and a membrane dry vacuum pump into a 1 L bottle with a blue cap, which was stored at room temperature.

2.8.7 50 mM NaAc pH 5

To stabilize the pH of the enzymatic reactions of the proteins and celluloses/hemicelluloses sodium acetate pH 5 was used utilized. Also for concentrating the proteins post-SEC to 2 mL to calculate their concentration.

Materials:

0,410g NaAc

MilliQ water

Method:

0,410g NaAc was placed within a 1 L beaker and around 800 milliQ water was added and mixed with a magnetic stirrer. The pH was adjusted with acetic acid to reach pH 5 and then it was transferred to a 1 L volumetric flask until the final volume of 1 L was reached, then

filtered using a 0.22 µm filter a membrane dry vacuum pump into a 1 L bottle with a blue cap, which was stored at room temperature.

2.8.8 SDS buffer

SDS buffer is utilized to prepare protein samples for SDS-PAGE. The buffer denatures the proteins and make them evenly charged to be able to read from the ladder.

Materials:

250 µL 4x sample buffer

100 µL reducing agent

650 µL milliQ water

Method:

250 µL 4x sample buffer, 100 µL 10x reducing agent and 650 µL milliQ water are mixed inside of a 2 mL Eppendorf tube and placed in the fridge at 4*C.

2.8.9 500 mM NaOH

Sodium hydroxide is a strong alkaline solution that was used to clean the Vivaflow machine. The high concentration of NaOH ensures that it destroys proteins and other biomolecules present in addition to microorganism in the vivaflow filter that are left behind are the up concentration of the desired proteins.

Materials:

20g NaOH

MilliQ water

Method:

20g NaOH is placed within a 1 L glass beaker instead of plastic due to the heating that occurs when it gets mixed with water. The beaker was filled with 800 mL milliQ water and mixed thoroughly with a magnetic stirrer. Afterwards it was transferred to a 1 L volumetric flask

until the final volume of 1 L was reached, then filtered using a 0.22 µm filter a membrane dry vacuum pump into a 1 L bottle with a blue cap, which was stored at room temperature.

2.9 Eluents for ICS-5000

For the ICS-5000 three eluents were utilized for the enzymatic reactions as shown in the table below.

Table 2.9.1

Eluent	**Components**
Eluent A	10.4 mL 50% (w/v) NaOH
	2 L MilliQ water
Eluent B	82.03g NaAc
	5.2 mL 50% (w/v) NaOH
	1 L MilliQ water
Eluent C	2 L MilliQ water

For each eluent their respective amount of milliQ water was measured using a volumetric flask besides eluent B where 82,03g NaAc was placed within a 1 L beaker and around 800 milliQ water was added and mixed with a magnetic stirrer. When it fully dissolves it is filtered with a 0,22 µm PES filter and then transferred to a 1 L volumetric flask and filled up to the desired volume.

Each eluent was placed in a special eluent bottle for ICS specifically and degassed using the Sonics Vibra-Cell Ultrasonic Processor for 20 minutes to remove any trace of carbon dioxide in the bottle.

After the degassing process the eluents get connected to their respective channels on the HPLC machine and nitrogen gas is utilized to exchange the air in the headspace. The bottles were opened, and the headspace was exchanged with nitrogen gas three times to ensure inertness.

Subsequently, 50% (w/v) NaOH was added to eluent A and B bottles in the appropriate amount as shown in Table X. The solution was carefully mixed and then reattached to the HPLC machine, with the air headspace exchanged again with nitrogen gas to maintain inert conditions

3. Methods

3.1 Cultivations of the expansin-like protein

Four uncharacterized *Z. tritici* proteins from Jean-Guy were utilized in this study. Through recombinant DNA the expansins gene from *Z. tritici* were cloned into the pPICZ alpha vector which was then transformed into Pichia X33 cells.

The promoter of the proteins will produce cells or proteins based depending on the carbon source which in this case is either glycerol which promotes cell growth or methanol which is the inducer for protein expression.

Materials

Pichia X33 + *Z. tritici* clone in glycerol

BMXY-base

BMGY

BMMY

50 mL centrifuge tubes

Cultivation flasks 100 mL, 1000 mL, 5000 mL sterilized

Multifuge X1R Refrigerated Centrifuge

TX-400 Rotor with Buckets, 15ml and 50ml Adapters

25% v/v methanol

Scale

Method

The cultivation is a five-day process. The first day 10 mL of 1 L BMGY is transferred over to a 100 mL sterilized cultivation flask. With an inoculation loop the Pichia X33 + *Z. tritici* clone in glycerol is inoculated into the medium and the organism will start to grow and divide

while using the nutrients in the medium. The organism is placed in an incubator with the temperature of 29°C at 250 rpm.

After 16-24 hours the inoculated BMGY is transferred over to a 5000 mL with the remainder of the BMGY which is roughly 990 mL and incubated at 29°C at 250 rpm.

Again, after 16-24 hours the BMGY medium is transferred over to 50 mL centrifuge tubes, evenly distributing them up until 40 mL and adjusting the weight with a scale to balance the tubes. The reason to have around 40 mL is to avoid spillage inside the machine. The centrifuge tubes are centrifuged in the Multifuge X1R Refrigerated Centrifuge using the TX-400 Rotor with Buckets, 15ml and 50ml Adapters at 1500g, 20°C for 10 min. The supernatant gets discarded, and the pellet is resuspended in 200 mL BMMY to promote protein expression in a 1000 mL cultivation flask. This is incubated at 20°C at 250 rpm.

Following this, yet again after roughly 16-24 hours the culture is supplemented with 25 mL 25% v/v methanol and further incubated at the same settings as the day prior.

After 16-24 hours the culture can either be supplemented with methanol again to produce more protein or it can be harvested and purified on the fifth day.

3.2 Purification

Several steps are taken to purify the four proteins after the cultivation process before they can be utilized in any experiments but prior to that they need to be harvested. The harvested proteins will be purified using Vivaflow, HisTag purification and SEC at the end.

3.2.1 Harvesting

P. Pastoris will secrete the desired proteins into the media when transitioning from BMGY to BMMY. The harvesting is achieved through centrifugation to separate the supernatant and the pellet, where the latter gets discarded, and the supernatant gets stored.

Materials

Cultivated media

Scale

50 mL centrifuge tubes

Multifuge X1R Refrigerated Centrifuge

TX-400 Rotor with Buckets, 15ml and 50ml Adapters

Method

The cultivation media is around 200-250 mL the last day and are transferred over to several 50 mL centrifuge tubes up until around 40 mL and not more to avoid spillage and loss of cultivation media during centrifugation. The tubes are balanced against each other and the difference between them are not larger than 0.1g. The centrifuge tubes are centrifuged in the Multifuge X1R Refrigerated Centrifuge using the TX-400 Rotor with Buckets, 15ml and 50ml Adapters at 3000g, 4°C for 10 min. A 250ml Millipore Stericup Filter Unit 0.45 µm (PES) Membrane gets attached to a 250 mL flask and gets filtered using a membrane dry vacuum pump to ensure that the supernatant and to get rid of any traces of cells and is stored in the fridge at 4°C until the supernatant gets concentrated by the VivaFlow machine.

3.2.2 Vivaflow

The VivaFlow machine serves multiple purposes including buffer exchange to attain desired pH for subsequent purification steps, desalting, purification of supernatant, and concentration.

Materials

VivaFlow machine

20% ethanol

500 mM NaOH

Buffer A pH 8 (50 mM Tris-HCl, 150 mM NaCl and 10 mM imidazole)

MilliQ water

Supernatant

Method

The Vivaflow machine is stored in 20% ethanol to prevent any sort of growth or presence of microbial growth when the machine is not operating. Prior to loading the protein into the VivaFlow machine it is rinsed with milliQ water twice for 5 minutes to remove any trace of ethanol from the cassette. MilliQ water gets discarded, and the supernatant from the

harvesting process gets loaded into the VivaFlow machine. The pump is set to 3 for two reasons. One to prevent the heat from the friction to wear down the tubes so fast, second is to have the pressure of the system below 2 bar.

When the supernatant is concentrated down to 100 mL, 250 mL of milliQ water gets added until it reaches 100 mL. This process is done three times. After the last addition of milliQ water, 250 mL of Buffer A is added for buffer exchange for HisTag purification. The supernatant is concentrated down to 100 mL and stored in the fridge at 4 °C.

The VivaFlow machine has to be rinsed with water twice again for 5 minutes each round and then washed with 500 mM NaOH to ensure that there is growth of any organisms and in addition to that it also denatures any proteins that remain in the cassette for 10 minutes. The final step is to run 20% ethanol in the VivaFlow machine so the system is stored in 20% ethanol at 4 °C.

3.2.3 Immobilized metal affinity chromatography (IMAC)

IMAC was the next step after concentrating the supernatant and performing buffer exchange with buffer A. The proteins are purified based on their affinity towards the immobilized metal ion nickel.

The HisTrap 5 mL column comes prepacked with Ni Sepharose High Performance, which is made up of cross-linked agarose beads embedded with an immobilized chelating group. These beads are charged with Ni^2+ ions, which serves as the stationary phase for IMAC.

The samples containing the His-tagged proteins will be loaded onto the column where the proteins will selectively bind to the stationary phase containing nickel ions.

The HisTrap 5 mL column is prepacked with Ni Sepharose High Performance that contains cross-linked agarose beads with an immobilized chelating group. The medium has been charged with Ni^{2+}-ions.

During the chromatographic process, the sample containing His-tagged proteins is loaded onto the Ni-NTA resin column that has been conditioned with the binding buffer. The proteins selectively bind to the immobilized nickel ions with the help of the binding buffer which will provide for stable conditions for the proteins, better binding conditions in addition to minimizing the chance of other molecules binding to the column.

A wash step after loading the protein to the column is performed to ensure that non-specifically bound proteins and contaminants elute while retaining the His-Tagged proteins to the column.

Proteins from the column are thereon eluted from the column using the elution buffer with high imidazole concentration. Imidazole and the His-tagged proteins compete for the binding site of the immobilized nickel. The higher the imidazole concentration is the less protein will be present in the column and more imidazole will bind to it. Through this the protein elute from the column, is collected, and analysed further.

Materials

MilliQ Water

Buffer A

Buffer B

Washing buffer

20% ethanol

Method

The NGC chromatography system is stored in 20% ethanol to prevent any presence of growth of any organisms, so the first step is to wash the whole system with milliQ water and the different buffers.

Next, the HisTrap HP 5 mL column is attached to the system with a low flow rate of 0.5 ml/min initially, gradually increasing to 2.0 mL/min. The column, pre-stored in ethanol, undergoes washing with milliQ water to remove residual ethanol. Following this, the column is conditioned with 5 CV of buffer A to ensure optimal binding conditions for the sample.

The sample gets loaded into the system through the sample tube and binds to the column until there is no more sample left to load. After this step washing buffer will run through the column either 5 CVs or until conductivity becomes stabilized to get rid of any nonspecific bound proteins and contaminants bound to the column.

A gradient will be utilized to elute the proteins from the sample using buffer B.The gradient ranges from 0% to 50% B over 20 minutes, followed by 50% to 100% B over 10 minutes to ensure complete elution. Proteins are expected to elute between 30% and 50%, where

fractions are collected based on UV chromatogram signals indicating protein presence. Throughout the process, the system flow rate remains at 2.0 mL/min to ensure that the column integrity and the protein binding conditions are maintained.

When the protein is done eluting the system and the column is washed with the binding buffer, milliQ water and 20% ethanol. The collected fractions thereafter analysed with SDS-PAGE to confirm presence of protein then pooled for the next purification step.

3.2.4 SEC

Post IMAC and verifying the protein presence in the fractions, the fractions get collected, pooled, and concentrated 1,5-2 mL with SEC buffer to buffer exchange prior to SEC considering the column is not compatible with imidazole in addition to also wanting to change the pH of the solution. Concentrating the proteins is one method to achieve better resolution for the chromatogram and they will be easier to detect.

SEC operates on the principle of separating molecules based on their size and shape. The stationary phase of the column contains porous beads made of materials such as agarose or dextran, with a range of defined pore sizes. As the sample is loaded onto the column, smaller molecules can enter the pores of the beads, whereas larger molecules are excluded and travel a shorter path through the column.

Materials

Amicon Ultra Centrifugal Filter 3 kDa MWCO

SEC buffer

MilliQ water

20% ethanol

Proteins

BD Emerald Sterile 2mL Syringe

Syringe 10 mL (12 mL) Henke-Ject

Microwave

Method

The proteins are first pooled together post His-Tag purification and centrifuged to concentrate the proteins down to 1,5-2 mL with Amicon Ultra Centrifugal Filter 3 kDa MWCO and then buffer exchanged with SEC buffer twice to the same amount as mentioned with the Multifuge X1R Refrigerated Centrifuge using the TX-400 Rotor with Buckets, 15ml and 50ml Adapters at 4500 rpm, 4°C until the desired volume is reached. After concentrating the proteins they are kept in 2 mL Eppendorf tubes until they are loaded into the machine.

The ÄKTA pure chromatography system and the ProteoSEC Dynamic 3-70 kDa HR resin column is stored in 20% ethanol to prevent any presence of growth of any organisms, so the first step is to wash the whole system with both milliQ water and SEC buffer for 1 CV of each which equates to 240 mL total with a flowrate of 1.6 mL/min. After this step the chromatogram should display a stable conductivity and the protein is ready to be loaded.

While waiting for milliQ water and SEC buffer to pass through the column, a needle is thoroughly washed with milliQ water twice, in the second wash the needle is put with milliQ water in the microwave to sterilize it. A 12 mL Henke-Ject syringe and a BD Emerald 2 mL syringe were conditioned with both milliQ water and SEC buffer prior to loading of the protein. Then around 12 mL buffer is then injected first into the loop on the load mode setting then, followed up shortly by the protein sample. During loading of buffer and sample the flow is set to zero.

After loading the sample, the flow is set to 1,0 mL/min and the fractionation starts when UV spikes are detected on the chromatogram with fraction size of 2 mL. The run takes either 2 hours due to the flow being 1,0 mL/min to have a better resolution and the column size, but if the protein detection is known prior to loading it is not necessary to wait for 2 hours though it will depend completely on protein size.

When done fractionating the system has to be stored in 20% ethanol. It is first purified with 1 CV of milliQ water and then 1 CV of 20% ethanol. The same thing applies to the loop that is washed with a 12 mL syringe.

The collected fractions thereafter analysed with SDS-PAGE to confirm presence of protein then pooled for the next purification step.

3.3 Concentration of proteins with buffer

After collecting the fractions from SEC they will be concentrated with NaAc until they reach the desired volume before further analysis.

Materials

50 mM NaAc buffer pH 5

Amicon Ultra Centrifugal Filter 3 kDa MWCO

Proteins

Method

The pooled fractions get concentrated to 2 mL with Amicon Ultra Centrifugal Filter 3 kDa MWCO and buffer exchange with 50 mM NaAc twice to 2 mL with the Multifuge X1R Refrigerated Centrifuge using the TX-400 Rotor with Buckets, 15ml and 50ml Adapters at 4500 rpm, 4°C. When the centrifugation is done, they are stored in 2 mL Eppendorf tubes in the fridge at 4°C until further analysis.

3.4 Measuring concentration at 280 nm and/or Bradford Method

Tyrosine and tryptophan are mainly responsible for the detection of proteins with the biophotometer at 280 nm. The formula is multiplied by 10^6 to get the concentration in µM. A is the measured absorption from the biophotometer and the molar extinction coefficient (ϵ) of the proteins is already established from Jean-Guy as their values are displayed from section 2.5.

$$Concentration\ (\mu M) = 10 * \frac{A}{\epsilon} 10^6$$

The Bradford assay is a colorimetric protein assay based on the binding of Coomassie Brilliant Blue G-250 dye to proteins, resulting in a color change proportional to the protein concentration. The concentration of BSA ranging from 0 to 20 mg/mL was utilized to obtain a standard curve. Multiple replicates were conducted to ensure accuracy, and any deviations were identified and treated as outliers.

Materials

Bradford Assay

50 mM NaAc pH 5

Protein

BSA standards

Method

For measuring the concentration with NanoDrop 1 mm 1 µL of 50 mM NaAc pH 5 is placed on the centre of the special cuvette first to blank the biophotomer. Thereafter the special cuvette is cleaned with a small paper towel and water between each sample and the absorption is given on the machine.

For the Bradford method, a bovine serum albumin (BSA) standard curve was prepared to measure protein concentration. Each well in a microtiter plate contained 159 µL of 50 mM NaAc pH 5 buffer, 1 µL of the sample/BSA, and 40 µL of Bradford assay which is thoroughly mixed and later on measured at 595 nm.

3.5 Analysis of the various polysaccharides with the expansin-like proteins with HPAEC-PAD

Expansins possess the ability to loosen the cell wall compartments and with the use of high-performance anion-exchange (HPAEC) in combination with pulsed amperometric detection (PAD) on with the Dionex ICS-5000 their potential activity was to be observed.

The actual activity these expansins-like proteins possess on the various substrates are currently unknown but with this experiment there are hopes to detect activity.

Materials

TGX

KGM

Beta-glucan

Beechwood Xylan

PASC

Whatman Filter paper

Aspergillius Niger biomass

F9

F12

F14

NaAc pH 5

MilliQ water

Eluent A

Eluent B

Eluent C

HPLC vial

Microtiter plate

Native COS standard DP2-DP6

Native XOS standard DP2-DP6

Method

In 2,0 mL Eppendorf tubes with a total reaction volume of 200 µL it was added 10 µM protein concentration, 20 mM buffer concentration of 50 mM NaAc, 1 g/L substrate concentration and the remaining volume would be filled up with water. Besides Aspergillius Niger Biomass, all polysaccharides were treated with and without PASC.

The samples were incubated at 37°C with for total of 48 hours. They would be sampled at 2 hours, 24 hours and 48 hours. The substrates had varying degree of rpm just to experiment with it, ranging from 300-800 rpm.

The samples were prepared as follows:

Substrate alone

Protein alone

Protein + substrate

Protein + substrate + PASC

After collecting the samples, they were exposed to heat to denature the protein to ensure all activity stopped. The samples were then filtrated using a microtiter plate with a filter and put inside an HPLC vial with a total for 50 µL so essentially 25 µL sample and 25 µL milliQ water to have a 2x dilution.

The samples were then analysed using HPAEC-PAD using the Dionex ICS-5000 as stated in the method “Chromatographic analysis of LPMO-derived products by HPAEC-PAD” (Østby et al., 2023).

The standards utilized were native COS DP2-DP6 and native XOS DP2-DP6 that were both made in house and had a concentration of 0.05 mg/mL.

3.6 N-deglycosylation

A common post-translational modification (PTM) is N-linked glycosylation, where oligosaccharides are attached to the side chain nitrogen of asparagine residues within the sequon motif N-X-S/T. N being the first amino acid of the sequon motif that is aspergine, X being any amino acid but proline ,and S/T being seeing either serine or threonine on the third amino acid. This can occur during or after the folding of the protein. The motif is recognized by the enzyme called oligosaccharyltransferase (OST) which is responsible for transferring the glycan to the side chain of the protein.

The enzyme EfEndo18A is an endo-β-N-acetylglucosaminidase which is a classified as a endoglycosidase. This type of enzyme hydrolyses the N-linked glycans between two GlcNAc residues.

The hydrolysis of the GlcNac is called deglycosylation and it’s a common method to observe whether there is glycosylation present or not. By removing the sugar chain, the theoretical weight of the protein is going to be lowerered, although by how much depends on how large the glycan chain is.

Materials

187 mM EfEndo18A

50 mM NaAc pH 5 buffer

F9

F12

F14

MilliQ water

Method

In a 1.5 mL Eppendorf tube 20 mM of the N-deglycosylating enzyme, 20 μM of proteins, and buffer gets added. In total the final volume of the mix is 100 μL. It is then incubated at 33°C for 24 hours and then analysed with SDS-PAGE analysis. To confirm whether there is an N-deglycosylation or not proteins without the enzyme was also incubated.

4. Results

4.1 Cultivation of the expansins-like protein

The four expansins from Jean-Guy cloned into the Pichia X33 expression vector were produced using the *P. Pastoris* expression kit as stated in section 3.

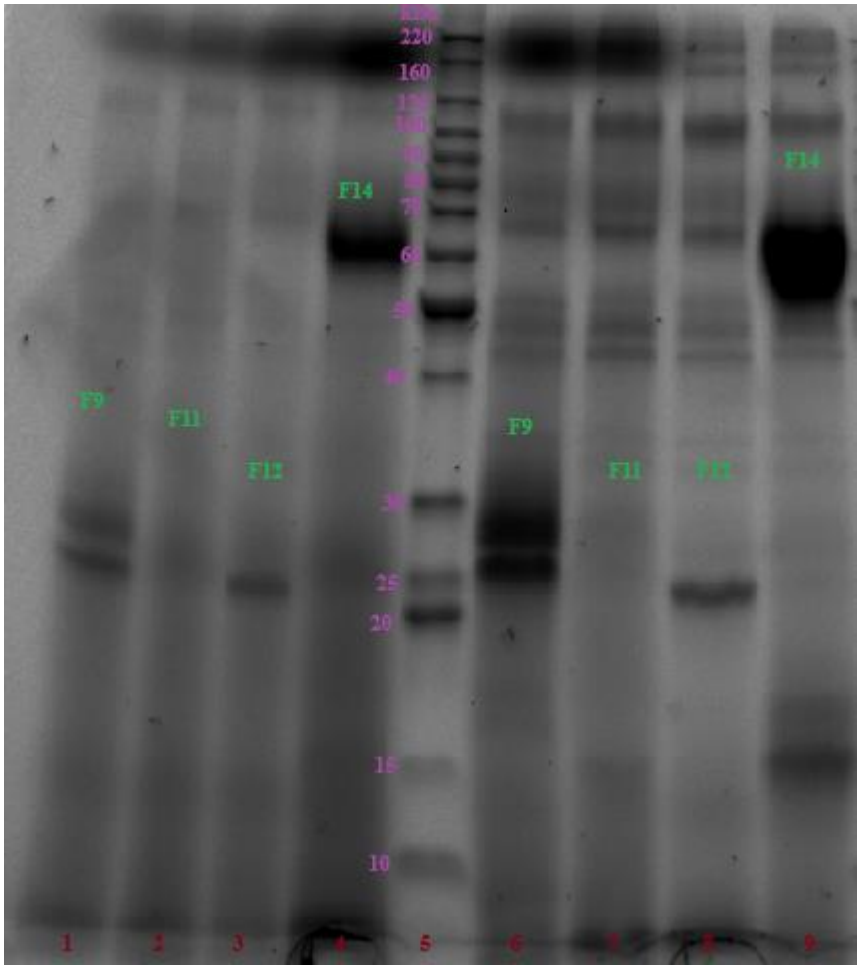


Figure 4.1.1. SDS-PAGE post- harvest and vivaflow. Left side of the benchmarker displays after harvesting while on the right after Vivaflow with the proteins annotated to their corresponding wells.

As observed in figure 4.1.1, the bands are clearer post-Vivaflow than pre-Vivaflow. Using Jean-Guys data, the size of protein F9 was estimated to be 25 kDa. The protein displays the formation of two bands. Protein F11 was expected to have a size of approximately 15 kDa, which is barely visible after Vivaflow. F12 was reported to be around 22 kDa, and F14 60 kDa, respectively.

4.2 Purification of the expansin-like protein

After the cultivation of the proteins and purification with Vivaflow where it underwent a buffer exchange it was ready to be purified with HisTag, followed up by SEC to be able to be utilized for further experiments. Due to formatting error the data for His-Tag purification was not saved for the proteins.

4.2.1 Purification of F9

The F9 protein eluted at a buffer B concentration of approximately 50-60%. The fractions that produced increased UV signal were collected and analysed further with SDS-PAGE. The protein was expected to have a molecular weight of 25 kDa.

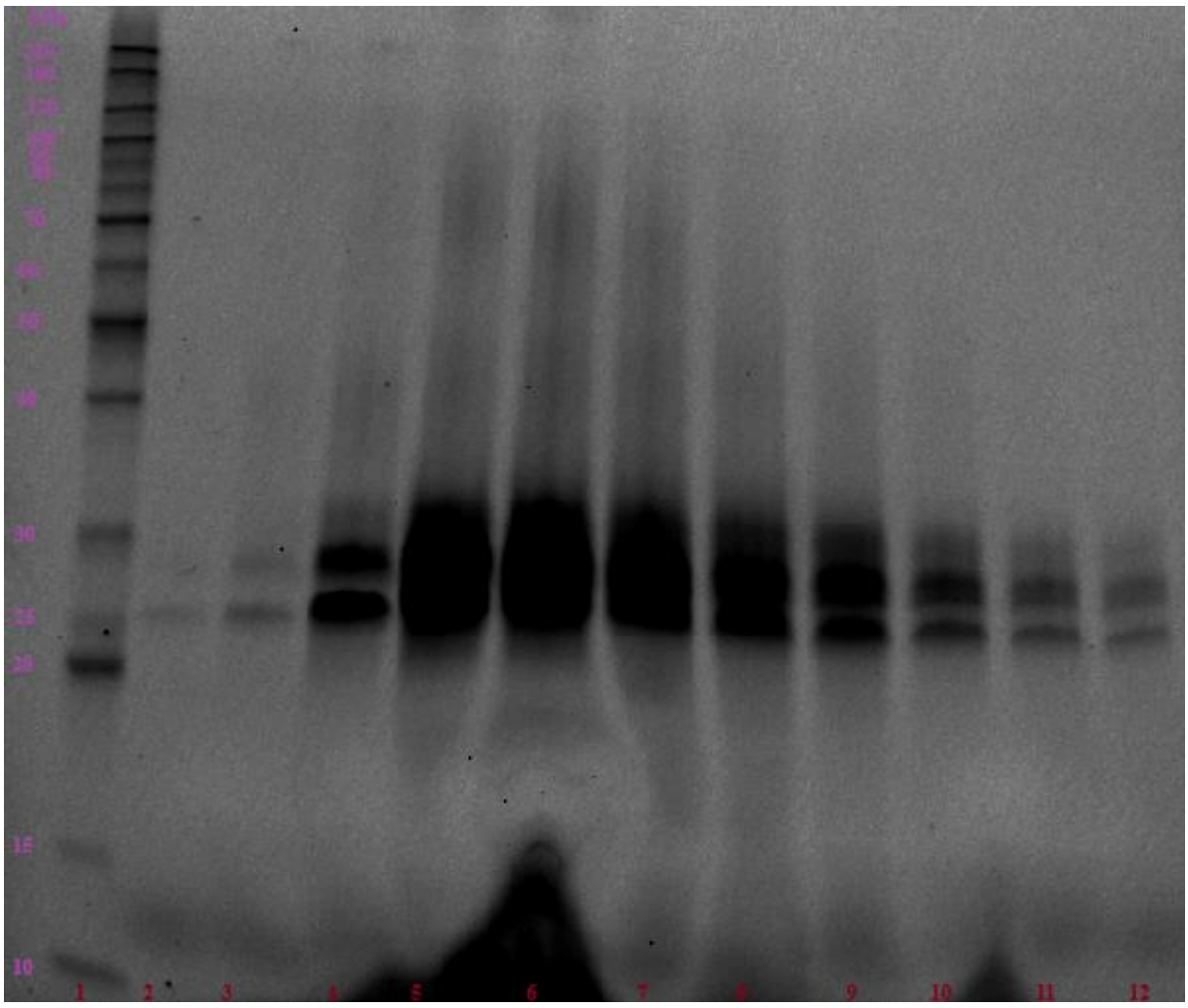


Figure 4.2.1 SDS-PAGE of the fractions from His-Tag purification of F9. The first well contains Benchmark ladder while well 2 and onwards contain the fractions of the proteins.

The SDS-PAGE from figure 4.2.1 shows that the fractions of protein F9 are more isolated although there are still signs of other proteins present. In addition to that the protein has two bands on the gel close to each other. This could be two different proteins or the same. The fractions from wells 2-11 were pooled together and concentrated to around 2 mL prior to SEC.

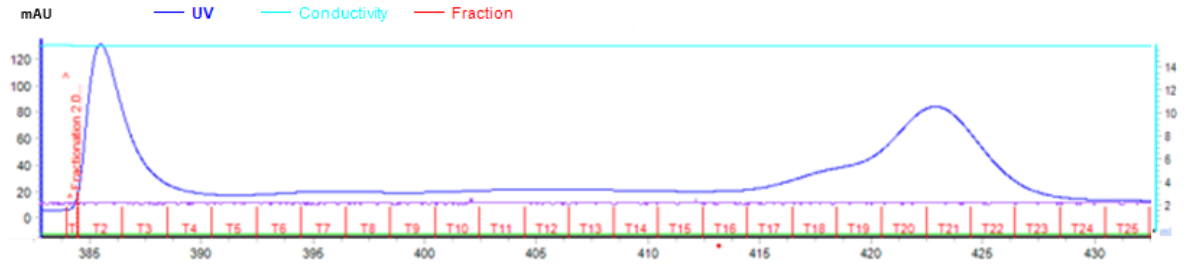


Figure 4.2.2 Chromatogram from the SEC of the F9 protein. It displays that F9 protein eluted twice, between 365 ml and and 390 ml in addition to around 415 ml and 426 ml with the fractions marked. The flowrate was set to 1 mL/min. The y-axis on the left displays UV in mAU which is displayed in blue on the chromatogram, the x-axis mL and conductivity on the right y-axis measured in mS/cm in light blue which is stable throughout the purification.

The two peaks from the chromatogram shown in figure 4.2.2 were analysed by SDS-PAGE. These were the fractions 1-3 and 16-24, as displayed.

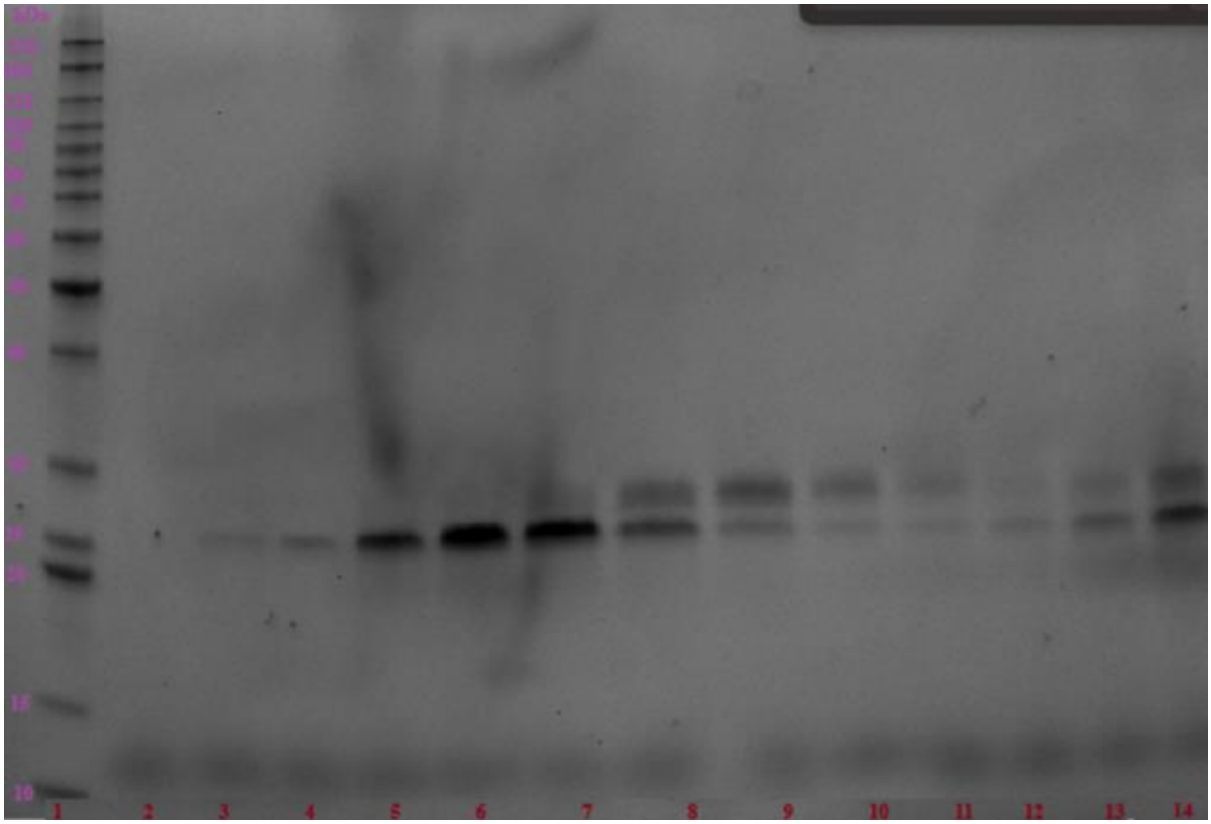


Figure 4.2.3 SDS-PAGE of the F9 SEC fractions. The first well is Benchmark ladder, well 2-4 contains fraction 1-3 and well 4-14 contains fraction 16-24.

After SDS-PAGE post His-Tag purification, the gel still displayed two bands close to each other even after SEC, which suggests that it's the same protein. All the fractions besides the one from well 2 were pooled together after concentrating it down to around 1.5 mL and exchanging the buffer with NaAc pH 5. Shortly after, the concentration was measured using both the Bradford method, as well as a biophotometer.

4.2.2 Purification of F11

F11 as displayed in figure 4.2.4 is barely visible, both prior and after Vivaflow, but the protein still underwent the buffer exchange prior to HisTag purification. The chromatogram displayed some activity through UV detector during His-Tag and the fractions from there

were collected and analysed through SDS-PAGE.

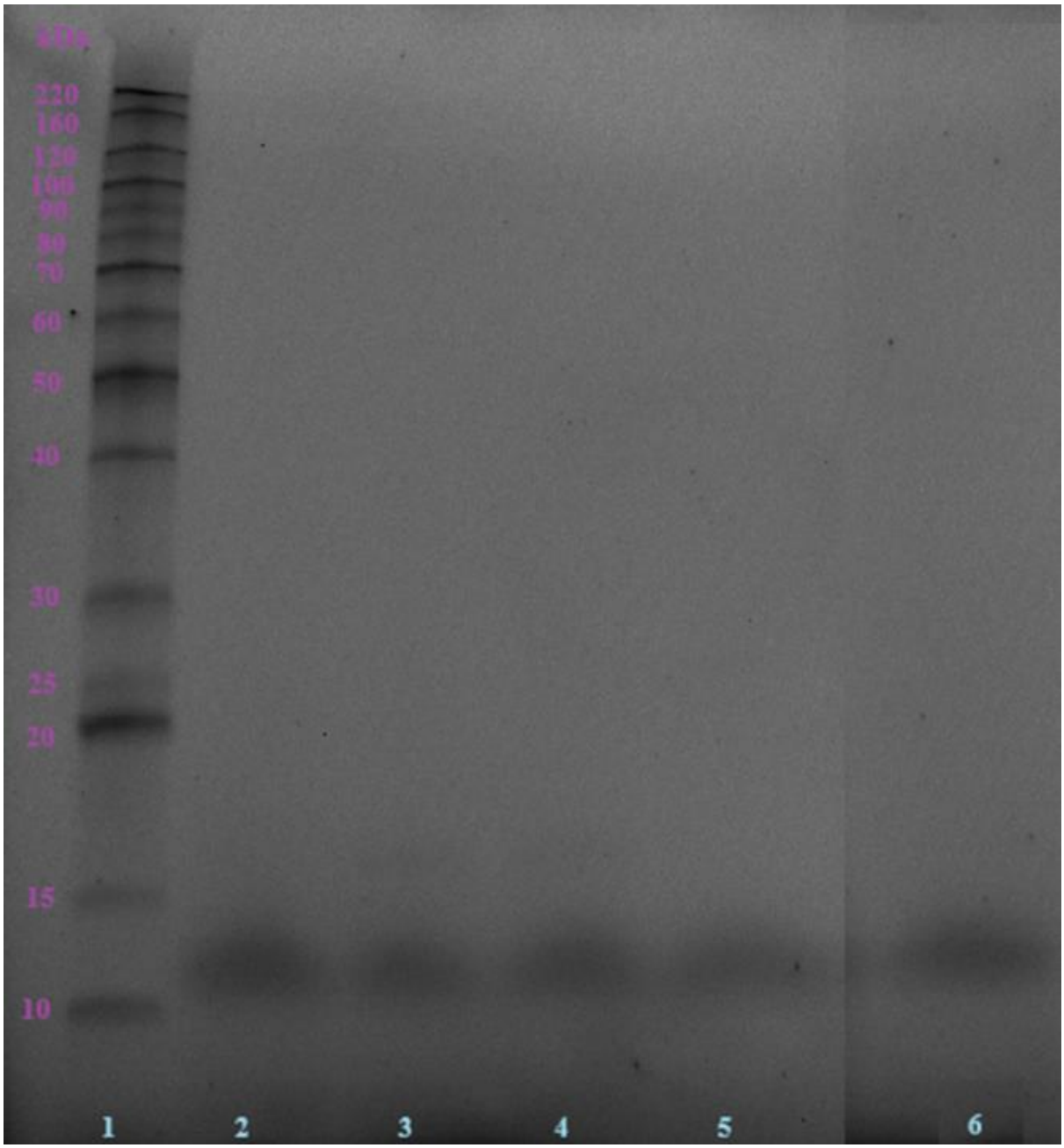


Figure 4.2.4 SDS-PAGE of the fractions from His-Tag purification of the F11 protein. The first well contains Benchmark protein while well 2-7 contains the fractions.

As shown in figure 4.2.4, there are barely any visible bands, if any at approximately 15 kDa. Considering the low abundance of protein in the sample there was no reason to proceed with SEC. F11 was cultivated and purified again with the same methods as the prior batch.

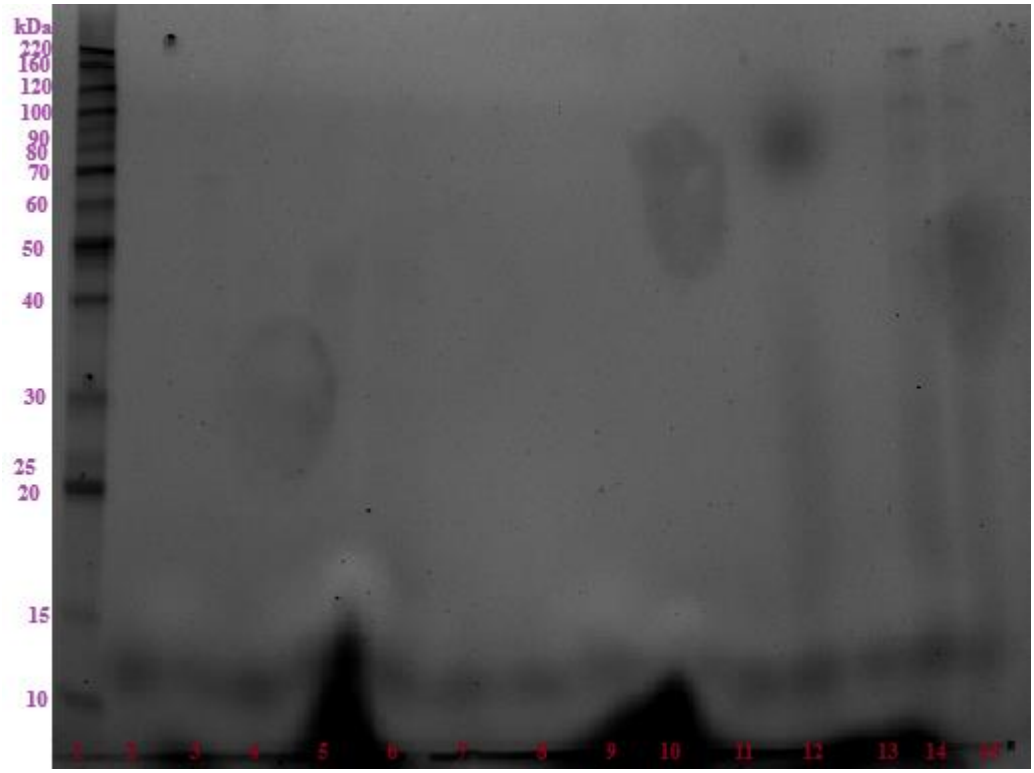


Figure 4.2.5 SDS-PAGE of recultivated and repurified F11 from scratch. Well one is Benchmark ladder. Well 2-11 are the fractions from His-Tag purification. Well 12 and 13 are post-Vivaflow and its flowthrough while 14 and 15 are flowthrough from the His-Tag purification.

According to the Jean-Guy data, the protein of interest was expected to have a molecular weight of approximately 15 kDa. This was not the case for either gels, considering the protein is not visible at all. Flowthrough from Vivaflow and His-Tag purification was stored and analysed on SDS-PAGE to rule out presence of it in the flowthrough but the protein was absent there as well.

There were two available F11 protein glycerol stocks in the -80°C freezer. Both were used for the cultivation process, but none yielded any significant protein for further purification and analysis, as shown in figure 4.2.6.

4.2.3 Purification of F12

F12 was expected to be around 23 kDa in molecular weight and underwent buffer exchange prior to His-Tag purification. It showed in figure 4.2.7 bands around between 20 and 25 kDa although still some other proteins present in the gel.

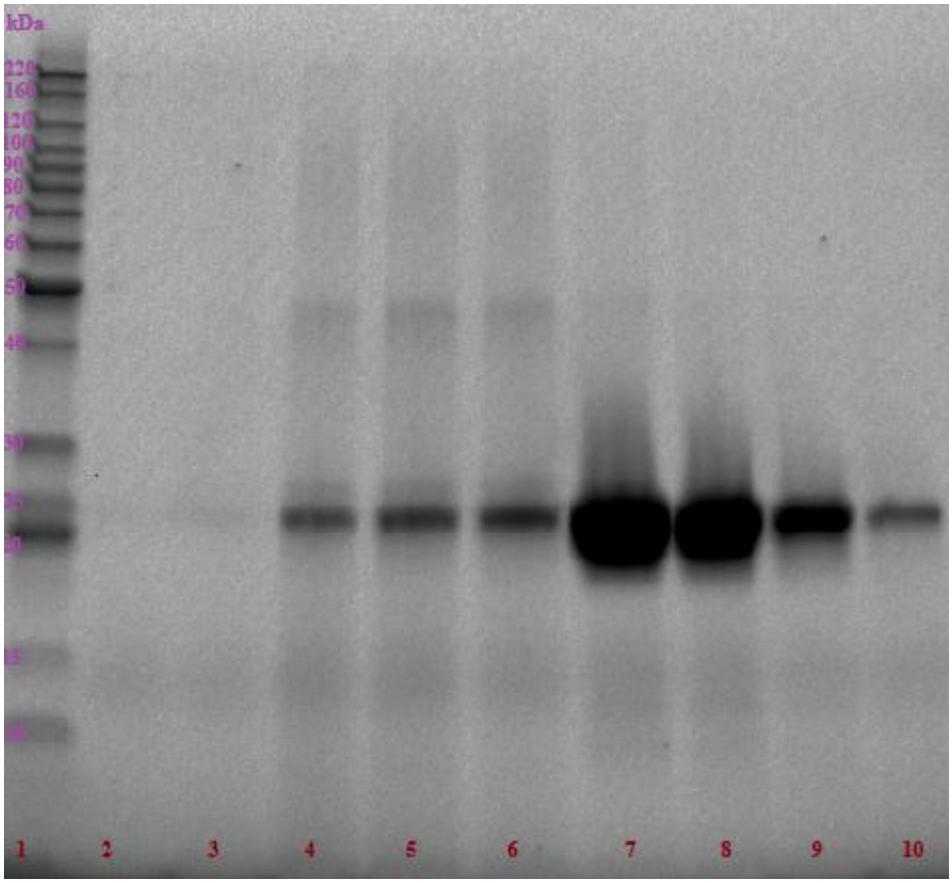


Figure 4.2.6 Fractions from HisTag purification for F12 protein. Well one contains Benchmark ladder, and the rest are fractions. As the gel shows there are still other proteins visible besides the desired one.

The fractions from well four to ten were pooled together after His-Tag purification since those are where the protein of interest is present. Still there are other proteins besides the one of interest in the gel, hence SEC was performed to ensure the protein was isolated and as pure as possible. The seven fractions that were pooled together and concentrated to around 2 mL while performing a buffer exchange with SEC buffer.

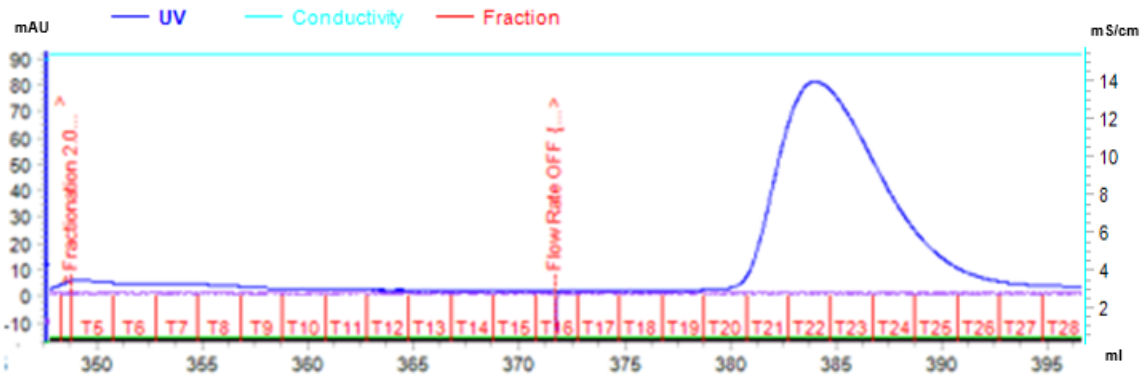


Figure 4.2.7 Chromatogram from the SEC of the F12 protein. The F12 protein eluted once between 380 mL and 390 mL. The flow rate was set to 1 mL/min. The chromatogram displays the UV (mAU) and conductivity (mS/cm) on the y-axis and the volume that has passed through the column.

From the chromatogram in figure 4.2.7, the protein starts eluting around the 380 mL mark which corresponds to fraction 20. Hence, fractions 19-27 were analysed on SDS-PAGE.

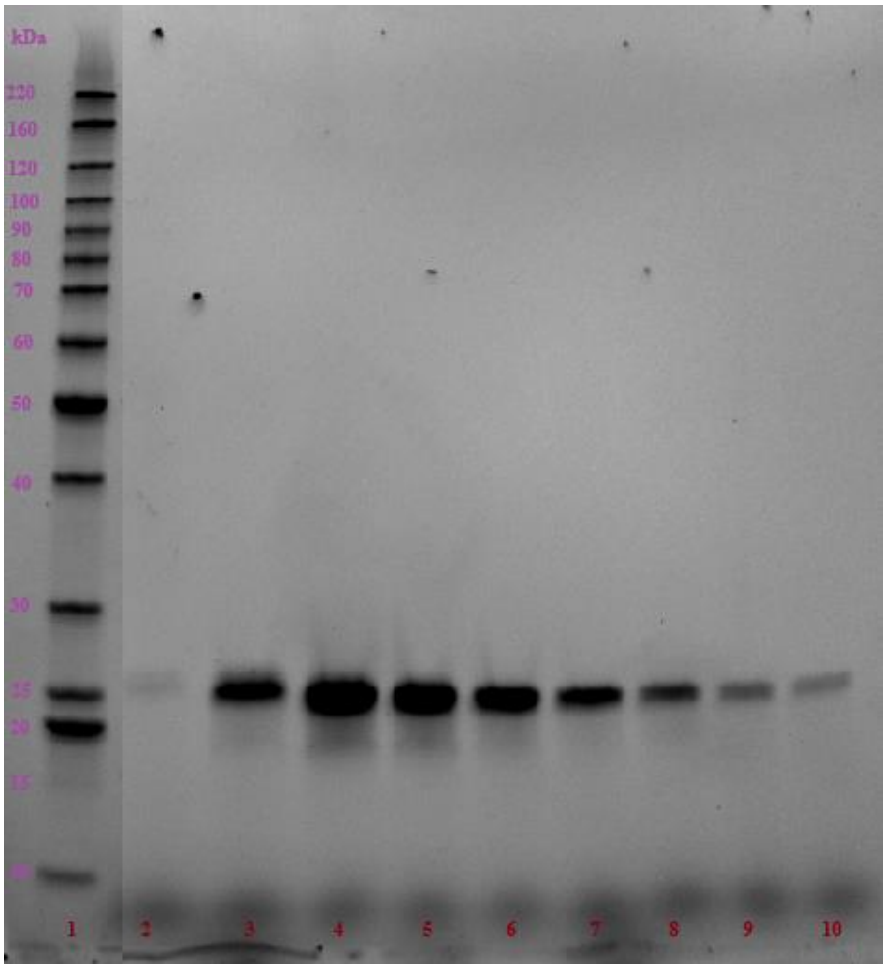


Figure 4.2.8. SDS-PAGE of the fractions from F12. Well one contains Benchmark ladder while the rest are the fractions of the protein.

As the gel on figure 4.2.8 shows, the protein is more pure compared to post His-Tag purification in figure 4.2.6. The bands were supposed to be approximately 22 kDa, but on the gel they were approximately 24 kDa.

4.2.4 Purification of F14

F14 was by far the largest protein of the four and underwent buffer exchange prior to His-Tag.

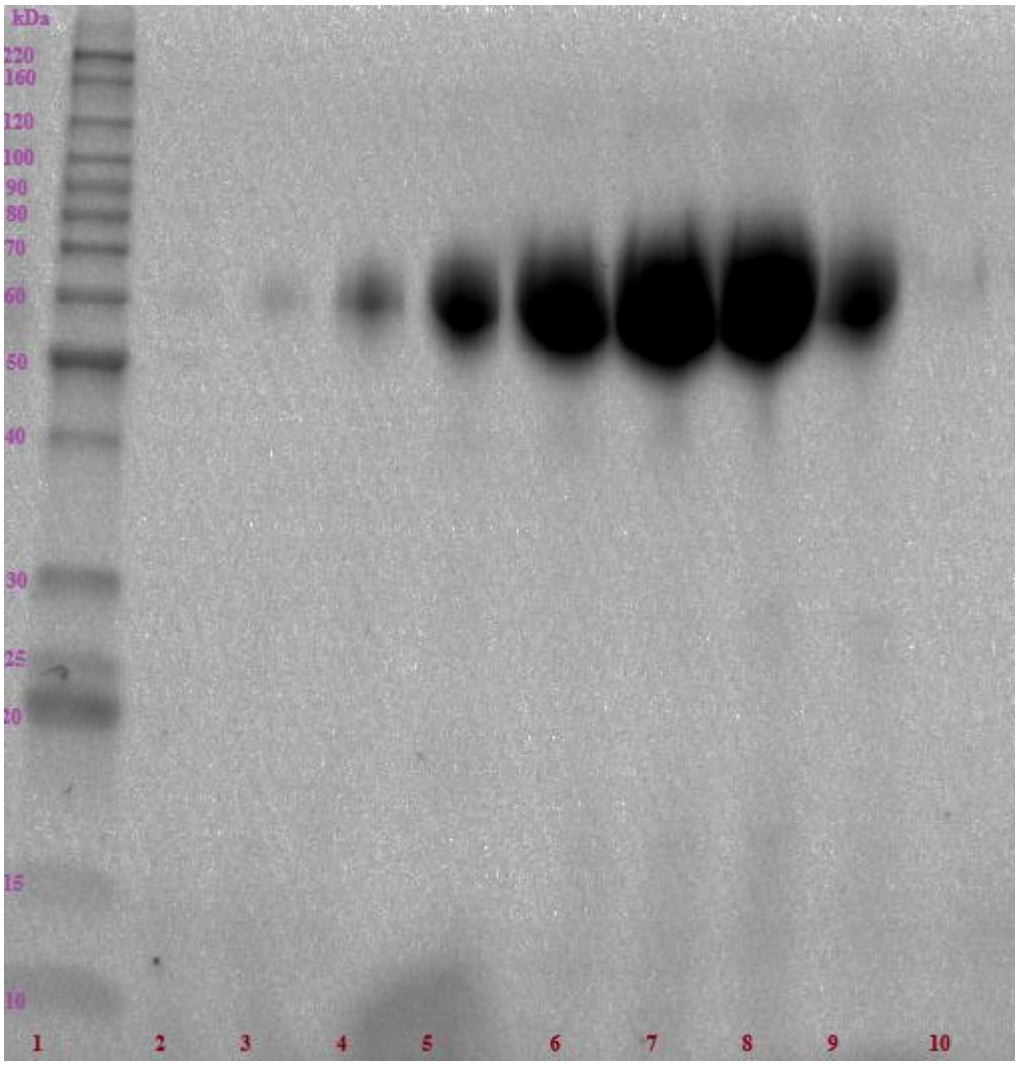


Figure 4.2.9 His-Tag purification of F14. Benchmark ladder is in the first well while the rest are fractions.

The protein bands of F14 were approximately 60 kDa, and the protein appeared quite pure. Regardless of this, it still underwent SEC to ensure the desired protein was isolated. It was concentrated to 2 mL prior to SEC. Concentration pellets formed due to the large size of the protein, but they were discarded.

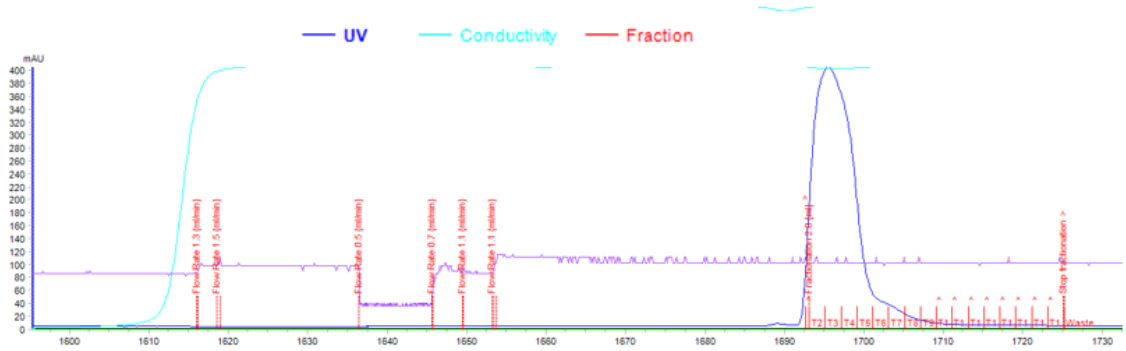


Figure 4.2.10 Chromatogram from the SEC of the F14 protein. It shows that F14 eluted between 1690 mL and 1710 mL as displayed on the chromatogram.

Despite what the runtime suggests, purifying the F14 protein took the least amount of time compared to the other two proteins, due to its size. Fractions 1-10 were analysed on SDS-PAGE shortly after.

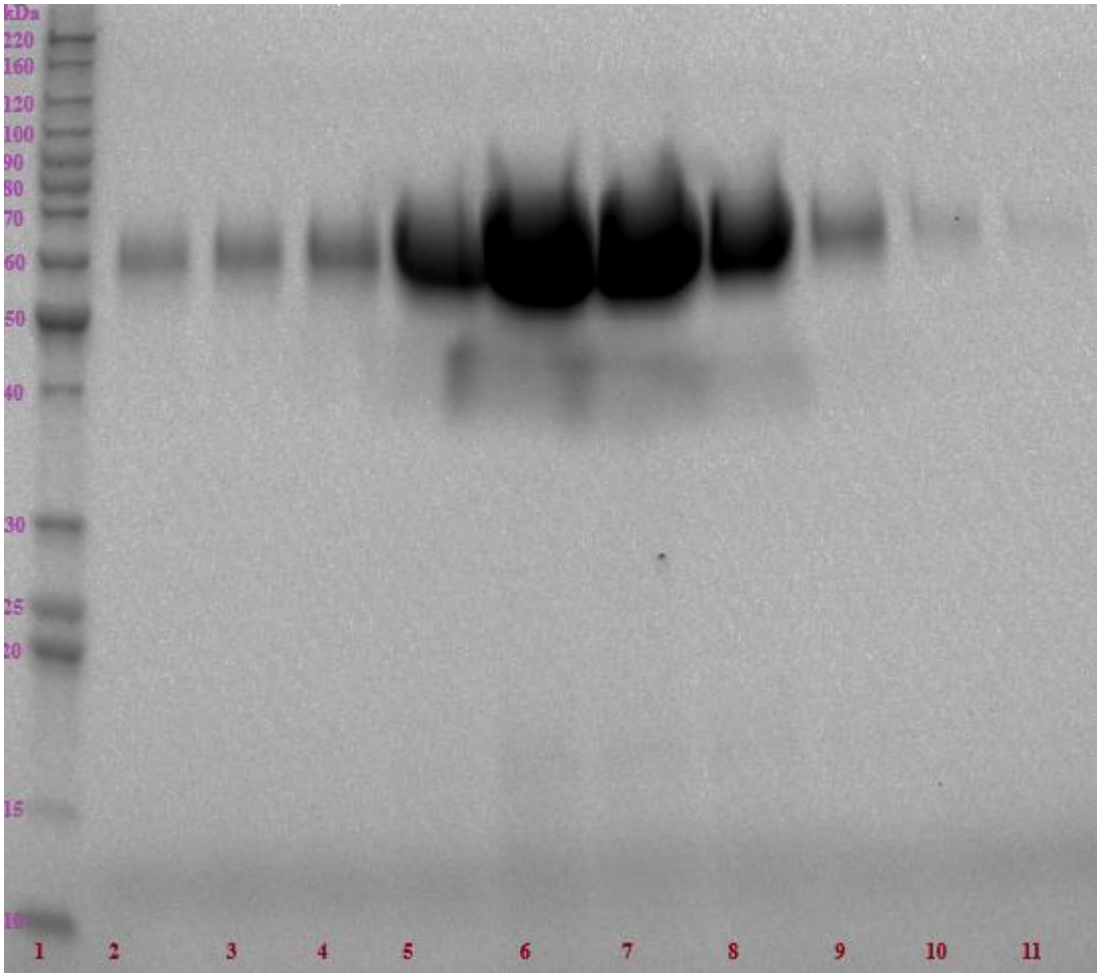


Figure 4.2.11 SDS-PAGE of the SEC fractions of F14. Well one is benchmark ladder while the rest are fractions from SEC.

Fraction 1-10 were pooled together, concentrated and performed a buffer exchange with NaAc pH 5.

4.3 Measuring concentration

Measuring concentration can be performed through either Bradford Method or direct UV measurements with the use of Beer-Lamberts law.

For Beer-Lamberts law the following equation was used.

$$Concentration\ (\mu M) = DF * \frac{A}{\epsilon} 10^6$$

Table 4.3.1 Protein concentration calculation table. The concentration of the different proteins was calculated using the dilution factor, absorbance and the molar absorption coefficient and multiplied by 10^6 to obtain the concentration in µM.

Proteins	Dilution factor	Absorbance 280 (A)	Molar absorption coefficient (ϵ)	Concentration (µM)
F9	10x	0,167	51825 L mol^{-1} cm^{-1}	32,2
F12	10x	0,129	28460 L mol^{-1} cm^{-1}	45,3
F14	50x	0,160	38640 L mol^{-1} cm^{-1}	207,0

Table 4.3.1 displays the different absorbances gained from measuring at A280 on the biophotometer as well as the dilution factor. The molar absorption coefficient was presented in section 2.6 but for the sake of clarity it's also included in this table.

As expected, considering how large F14 was the concentration is high compared to the other two. An important thing to note is that the proteins were cultivated several times to perform the enzymatic reactions. The absorbance and concentration of the proteins will fluctuate, but it's accounted for in the calculations.

As stated in methods section 3.4, the concentration was also measured with Bradford method, but due to underestimating the actual protein concentration these results were not utilized.

4.4 Analysis of the effect of expansins on various substrates with HPAEC-PAD

The various substrates listed in section 3.5 were exposed to enzymatic reactions from the different expansin-like proteins to assess their potential effect on them.

4.4.1 Analysis of polysaccharide substrates

In this experiment the substrates were subjected to the presence of expansin-like proteins. The polysaccharides utilized were KGM, TXG and beta-glucan. Samples with and without the presence of PASC were made to check whether PASC would impact the results.

Figure 4.5.1 shows the behaviour of beta-glucan without PASC and how the expansins affect it with the use of the native COS DP2-DP6 standard. Around the 17m marker some activity is detected on F9+betaglucan.

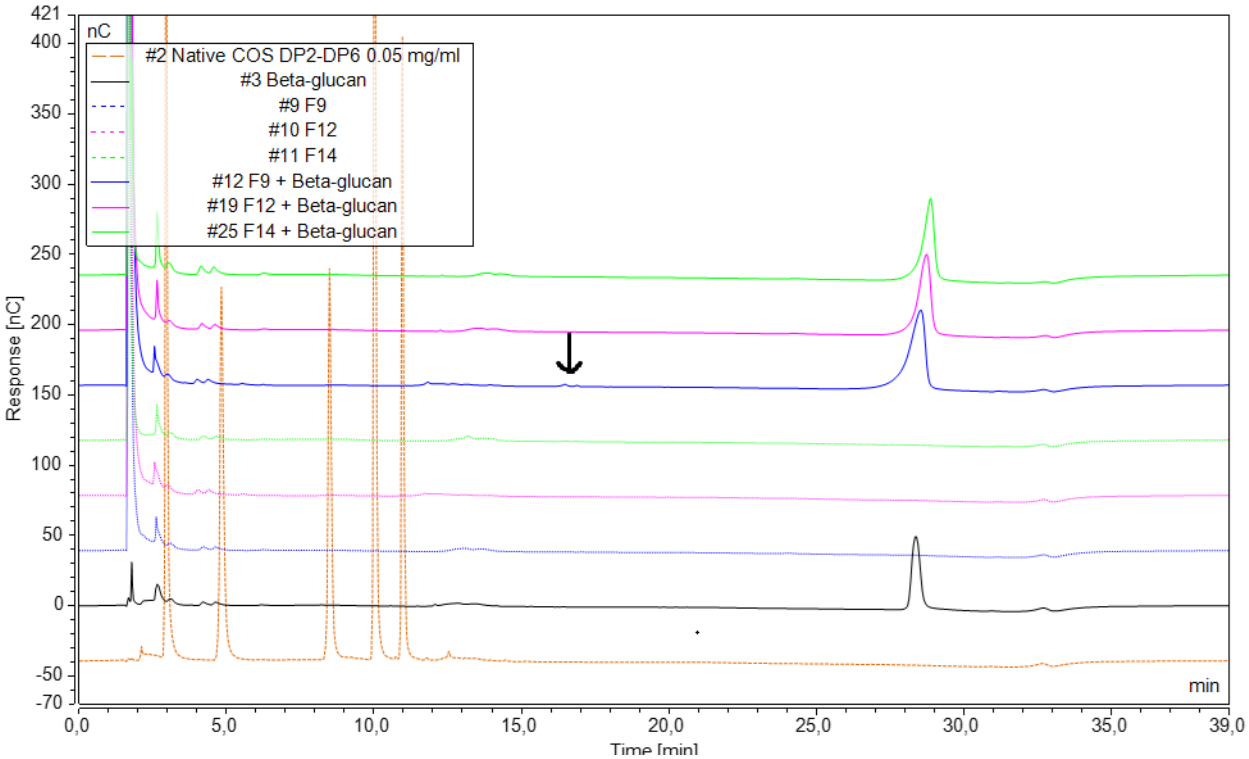


Figure 4.5.1 Chromatographic Analysis of Enzymatic Activity on Beta-Glucan. The graph illustrates the behavior of the polysaccharide under treatment by proteins, the proteins in isolation, beta-glucan alone and with the known standard COS DP2-DP6. Marked with an arrow around 17m on the chromatogram.

Figure 4.5.2 is a magnification of the highlighted section with the arrow in the previous chromatogram. It displays two peaks forming between 16.5-17m solely on F9+beta-glucan which is neither present on the beta-glucan and F9.

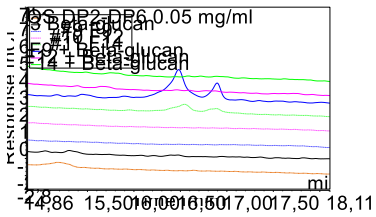


Figure 4.5.2 Magnification of the previous graph. Shows slight activity around the 16-17m marker.

For beta-glucan+PASC alongside treatment of the various expansin-like proteins did not show any potential activity, as shown in figure 4.5.3

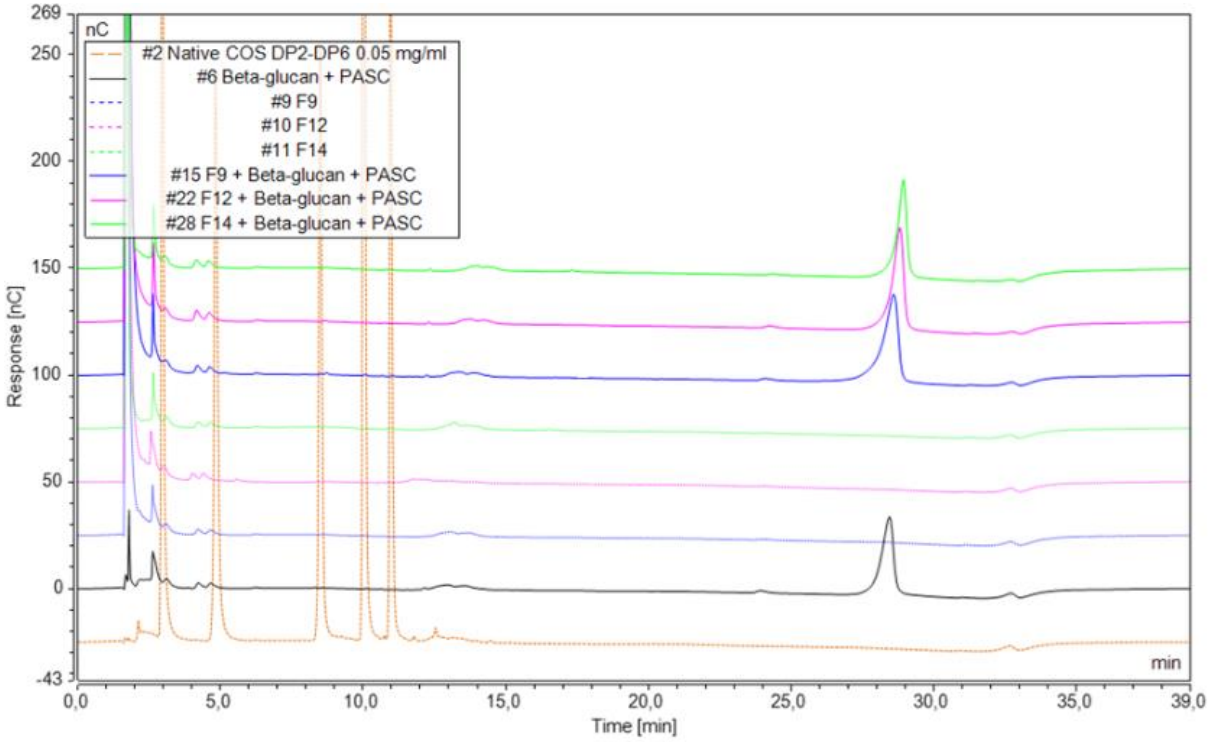


Figure 4.5.3 Chromatographic Analysis of Enzymatic Activity on Beta-Glucan in combination with PASC. The graph illustrates the behavior of the polysaccharide under treatment by proteins and PASC, the proteins in isolation, beta-glucan alone and the standard COS DP2-DP6.

For the polysaccharide TXG, again protein F9 did display some activity around the 17m marker, but not for F12 or F14.

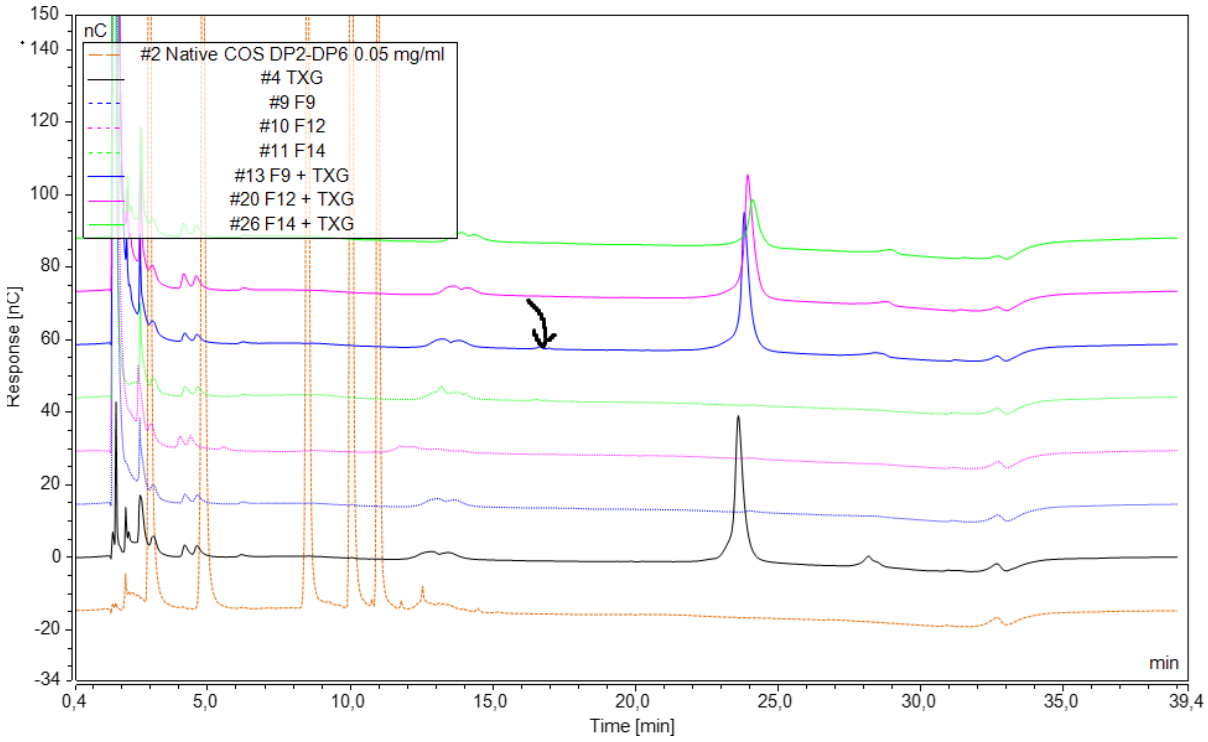


Figure 4.5.4 Chromatographic Analysis of Enzymatic Activity on TXG. The graph illustrates the behaviour of the polysaccharide under treatment by proteins, the proteins in isolation, the polysaccharide alone and the standard COS DP2-DP6.

Figure 4.5.5 is a magnification of figure of 4.5.4, which displays the potential activity on F9 a bit closer. Both F9 and TXG by themselves do not possess this potential activity which could be something to look further into.

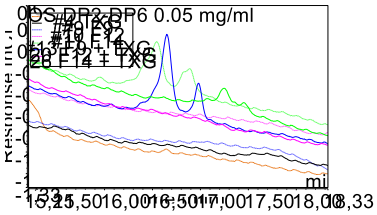


Figure 4.5.5 Magnification of the previous graph. Small peaks between 16 and 17 minutes of the chromatogram on F9 + TXG.

For TXG treated with PASC there was no potential activity spotted on the chromatogram on figure 4.5.6.

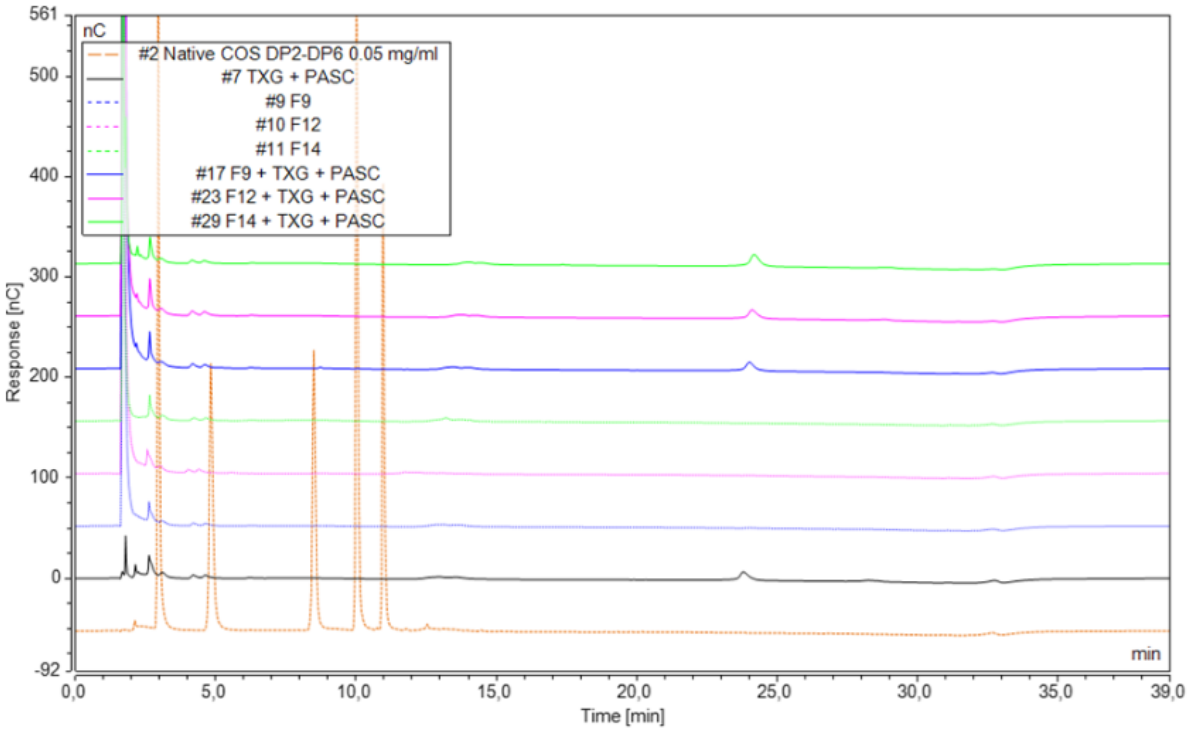


Figure 4.5.6 Chromatographic Analysis of Enzymatic Activity on TXG in combination of PASC. The graph illustrates the behavior of the polysaccharide under treatment by proteins, the proteins in isolation and TXG + PASC in isolation with the known standard COS DP2-DP6.

The next polysaccharide in question was KGM which had some peaks between the 8–11-minute mark. Figure 4.5.7 displays the overall activity of the chromatogram.

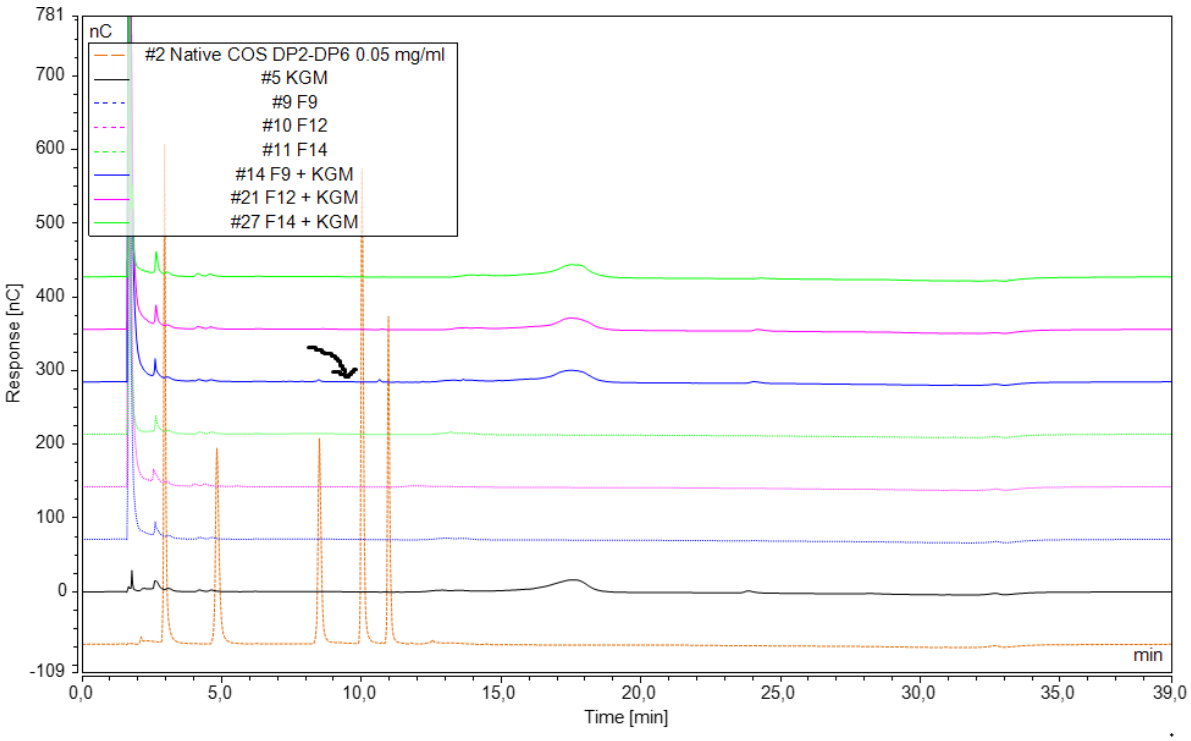


Figure 4.5.7 Chromatographic Analysis of Enzymatic Activity on KGM. The graph illustrates the behavior of the polysaccharide under treatment by proteins, the proteins in isolation and KGM, with the known standard COS DP2-DP6.

Magnifying the graphs, as displayed in figure 4.5.8, reveals there was activity for the various proteins. Some of them overlap with the standard for DP4.

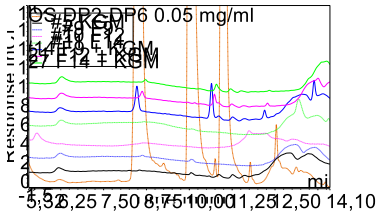


Figure 4.5.8 Magnified picture of the previous chromatogram. Three peaks form around 8.75, 11 and 12m.

For F9+KGM+PASC one substrate top was completely missing, in comparing the substrate and substrate+protein as shown in figure 4.5.9.

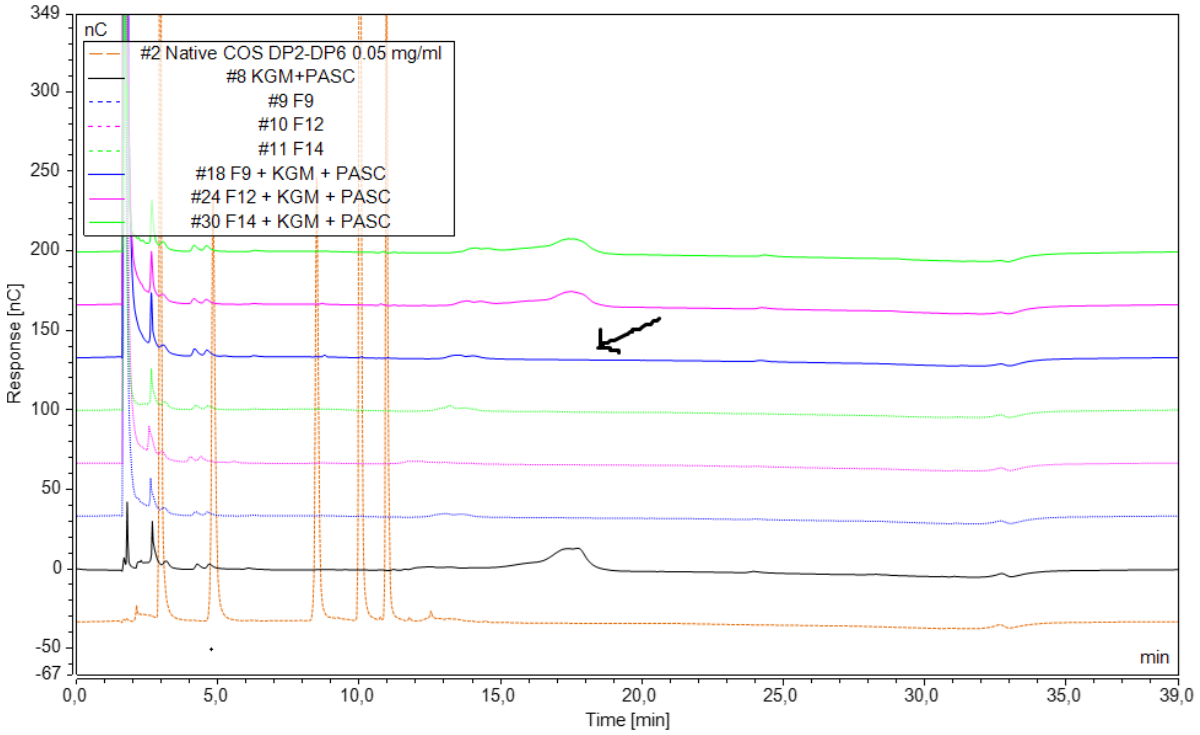


Figure 4.5.9 Chromatographic Analysis of Enzymatic Activity on KGM+PASC. The graph illustrates the behavior of the polysaccharide under treatment by proteins and PASC, the proteins in isolation and KGM+PASC, with the known standard COS DP2-DP6.

The peak on a closer magnification at around the 17m mark was missing and it does not reappear later either on the graph as it would if new products would have been formed. As illustrated the peak does appear on both F12+substrate and F14+substrate and substrate by itself.

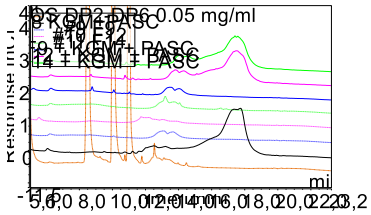


Figure 4.5.10 Magnification of the previous graph. Peak around 17m on F9+KGM+PASC is missing.

4.4.2 Analysis of Aspergillius Niger biomass

Aspergillius Niger biomass was utilized in combination of the expansin-like protein which contains various polysaccharides and other substrates which are naturally present in fungi to determine whether the proteins if the proteins from fungi could affect the fungi cell wall components. The samples underwent a 2 hours, 24 hoursand 48 hours treatment. Aspergillius Niger was not treated with PASC compared to the other substrates.

Figure 4.5.11 displays the behaviour of ANB after 48 hours of treatment and how the expansins affected the substrate. Native COS DP2-DP6 was utilized as the standard.

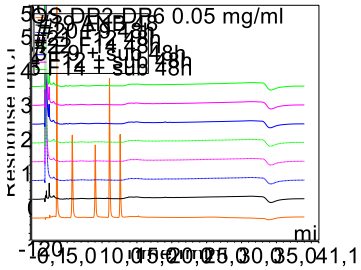


Figure 4.5.11 Chromatographic Analysis of Enzymatic Activity on ANB. The graph illustrates behavior of the ANB under treatment by proteins, proteins in isolation and ANB alone. Also, with the known standard COS DP2-DP6. All the samples besides the standard were incubated for 48 hours.

4.4.3 Analysis of Whatman filter paper

Whatman filter paper was the only cellulose structure utilized with the expansin-like protein to determine if the proteins could have an impact on them or not. The samples underwent a 2 hours and 48 hours treatment.

Figure 4.5.12 displays the behaviour of Whatman filter paper after 2 of treatment and how the expansins affected the substrate. Native COS DP2-DP6 was utilized as the standard.

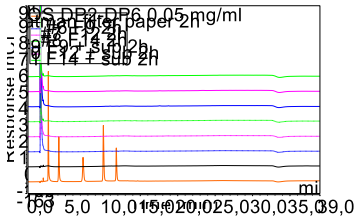


Figure 4.5.12 Chromatographic Analysis of Enzymatic Activity Whatman filter paper. The graph displays behaviour of Whatman filter paper under treatment after 2 hours with the proteins, the proteins alone and the substrate alone. Standard applied was COS DP2-DP6.

Figure 4.5.12 is a zoom in of the previous chromatogram and it displayed potential activity around 16-18m solely on F9+substrate on the samples that had been treated for 2 hours.

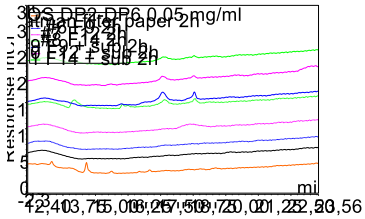


Figure 4.5.13 Zoom in of previous chromatogram. Potential activity between 17-19m.

The same was done with the samples that were treated for 48 hours and they displayed similar activity.

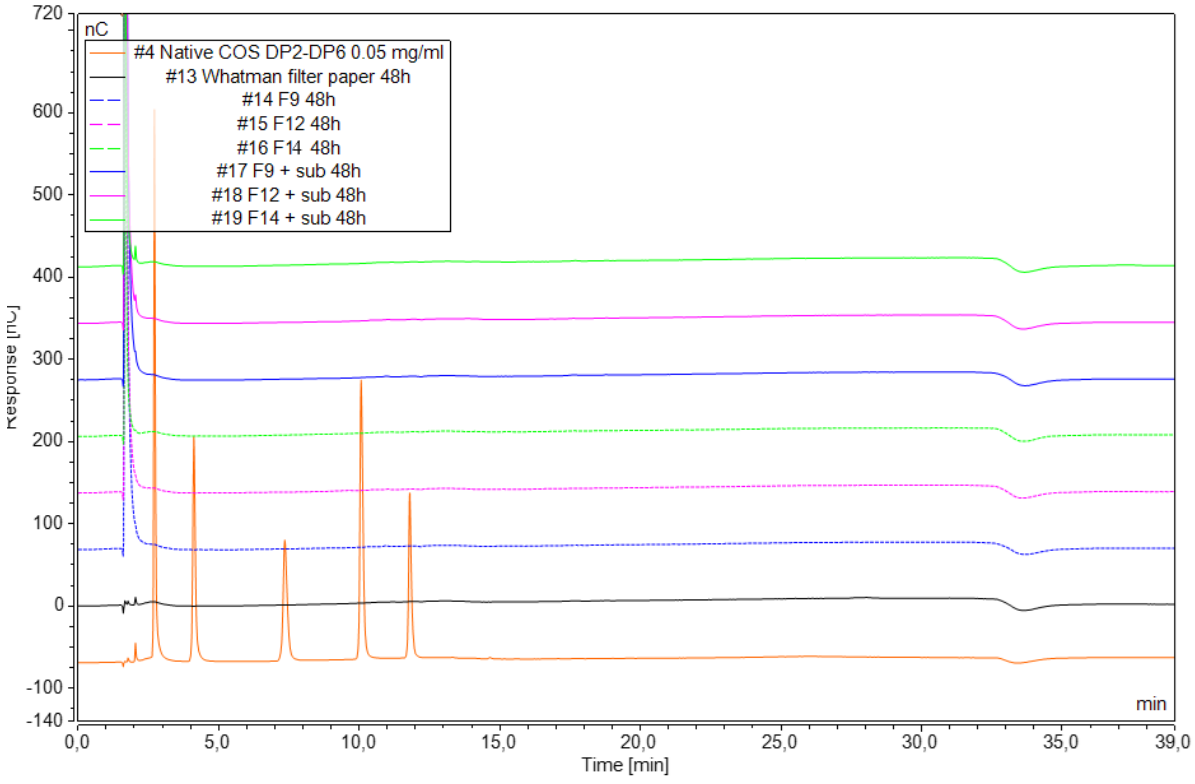


Figure 4.5.14 Chromatographic Analysis of Enzymatic Activity Whatman filter paper. The graph displays behaviour of Whatman filter paper under treatment after 48 hours with the proteins, the proteins alone and the substrate alone. Standard applied was COS DP2-DP6.

Only on F9, some potential activity was picked up by the chromatogram on the samples that were treated for 48 hours, as shown in graph 4.5.15. The similar peaks do not appear in F9+sub 2 hours treatment.

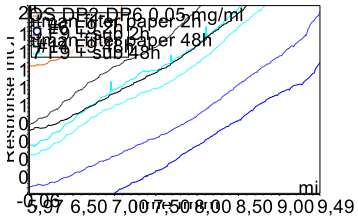


Figure 4.5.15 Chromatographic Analysis of Enzymatic Activity Whatman filter paper. The graph displays behaviour of Whatman filter paper under treatment after 2 and 48 hours in the presence of F9 only, the proteins alone and the substrate alone. Standard applied was COS DP2-DP6. Some activity is spotted on F9 + sub 48 hours.

4.4.4 Analysis of xylan

There were done two different set of experiments on xylan (in both they were treated alongside PASC). For the first set of experiment, different set of concentrations were utilized for the F14 protein. The concentrations utilized were 1 µM, 10 µM and 50 µM. This was to observe if concentration would impact the effect on the substrate. All samples were treated 48 hours.

As observed in figure 4.5.17, the concentration on xylan+PASC did not impact xylan at all after 48 hours of incubation.

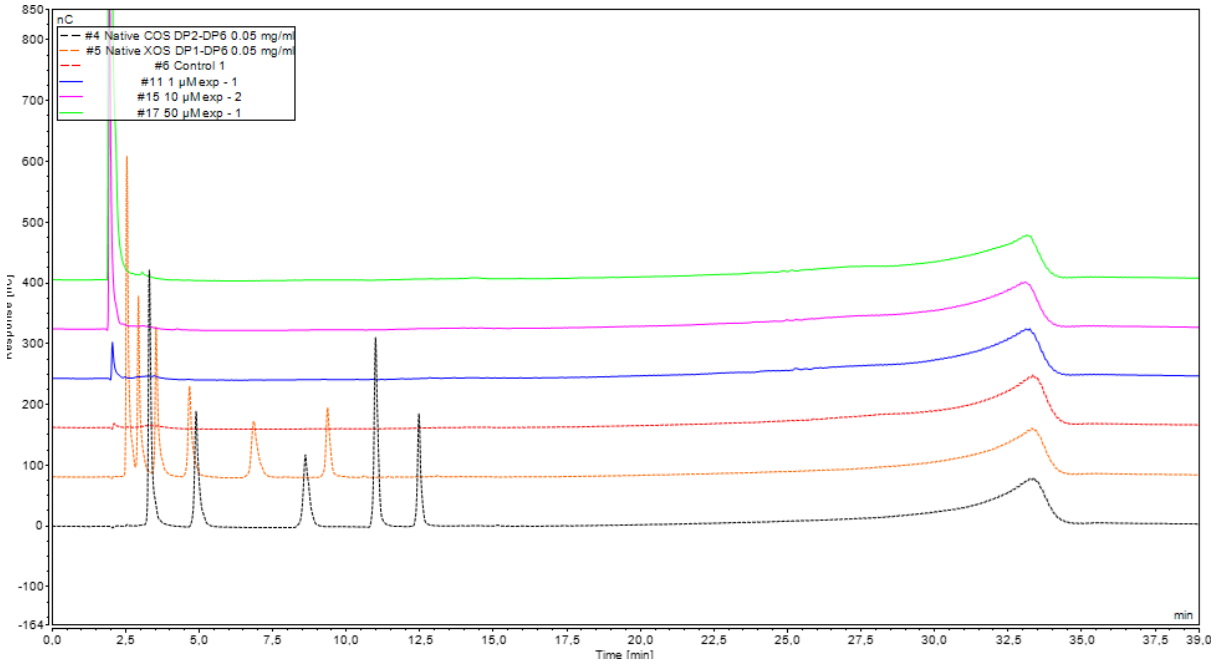


Figure 4.5.17 Chromatographic Analysis of Enzymatic Activity on Xylan+PASC. The graph displays behaviour of Xylan+PASC treated for 48 hours at different concentrations with the presence of F14 only.

For the other experiment protein F9 and F12 concentration remained, and the treatment time varied by having samples treated for 2 hours, 24 hoursand 48 hours. The standards used were native XOS DP2-DP6 and native COS DP2-DP6.

Figure 4.5.21 displays the treatment time for F9+substrate. There was no difference between xylan+PASC for the different treatment times so xylan+PASC 24 hours was utilized for the graphs. The same thing applies to the protein treatment time, no deviation there.

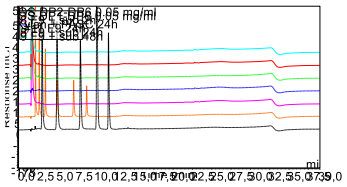


Figure 4.5.18. Chromatographic Analysis of Enzymatic Activity on with the presence of F9 only. Standards utilized were native XOS DP2-DP6 and native COS DP2-DP6.

Figure 4.5.22 shows a zoomed in version of graph 4.5.21. Some activity was picked up around the 15m marker for F9+sub for the 24 hours and 48 hours treatment.

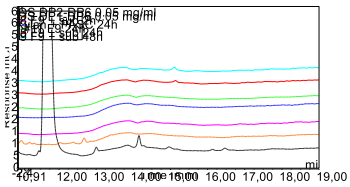


Figure 4.5.19. Zoomed in version of previous chromatogram. Potential activity around the 15m mark.

Protein F12 had the same procedure as protein F9 in terms of method. Xylan + PASC with 24 hourstreatment was utilized with the standards XOS DP2-DP6 and native COS DP2-DP6. No difference between 2 hours, 24 hoursand 48 hours treatment of the protein. Figure 4.5.23 displays if there is any activity on the substrate.

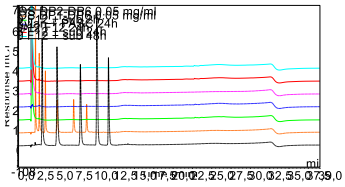


Figure 4.5.20. Chromatographic Analysis of Enzymatic Activity on with the presence of F12 only. Standards utilized were native XOS DP2-DP6 and native COS DP2-DP6.

No noticeable activity was detected on the F12 protein compared to F9.

4.5 MALDI-TOF

The expansin-like protein F9 showed the highest potential activity, so samples treated with F9 combined with polysaccharides like beta-glucan, TGX, KGM, and KGM+PASC were further analyzed using MALDI-TOF. This analysis was aimed to identify specific polysaccharide structures that were fragmented due to the treatment of F9.

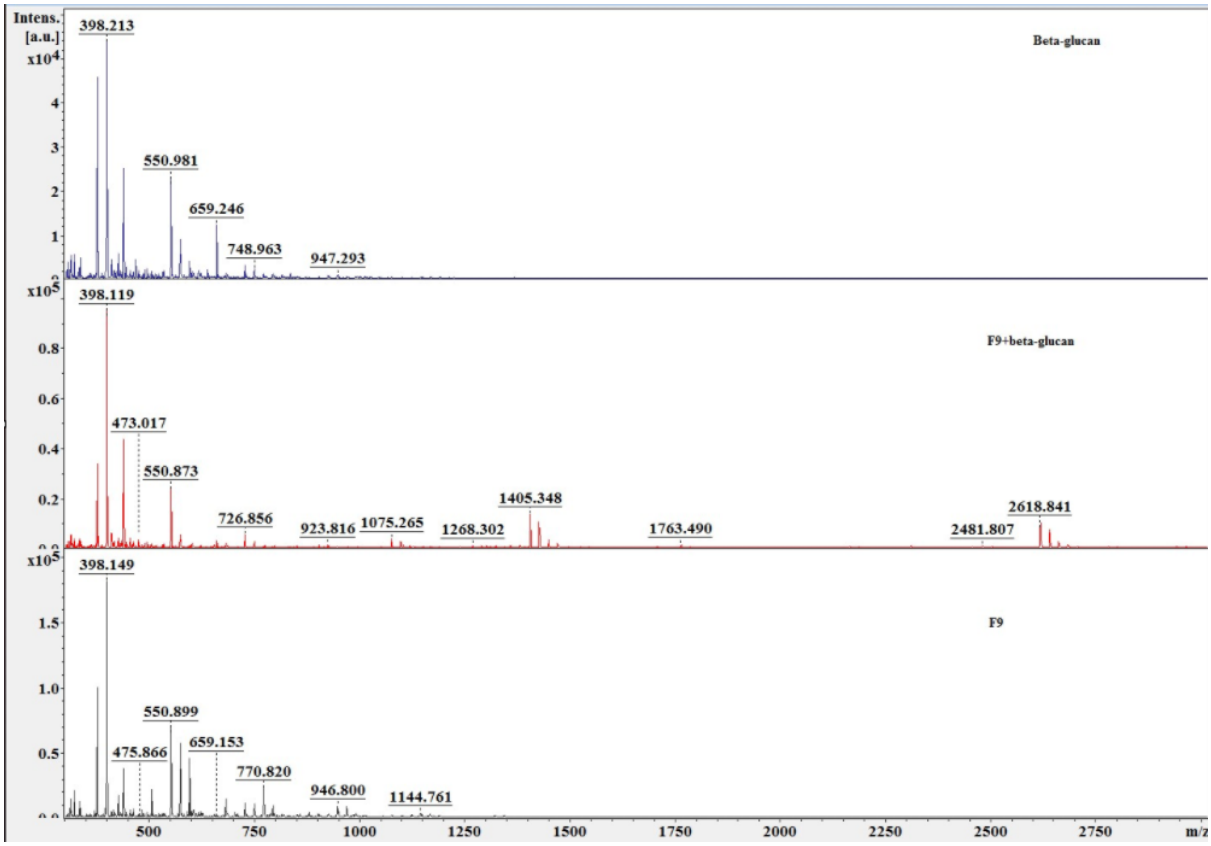


Figure 4.6.1. MALDI-TOF spectra displaying the molecular masses of beta-glucan treated with F9, beta-glucan alone, and the protein alone. Peaks correspond to various fragmentation patterns observed in the samples.

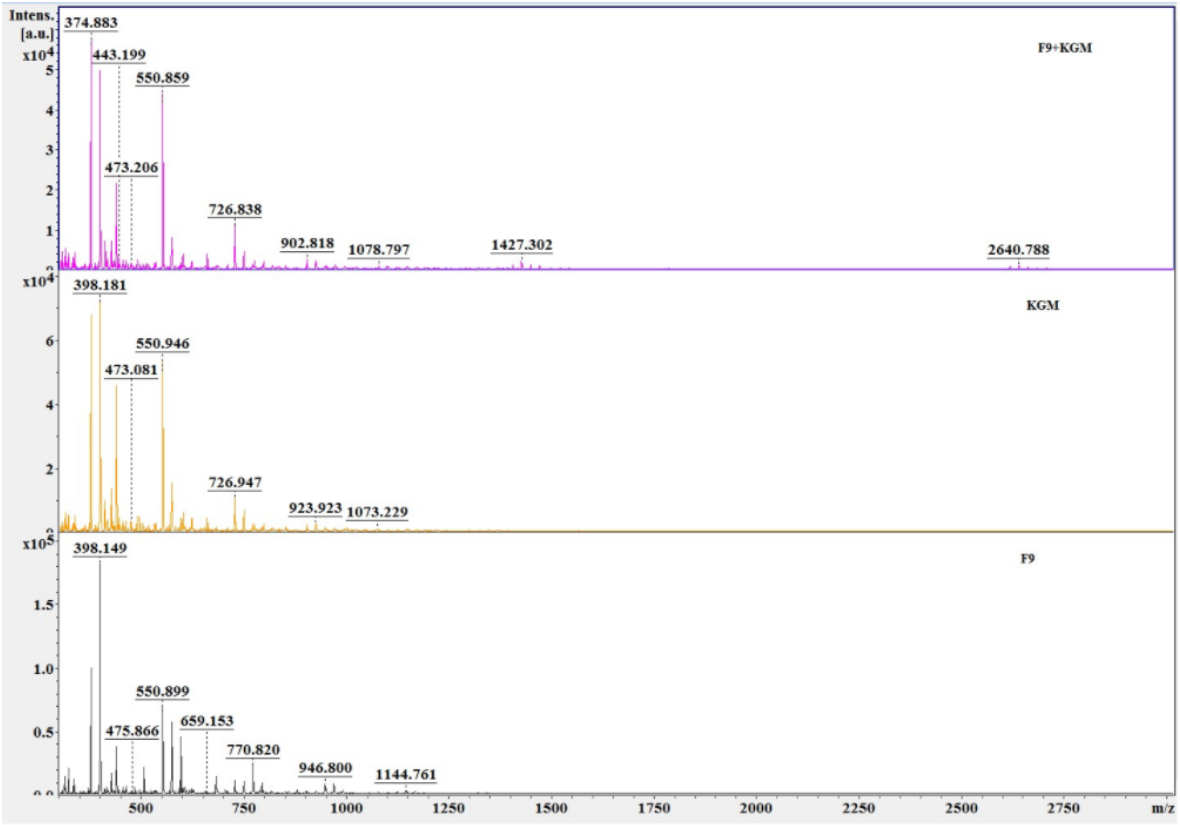


Figure 4.6.2. MALDI-TOF spectra displaying the molecular masses of KGM treated with F9, KGM, and the protein alone. Peaks correspond to various fragmentation patterns observed in the samples.

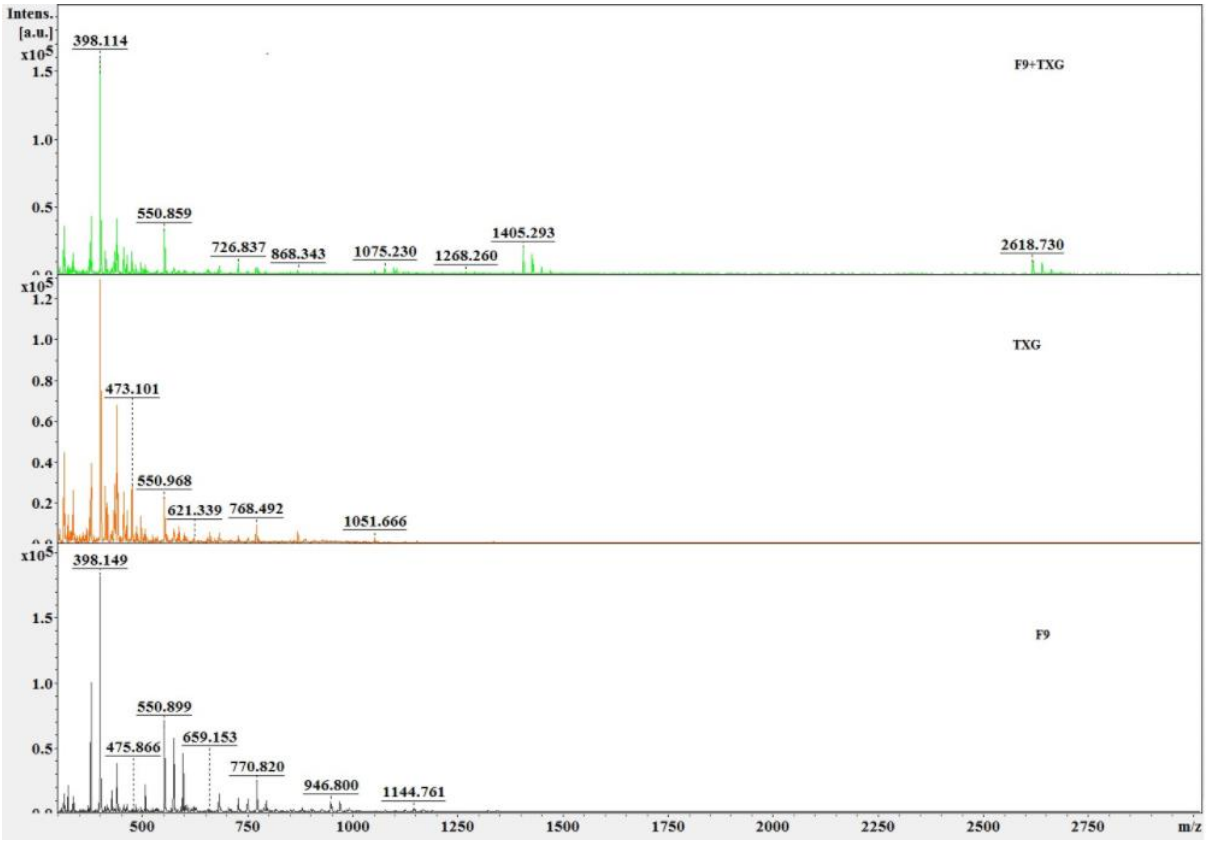


Figure 4.6.3. MALDI-TOF spectra displaying the molecular masses of TXG treated with F9, TXG, and the protein alone. Peaks correspond to various fragmentation patterns observed in the samples.

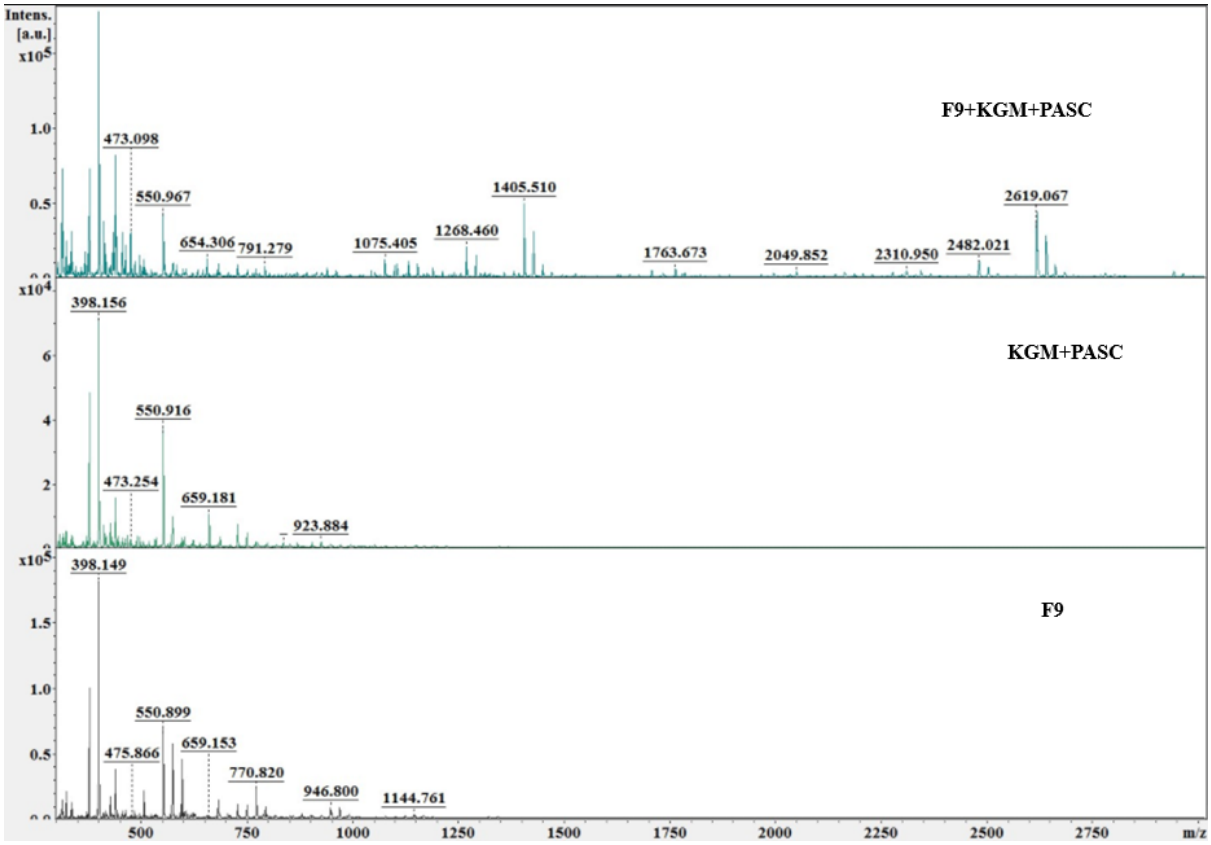


Figure 4.6.4. Figure 4.6.2. MALDI-TOF spectra displaying the molecular masses of KGM treated with F9 and PASC, KGM+PASC, and the protein alone. Peaks correspond to various fragmentation patterns observed in the samples.

4.6 N-Deglycosylation of F9, F12, and F14 by EfEndo18A Enzyme

The first thing in order is to check whether the protein has N-linked glycosylation potential in the amino acid chain which was done by using NetNGlyc – 1.0 to detect the sequon motif and it's potential to being glycosylated. The sequence used for detecting the N-glycosylations were the recombinant ones expressed in *P. Pastoris* from section 2.6.

The entire protein sequence, including the signal peptide, was analyzed for potential N-glycosylation sites. It's crucial to note that the signal peptide is cleaved off from the nascent polypeptide chain during protein maturation. As a result, N-glycosylation sites predicted within the signal peptide are not relevant to the mature protein and its PTMs.

Output for 'Sequence'

```
Name:  Sequence         Length:  318
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGV      80
SLEKREAEASVLPGITENKSHSVRRDLKTGLGTRYGKNCKEEDCWQSGACAFTNYKLPSTIDGSTCVSEAIWDSSAHCGG     160
CIQVTYKGKTITIMVTNKTDGDANHLDMPPATWSKLTNGMTGGGVDGIEWDWVTCPITAPLTIHMHGGSSQYWFAATVEN     240
ATLRTTKMEVSTDNGKTWKGTERDINNFFVVDGVLPTTSAWVKVTSESGSQVVVENVKLESGKSTVASENYAHHHHHH
......................N.................................N.........N.............      80
.................N.....................................................................     160
.................N.....................................................................     240
.....................................................................................      320

(Threshold=0.5)
--------------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                  agreement result
--------------------------------------------------------------------------
Sequence       23 NTTT   0.6687     (9/9)   ++
Sequence       57 NSTN   0.6283     (8/9)   +
Sequence       67 NTTI   0.6474     (9/9)   ++
Sequence       98 NKSH   0.6440     (8/9)   +
Sequence      177 NKTD   0.5629     (6/9)   +
Sequence      240 NATL   0.4818     (5/9)   -
--------------------------------------------------------------------------
```

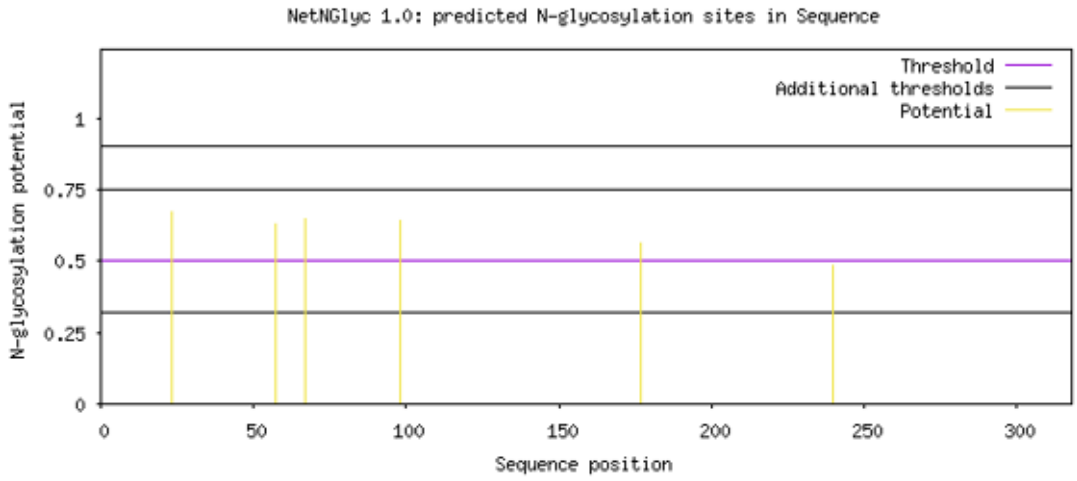


Figure 4.6.1. Potential N-glycosylation sites for F9 protein. In total six sites were predicted to potentially be N-glycosylated.

As figure 4.7.1 illustrates, F9 has six potential sites for N-glycosylation, but only three of them are located after the signal peptide, which are the amino acids at position 98, 177 and 240.

Output for 'Sequence'

```
Name:  Sequence          Length:  227
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGV      80
SLEKREAEATTVSYDPGYDKANNPMTGVACSDGNNGLITRYGYQTYGSIPRFPYIGGSSDIAGYNSDQCGQCYSVSYNGG     160
QPIYILAIDHTLEGLNISEEAMNALTGGQAEAVGRVDAQVTKVGVDMCGLAPRKRAVEFLAHHHHHH
.......................N.................................N.........N.............      80
.....................................................................................     160
...............N.....................................................                 240

(Threshold=0.5)
-----------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                   agreement result
-----------------------------------------------------------------------
Sequence       23 NTTT   0.6663     (9/9)   ++
Sequence       57 NSTN   0.6190     (8/9)   +
Sequence       67 NTTI   0.6358     (9/9)   ++
Sequence      176 NISE   0.6074     (8/9)   +
-----------------------------------------------------------------------
```

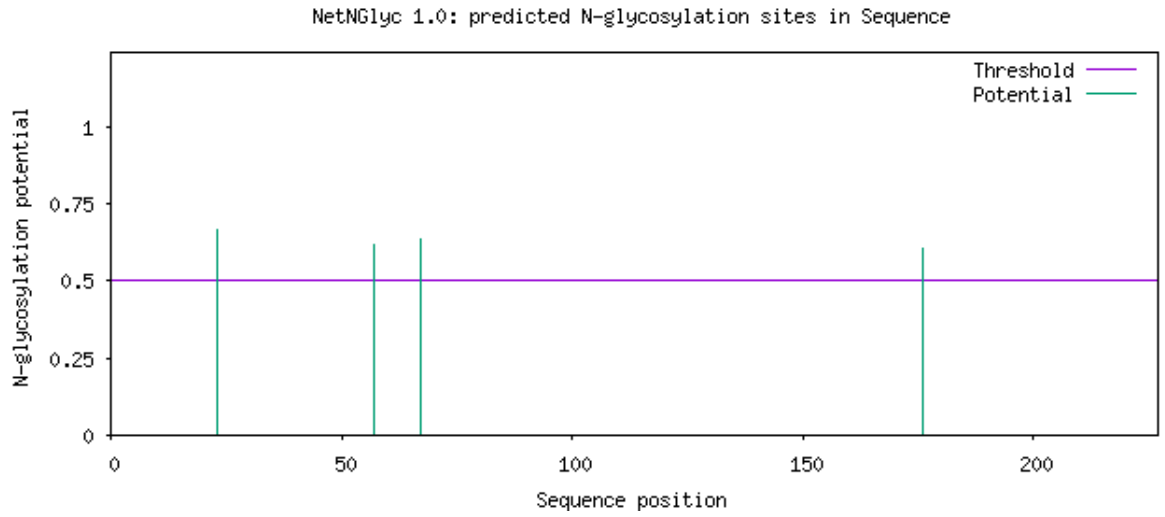


Figure 4.6.2. Potential N-glycosylation sites for F11.

Although the production of F11 was not possible in this study it is still in interest to see whether the protein has potential N-glycosylation sites. It has the recognized sequon motif once after the signal peptide in the sequence with the amino acids NIS as shown in figure 4.7.2.

Output for 'Sequence'

```
Name:  Sequence         Length:  292
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGV      80
SLEKREAEAFTGRGTFYGGNVQGGMCSFASYTLPTGIDGTAISKLDWAGSGVCGACIKVTGLRGSTISMIVDQCPECPPH     160
SLDLFQNSFGKIDDPQKGIIQLSWEFVDCPLNGLIYFRMKEGVSANWFSVQAVNASKRVKDIQVSTDHGATWQSGLTRMD     240
YNFFQKSAGFGVDVVDLKVISIDGKERIAKNCQVIGGNTCNADGNFHHHHHH
.....................N..................................N.........N.............      80
.....................................................................................     160
.....................................................N.......................N.....     240
.....................................................                                  320

(Threshold=0.5)
---------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                   agreement result
---------------------------------------------------------------------
Sequence       23 NTTT   0.6678     (9/9)   ++
Sequence       57 NSTN   0.6261     (8/9)   +
Sequence       67 NTTI   0.6444     (9/9)   ++
Sequence      214 NASK   0.6076     (8/9)   +
---------------------------------------------------------------------
```

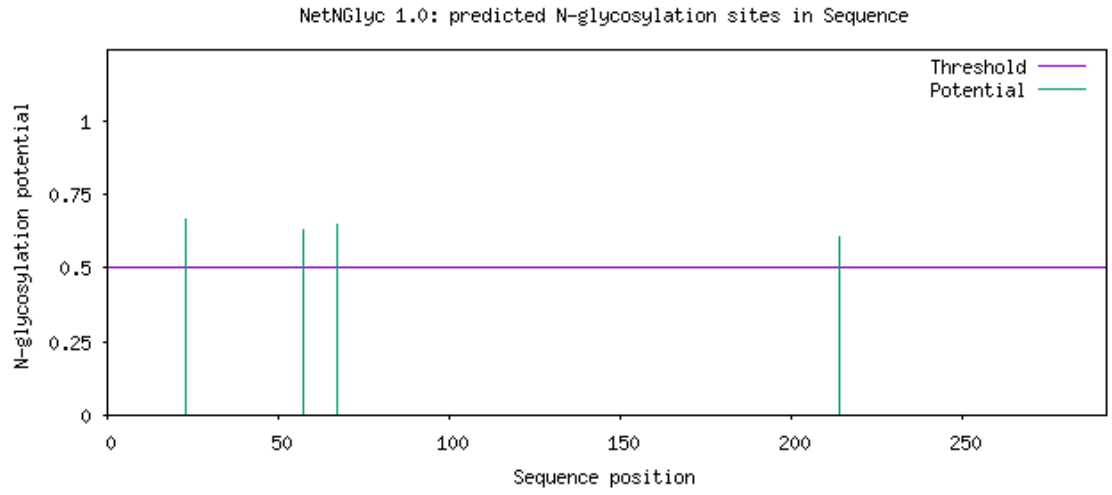


Figure 4.6.3. Potential N-glycosylation sites for F12.

For protein F12 only one observable N-glycosylation site is detected within the sequence after the signal peptide. The amino acid sequence is NAS.

Output for 'Sequence'

```
Name:  Sequence          Length:  373
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGV      80
SLEKREAEAVPKPELQHHHQHHPKRDIVWVTEYQEVVETIAVTKTVWVKPSQTQATPFSTQEVKQVQPAAYSPAPVAAPA     160
YVAPAAQSEPAAAPAYVAPVSSEPAAAYVAPVSSEAAAVPTSTYVPPPAPYVAPVASSSAAAAPASSSAAAPSYNSPSDS     240
GSSSGGQSYSGDFTWFDVGMGACGFTSTSDQDVVAVSHVLFDKVSTGNSNTNPLCKKIIYLIGADGNTYPGEIVDRCPSC     320
AEGSLDLSESFFKKVTSNGDGRVLNMKWHMPLEQKLISEEDLNSAVDHHHHHH
.....................N.................................N.........N.............      80
...............................................................................     160
...............................................................................     240
...............................................................................     320
.....................................................                              400

(Threshold=0.5)
--------------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                  agreement result
--------------------------------------------------------------------------
Sequence       23 NTTT   0.6694     (9/9)   ++
Sequence       57 NSTN   0.6317     (8/9)   +
Sequence       67 NTTI   0.6513     (9/9)   ++
--------------------------------------------------------------------------
```

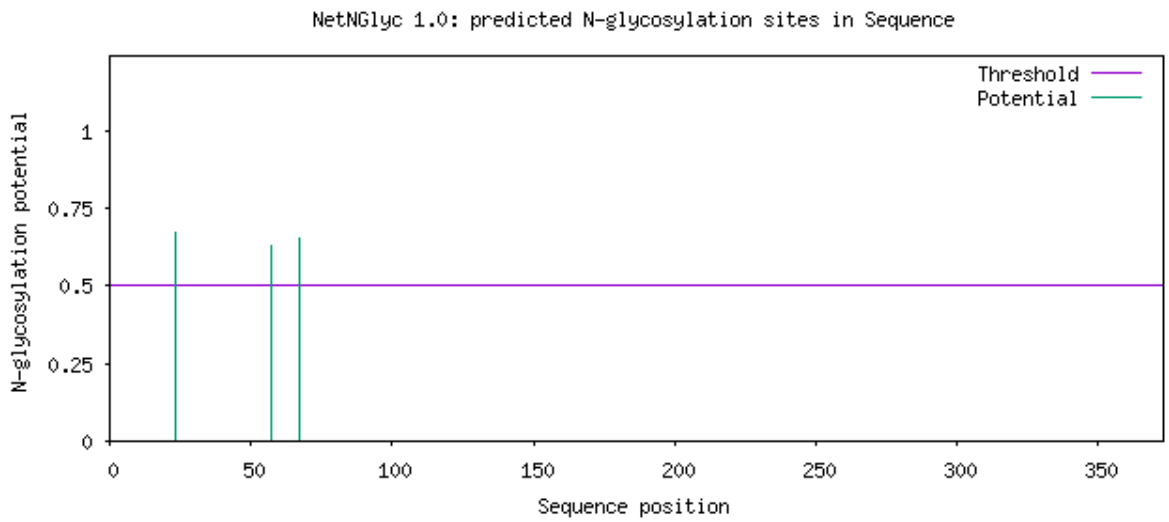


Figure 4.6.4. Potential N-glycosylation for F14.

The expansins-like protein, F14 did not display any possible N-glycosylation sequences in the amino acid chain after the signal peptide.

The proteins were analyzed on SDS-PAGE following treatment with the *Ef*Endo18A enzyme to determine the presence or absence of N-glycosylation, which could indicate whether this PTM occurred or not, as shown in figure 4.7.5.

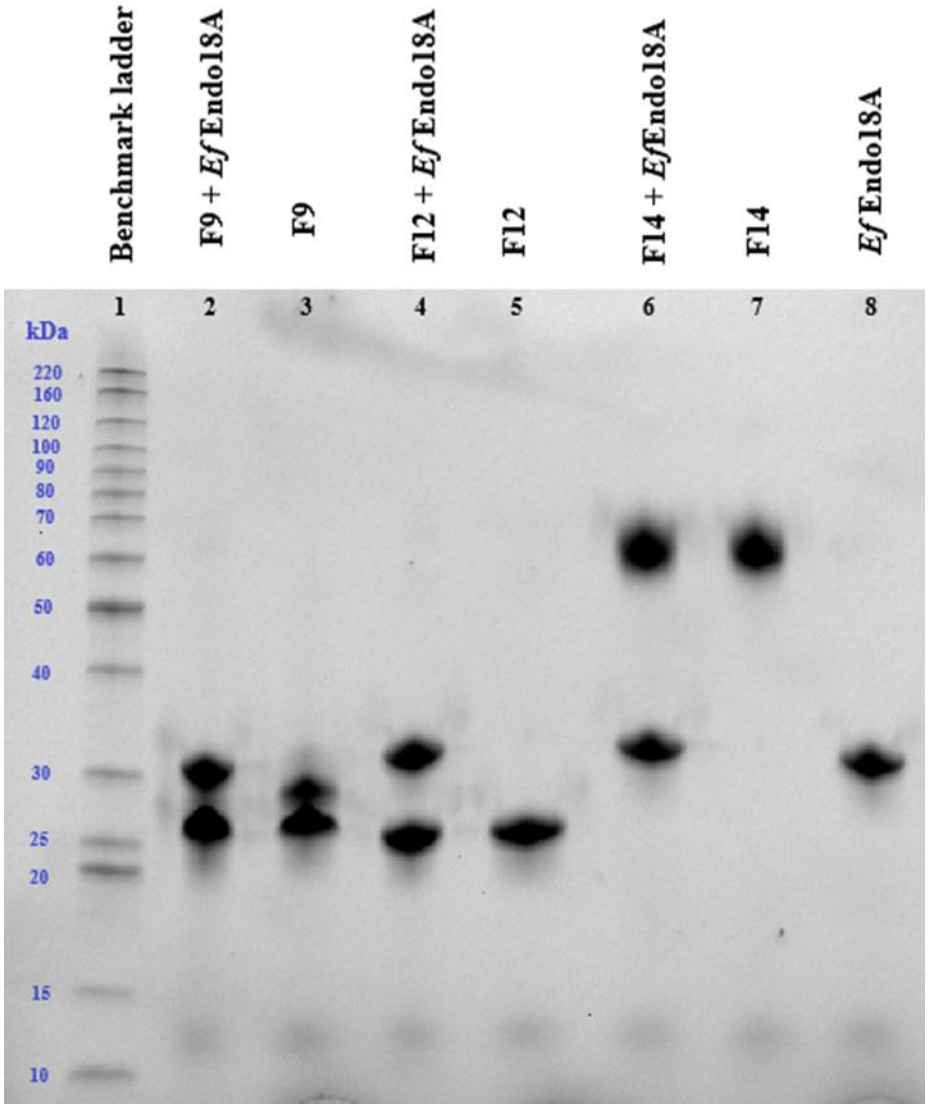


Figure 4.6.5. Analysis of the different proteins by the *Ef*Endo18A enzyme. First well contains Benchmark ladder while the rest are either protein + enzyme, protein alone and enzyme alone.

Assessing whether there has been any deglycosylation based on the results of this gel is quite hard to determine considering the bands haven't changed too much in size. The protein with the most likelihood of glycosylation was F9, but the band is roughly the same size, although it doesn't show two bands when comparing well 2 and 3.

*Ef*Endo18A was used to hydrolyse different type of substrates in a study and was compared to this to observe difference in bands.

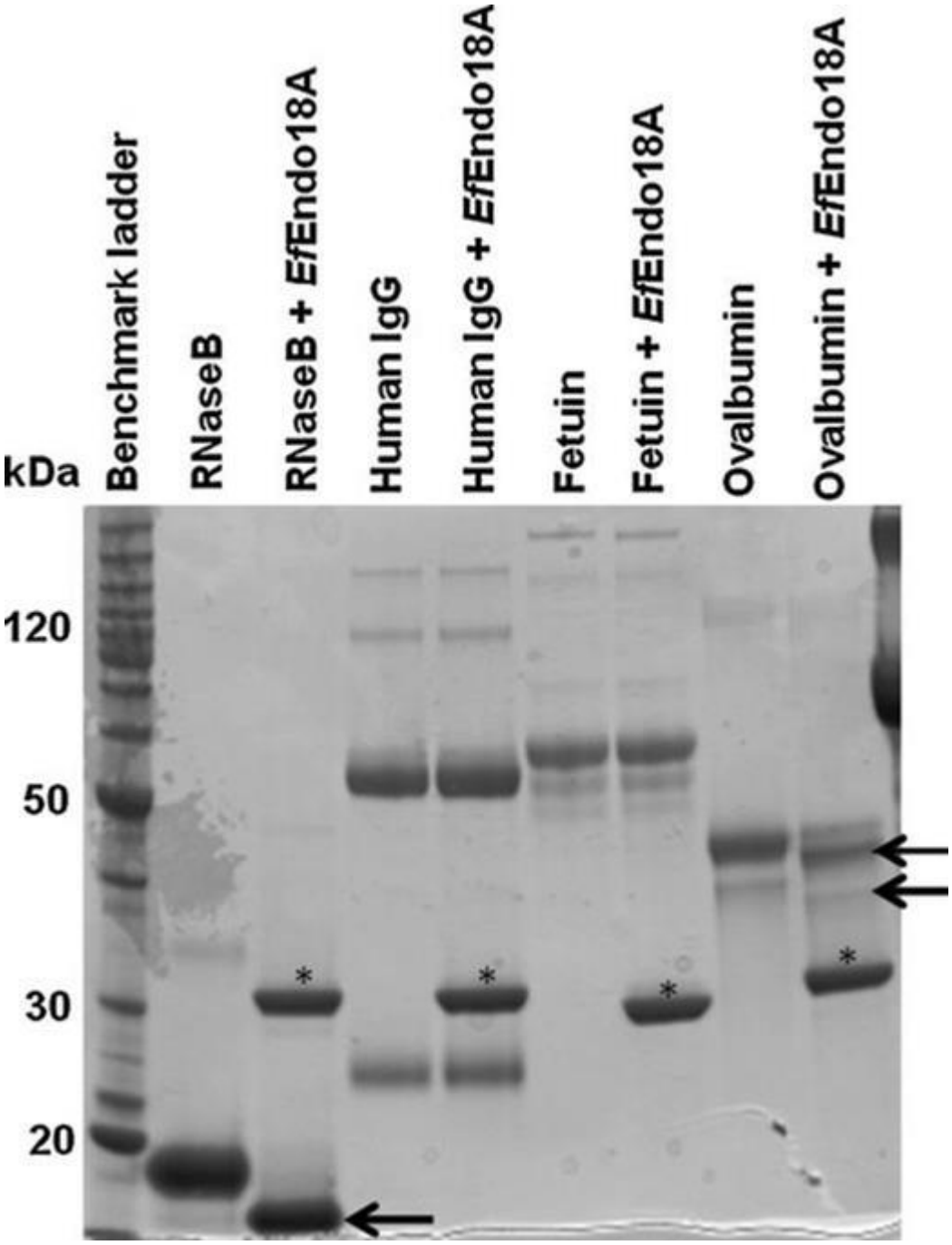


Figure 4.6.6. SDS-PAGE analysis of different glycoproteins subjected to treatment with the *Ef*Endo18A enzyme.

5. Discussion

5.1 Cultivation and purification of the expansins-like protein

The cultivation and purification of the expansin-like protein were achieved for all the proteins but F11, which process proved to be challenging. Except for the F14 protein, their respective sizes were in accordance what was to be expected according to Jean-Guys data.

Protein F9 was successfully cultivated as displayed on figure 4.1.1. The bands produced by F9 were between 20-25 kDa, both after harvesting and using Vivaflow. These thick bands being observed suggests that the protein was overexpressed.

After employing His-Tag purification the bands still contained some background protein, as displayed on gel 4.2.1, as well as in the two double bands close to each other, which is why SEC was utilized to try to separate the proteins based on size. The chromatogram from figure 4.2.2 displays two peaks with significant time between them. This could be due to residual proteins in the column which would imply that perhaps the column was not properly stored in ethanol and eluted when the elution buffer started running through the column.

The fractions collected from the SDS-PAGE analysis from figure 4.2.3 were pooled together and concentrated with NaAc pH 5 buffer. Despite the SEC purification step, the double bands on the gel remained, which implies that the two bands are produced by the same protein, and that the protein was not reduced despite treatment with the reducing agent.

F11 was expected to be 14.768 kDa. SDS-PAGE analysis pre- and post Vivaflow indicates the presence of proteins in the sample, albeit in low abundance, as illustrated in Figure 4.2.1. On the gel, there appears to be a protein around the 15 kDa mark, which could be the protein of interest, but it's unclear if it's the same protein or not.

SDS-PAGE analysis of F11 after His-Tag purification shows no proteins on the gel besides bands between 10-15 kDa, consistent with other P. Pastoris proteins, suggesting it's not F11. F11 was cultivated again with the same method as in sections 3.1 and 3.2, but using a different glycerol stock, yielding no different results as shown in Figure 4.2.4 and 4.2.5. The gel included flowthrough between the steps on figure 4.2.5 to assess if the protein passed through the Vivaflow filter or failed to bind to the His-Tag column, but there appeared to be an absence of the protein here as well.

The most prominent issue might have been the overall low concentration of the protein. Loss of protein between purification steps is expected, and with the low initial concentration, the protein can become undetectable.

One possible additional step to include is inducing the protein with methanol for an additional day to see if it increases protein yield. This applies to other proteins as well,and can be attempted to compare cultivation for five and six days, respectively.

Assuming the spotted protein at the 15 kDa mark is F11, the overall expression level appears low. Protein degradation and misfolding might have occurred (Jackson & Hewitt, 2016), possibly due to protease action within the protein, leading to fragmentation. This could explain why the protein doesn't bind to the His-Tag column during purification. Fragmentation also complicates size quantification.

The protein has been cultivated and purified prior to this study, so it's not the first time it's been expressed, but it's possible that the method is not sufficiently robust.

Two different stocks were used to produce F11, but neither worked. Perhaps the glycerol stocks have degraded over time, explaining the lack of protein production.

Despite storing the glycerol stocks at -80°C for optimal stability, factors like constant freeze-thaw cycles could still lead to degradation of P. Pastoris cultures. Contamination is another potential issue, especially if stocks weren't handled under sterile conditions, affecting recombinant strain function and protein expression (Plantz & Andersen, 2003).

Storing proteins at -80°C helps maintain stability, but genetic instability in recombinant strains could occur over time. This depends on post-translational modifications, protein size, and structure. Considering that the protein around the 15 kDa mark didn't bind to the His-Tag column, it's possible that the His-Tag residue was cleaved off during maturation or that it wasn't coded for.

F12 was expected to be around 23 kDa in molecular weight. This is in agreement with figure 4.1.1. The purification of F12 was more straightforward than F9. After purifying it with Vivaflow it underwent His-Tag purification as displayed on figure 4.3.2, and the fractions from the gel looks relatively clean, with just a few bands noticeable around 40 kDa. This protein also underwent SEC to ensure sufficient purity. The chromatogram on figure 4.2.7 shows only one peak, which was analysed with SDS-PAGE as displayed on figure 4.2.8. The proteins looks pure on the gel.

F14 was expected to have a theoretical molecular weight of 30 kDa, but the protein was double the size when observed after cultivation on figure 4.1.1 and after Vivaflow purification. The protein instead had a molecular weight of 60 kDa. It also underwent His-Tag purification to remove any other possible proteins present in the sample. The fractions were collected from this purification step as shown in figure 4.2.10 and this protein appeared similar in purity as the other proteins do after SEC, but nonetheless SEC was still performed on it considering the two proteins prior had undergone the same procedure.

The chromatogram from F14 as displayed on figure 4.2.10 had a very sharp peak and the fractions were collected and analyzed by means of SDS-PAGE as shown in figure 4.2.11. The fact that the protein appeared double its projected size was unexpected. The most likely explanation for this is that PTMs, such as glycosylations, for instance, had occurred, or perhaps that the protein did not fully denaturate prior to SDS-PAGE analysis.

5.2 Measuring concentration

As stated in section 4.3, the only method to determine the protein concentration was by measuring absorbance at 280 nm. This was due to the protein concentration from the Bradford method always underestimating the actual concentration by a large margin. Ideally, both results should be utilized, both from Bradford method and absorbance at 280 nm by adding the two of them and then divide by two. This would give the most accurate representation of the actual concentration.

5.3 Analysis of the effect of expansins on various polysaccharides with HPAEC-PAD

Activity was observed in graph 4.5.2 without the presence of PASC around the 16.5m window when treated with F9. The two peaks formed on the beta-glucan+F9 did not appear for either the substrate or the protein by itself, and they were visible under these conditions, which are mentioned in section 3.6. The amplitude of the peak is about 1.5 nC, which is quite low, but is still an indication of detected activity. However, determining the potential activity and its implications is challenging. A possible explanation is that the expression host *P. Pastoris* for F9 could have produced host cell proteins (HCPs) that could be abundant in the samples (Chen et al., 2013). *P. Pastoris* naturally secretes various enzymes during cultivation that could potentially have been co-purified with the overexpressed protein F9 and could all together have been undetected if they were of similar size to the protein or low in

concentration so they would go unnoticed in the SDS-PAGE gel analysis during the purification steps. Enzymes that could potentially target beta-glucan are such as beta-glucanases, beta-glucosidases, endoglucanases, and/or lyases.

Something worth mentioning is that the expansin F9 potentially also could have a synergistic effect with these HCPs which could have caused the activity detected (Cosgrove, 2017). Proteins, such as expansin-like proteins, have been reported to facilitate the hydrolysis of cellulose, which is a component in the cell wall, and it's possible that they could produce a synergistic effect with enzymes such as cellulases (Bunterngsook et al. 2015). The main goal of this study was to determine whether the expansin-like proteins cultivated in the lab could affect the cell wall structure. Although not conclusive, the findings suggest that these proteins could have a synergistic effect with cell wall-hydrolyzing enzymes. Expansin-like proteins from fungi possess both sequence and structural similarities to expansins (Cosgrove, 2017).

The samples containing pure protein did not possess any significant change after being incubated for 2 hours, 24h, and 48 hours with the buffer and shaking. The buffer did not impact the proteins under these conditions. This seemingly suggests that the proteins are relatively stable in the buffer solution regardless of incubation time, and that they are quite robust under these conditions described in section 3.6. This could prove useful for when setting up future experiments to not use up all the protein considering large amounts get utilized when setting up these reactions. Cultivating and purifying these proteins is time-consuming and costly. This does only seem to apply to the conditions stated in section 3.6; different factors and treatments could alter the protein stability. Changes in temperature, pH, ionic strength, and time courses over 48 hours could alter these results, by themselves or in combination with the factors stated. The various substrates utilized in these experiments did not show significant change between treatment times either.

The most surprising result is what was observed in graph 4.5.9 where a peak that should be detected between 14-19 min has disappeared. The peaks with an amplitude of 10 nC, which were detected for both the KGM+PASC, F12+KGM+PASC, and F14+KGM+PASC, does not appear on F9+KGM+PASC, which was unexpected. The absence of a peak may suggest that a new product has been formed by the protein-substrate interaction. However, when this occurs, usually new products are detected on the chromatogram during its runtime, which was not the case in this instance, unless the products were difficult to be quantify due to their

small size. Another, and a more plausible explanation for this could be human error, in that KGM simply was not added to the sample.

In total, there were 27 unique samples in the run without additional replicates, even for the samples with protein+substrate. It's important to be critical of the results and what the potential activities could be due to, and whether they can be reproduced. Due to how long it took to produce the expansin-like proteins no additional replicates were added to the mix, but for more consistent results and to avoid any outliers, it would be beneficial to run this set of experiments again with several replicates.

ANB was not treated with PASC, as opposed to the other substrates, and it did not display any potential activity as shown in figure 4.5.11. It also showed no difference between treatment time after 2 hours and 48 hours with or without the presence of proteins. Perhaps the samples have to be further processed or the parameters such as temperature, pH, and shaking have to be adjusted.

Whatman filter paper was subjected to 2 hour and 48 hour treatment without PASC as shown in figure 4.5.12 and 4.5.13. No difference between the substrate treatment for 2 hours or 48 hours was observed.

There was difference between treatment time for any of the proteins based on time, besides F9, where some activity was detected for F9+substrate that had been treated for 48 hours, as shown in graph 4.5.15. This activity could also be due to HCPs, as mentioned for beta-glucan and the other polysaccharides. If there was background activity it was most likely be due to the cellulase enzyme. It has been reported that endoglucanase activity has been present in recombinantly expressed *E. coli* due to the HCPs from the expression host, which was purified with adding the additional step of HPLC (Cosgrove, 2017).

Xylan had two treatment plans, one where the samples treated with F14 were incubated for 48 hours with different concentrations (1 μM, 10 μM and 50 μM) in combination with PASC present in all samples. The treatment did not show any signs of activity as shown in figure 4.5.17.

The other treatment was similar to the other polysaccharides, where the samples were treated for 2 hours, 24 hours and 48 hours in combination with PASC. This applies to protein F9 and F12. As figure 4.5.20 shows, the was no activity on xylan+PASC treated with F12.

On the other hand, for xylan+PASC+F9 that were treated for 24 hours and 48 hours did display some activity reminiscent of the COS DP2-DP6 standard around 15 minutes as shown in graph 4.5.19. It is hard to determine if this actually is product created from the substrate that has been treated or not and also if it is similar to the standard.

As stated, these proteins were produced with *P. Pastoris* which does produce higher yield (Karbalaei et al., 2020), but something that could potentially be done is to express the expansin-like protein gene with *E. coli*. Something that could be evaluated is to add additional purification steps such as hydrophobic interaction chromatography, ion exchange chromatography or even HPLC. It is important to note that with each additional purification step, there is going to be a loss of protein and potential contamination (Lee C.H, 2017); however, it is vital to have pure samples to ensure high-quality results, especially for proteins that are uncharacterized.

Something to keep in mind that more suitable standards could have been utilized for these experiments. In figure 4.5.8 some peaks overlap with the standard used, and the same applies to figure 4.5.10. Saying that these are the same products due to retention time being similar is quite the stretch its something that is worth looking into. More standards would make it easier to discern what the potential cause of the activity could be.

5.4 N-deglycosylation of F9, F12 and F14

Figure 4.5.5 displays the impact of the deglycosylation utilizing the enzyme *Ef*Endo18A on the various proteins. As the figure shows, only the well containing F9 in combination with the enzyme *Ef*Endo18A showed signs of change on the gel, in contrast to the two other proteins. The results were compared to a previous study using the same enzyme but on different proteins as shown in figure 4.6.6.

The double band on F9 appeared to have merged, resulting in a possible partial deglycosylation. F9 had three potential N-linked glycosylation sites on the sequence at the amino acid positions 98, 177 and 240, with the sequences NKS, NKT and NAT, which fulfilled the N-X-S/T sequon motif criteria (Tabish et al., 2011). The deglycosylation had the highest probability on the 98th residue compared to the other two, but they were possibly deglycosylated too as shown in figure 4.7.1.

F14 did not show signs of deglycosylation on the gel analysis in figure 4.5.5. By comparing the control and the F14 sample with the enzyme it appears there is no reduction of molecular weight. This suggests that F14 most likely did not undergo N-deglycosylation.

Analysis of the sequence shows that the protein lacks the N-X-S/T sequon motif for N-linked glycosylation as shown in figure 4.5.4, which is vital for the glycosylation to occur. This suggests that the protein in its mature state is absent of N-linked glycosylation. Notably, the signal peptide of F14 does show potential N-glycosylation sites, implying that glycan attachment might have occurred at an earlier stage.

Although the protein is likely free of N-linked glycans, it might still be glycosylated through alternate means, such as O-linked glycosylation. The glycans are attached to serine or threonine residues and are affected by nearby amino acids.

F12 did also not display any signs of deglycosylation on the SDS-PAGE in figure 4.5.5. From the control and the sample it appears that the molecular weight of the protein did not show significant deviation.

As opposed to F14, the F12 protein appear to have one site with potential N-glycosylation in amino acid position 214 with the sequence NAS as shown in figure 4.5.3, which fits the criteria of the N-X-S/T sequon motif after the signal peptide sequence. It was determined by NetNGlyc that the probability of N-linked glycosylation was 0.6076. The N-deglycosylation experiment was only performed once. As a future perspective, there are several parameters that can be optimized for this experiment such as temperature, concentration of enzyme, incubation, using a different set of deglycosylation enzyme altogether, that are likely to improve this probability.

Whether or not F9 and F12 is N-linked glycosylated is a question that can be tested and examined in closer detail. For F9 specifically, a time assay with the *Ef*Endo18A enzyme can be executed. The effect of the enzyme should be sampled every five minutes to begin with, until reaching the 30-minute mark and then sample it after two hours and then overnight. This could prove crucial for several reasons because it would indicate how the band shifts over time and by how much if there is any deglycosylation present at all.

To determine whether there is deglycosylation present or not, the samples from the experiments can be analysed through MALDI-TOF MS to observe shifts in spectra with and without the enzyme on the protein.

Temperature can affect the optimal enzymatic activity of EfEndo18A. In this experiment the incubation was set to 33°C, while in another study the temperature it was set to 37°C. This could lead to reduced enzymatic activity and slower deglycosylation, possibly altering the efficiency of the deglycosylation. Furthermore, having the temperature set to 33°C does entail that the time for completing the deglycosylation is longer as opposed to having it set to 37°C, due to the fact that the product formation rate will be slower.

Although in the previous study the time it took to deglycosylate RNaseB was no longer than 30 minutes (Bøhle, L.A. et al, 2011), it's important to note that the characteristics of the proteins in this study differs significantly. Given how much is unknown about the expansin-like proteins and understanding the optimal temperature, it might be advisable to keep them at lower temperatures to reduce the likelihood of denaturation.

The concentration of *Ef*Endo18A can influence the deglycosylation process. Higher enzyme concentrations accelerate deglycosylation, as more enzyme molecules are available to catalyze the reaction. However, the same applies to the substrate, in this case being the expansin-like proteins. It's important to note that at a certain point, either the enzymes or the proteins will become saturated, limiting further increases in reaction rate.

Finding the optimal ratio of enzyme and expansin-like protein concentrations is vital to avoid enzyme or protein waste. Producing enzymes and proteins is time-consuming and costly, making efficient resource utilization crucial.

As stated, the enzyme used in this experiment was EfEndo18A, which is an endo-β-N-acetylglucosaminidase. EfEndo18A showed that it does not have the ability to deglycosylate O-linked glycans and N-linked glycans of the complex type (Bøhle, L.A. et al, 2011). This is something worth investigating further to be able to fully understand and characterize the expansin-like proteins.

The enzyme PNGase F also cleaves the GlcNAc just like endo-β-N-acetylglucosaminidases (Fischler & Orlando, 2019). However, PGNase cleaves at the innermost GlcNAc and not between two GlcNAc units. PNGase F is more robust and has broader use than EfEndo18A, and can even cleave the complex N-linked glycans. PNGase F is a more standardized enzyme for glycoprotein analysis, and it would be better to start off deglycosylation with a very standardized enzyme.

Considering that these proteins are fungal proteins perhaps looking into using fungal enzymes could be food for thought. The expansin-like proteins were produced through recombinant expression with P. Pastoris, which is a methylotrophic yeast. The source of the deglycosylation enzyme in this experiment was the bacteria Enterococcus faecalis.

Using a fungal enzyme for N-deglycosylation could be advantageous due to the different glycosylation patterns other organisms have. Fungal protein has different types of glycan structures compared to other organisms. Fungal enzymes might recognize fungal proteins more easily, which could in turn lead to a greater likelihood of deglycosylation. Although not much is known about these proteins, it could be a potential avenue to explore and lead to a deeper understanding of the glycan structures and functional properties of the expansin-like proteins. However, it should be noted that this is purely speculative.

Another possibility is to fragment the protein using trypsin digestion to obtain smaller peptides from the protein while retaining the potential glycosylation sites. Post-fragmentation, the peptides can then again be treated with EfEndo18A or any other kind of N-deglycosylation enzyme to test whether deglycosylation is achieved. The F9 protein had three potential deglycosylation sites and by fragmenting the sequence it might be possible to determine where the deglycosylation is located, if there is any.

After testing N-deglycosylation on the peptides they can be further analysed with mass spectrometry. The samples could then be compared to the spectra without the N-deglycosylation enzyme present to quantify the mass shift to determine if there is mass differences.

The enzyme for this experiment might not have been compatible with the proteins in question but one thing to keep in mind is that there are several PTMs present after the maturation of the protein. Another possibility is the presence of O-glycosylation, which a different set of enzymes must be utilised to determine.

Another thing to keep in mind is that these set of proteins are uncharacterized. Exactly how they are impacted by deglycosylation remains unknown. The loss of the glycans could lead to instability and loss of function, considering they could be vital for structural stability.

6. Conclusion

Based on the results obtained in this study, it is inconclusive whether expansin-like proteins from Z. tritici impact the polysaccharides present in the cell wall structure. Further research is required to determine their effects on the various polysaccharide substrates.

Although some activity was observed in the analysis of the substrates, what the actual activity is remains unclear without further data gathered. More replicates and more standards must be utilized to determine what this activity is. Whether the observed activity originates from HCPs, synergistic effects between HCPs and expansin-like proteins, the expansin-like proteins or other factors.

Protein F14 was expected to be around 30 kDa but it resulted in being 60 kDa, which is twice the size. It could be due to potential PTMs that did occur after the maturation of the protein. Most likely not N-glycosylation as assessed during the discussion but could be other types of glycosylation. A more robust and general enzyme such as PNGnase should be utilized to determine if there is N-glycosylation present for the cultivated proteins.

Protein F9 did possess the double bands on the SDS-PAGE analysis gels despite all the purification attempts, and F11 was not able to be cultivated at all despite using different glycerol stocks, which can be interpreted as a result of potential degradation or misfolding of the protein.

If the activity picked up by the chromatograms are due to background enzymes further purification steps should be utilized to prevent HCPs. Both HIC and IEC are potential candidates

A lot of time was spent on the production of these expansin-like proteins. The yield post-purification was not the highest. Optimizing analysis methods to reduce waste and minimize production time would be highly beneficial considering how long it takes to produce the expansin-like proteins.

Several experiments such as trypsin digestion and further analysis of the activity with MALDI-TOF in addition to study synergetic effects with other enzymes are potential avenues to explore and they could lead to a further understanding of these uncharacterized proteins.

7. Reference

Breitling, J. and Aebi, M. (2013) N-linked protein glycosylation in the endoplasmic reticulum, Cold Spring Harbor perspectives in biology. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3721281/ (Accessed: 15 April 2024).

Bunterngsook, B. et al. (2015) 'Binding characteristics and synergistic effects of bacterial expansins on cellulosic and hemicellulosic substrates,' Bioresource Technology, 176, pp. 129–135. https://doi.org/10.1016/j.biortech.2014.11.042.

Bøhle, L.A. et al. (2011) An endo-β-n-acetylglucosaminidase from Enterococcus faecalis V583 responsible for the hydrolysis of high-mannose and hybrid-type N-linked glycans, FEMS microbiology letters. Available at: https://pubmed.ncbi.nlm.nih.gov/22093069/ (Accessed: 15 April 2024).

Chen, F. et al. (2017) Host resistance and temperature-dependent evolution of aggressiveness in the plant pathogen Zymoseptoria tritici, Frontiers. Available at: https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2017.01217/full (Accessed: 15 April 2024).

Chen, Y. et al. (2013) Optimized expression in Pichia pastoris eliminates common protein contaminants from subsequent his-tag purification - biotechnology letters, SpringerLink. Available at: https://link.springer.com/article/10.1007/s10529-013-1411-3 (Accessed: 15 April 2024).

Cosgrove, D. (2015) Plant Expansins: Diversity and interactions with Plant Cell Walls, Current opinion in plant biology. Available at: https://pubmed.ncbi.nlm.nih.gov/26057089/ (Accessed: 15 April 2024).

Cosgrove, D. (2023) Structure and growth of Plant Cell Walls, Nature reviews. Molecular cell biology. Available at: https://pubmed.ncbi.nlm.nih.gov/38102449/ (Accessed: 15 April 2024).

Cosgrove, D.J. (2017) Microbial Expansins, Annual review of microbiology. Available at: https://pubmed.ncbi.nlm.nih.gov/28886679/ (Accessed: 15 April 2024).

Department of Jobs, Precincts and Regions (2022) Septoria tritici blotch of wheat, Agriculture Victoria. Available at: https://agriculture.vic.gov.au/biosecurity/plant-diseases/grain-pulses-and-cereal-diseases/septoria-tritici-blotch-of-wheat (Accessed: 15 April 2024).

Fagundes, W., Hauisen, J. and Stukenbrock, E. (2020) Dissecting the biology of the fungal wheat pathogen Zymoseptoria tritici: A laboratory workflow, Current protocols in microbiology. Available at: https://pubmed.ncbi.nlm.nih.gov/33175475/ (Accessed: 15 April 2024).

Fischler, D. A., & Orlando, R. (2019). N-linked Glycan Release Efficiency: A Quantitative Comparison between NaOCl and PNGase F Release Protocols. Journal of Biomolecular Techniques, 30(4), 58-63. doi: 10.7171/jbt.19-3004-001. PMID: 31598098; PMCID: PMC6774468.

Igrejas, G., Ikeda, T. and Guzmán, C. (2020) Wheat quality for improving processing and human health, SpringerLink. Available at: https://link.springer.com/book/10.1007/978-3-030-34163-3 (Accessed: 15 April 2024).

Jackson, M.P. and Hewitt, E.W. (2016) Cellular proteostasis: Degradation of misfolded proteins by lysosomes, Essays in biochemistry. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5065703/ (Accessed: 15 May 2024).

Karbalaei, M., Agha-Amiri, S., Marashi, S. A., & Saghiri, R. (2020). Pichia Pastoris: A Highly Successful Expression System for Optimal Synthesis of Heterologous Proteins. Journal of Cellular Physiology. Retrieved from www.ncbi.nlm.nih.gov/pmc/articles/PMC7228273/

Lee, C. H. (2017). A Simple Outline of Methods for Protein Isolation and Purification. Endocrinology and Metabolism (Seoul, Korea). Retrieved from www.ncbi.nlm.nih.gov/pmc/articles/PMC5368116/

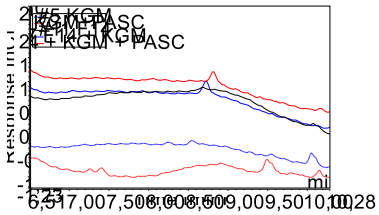
Marowa, P., Ding, A. and Kong, Y. (2016) Expansins: Roles in plant growth and potential applications in Crop Improvement, Plant cell reports. Available at: https://pubmed.ncbi.nlm.nih.gov/26888755/ (Accessed: 15 April 2024).

McQueen, M. and Cosgrove, D. (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension, Proceedings of the National Academy of Sciences of the United States of America. Available at: https://pubmed.ncbi.nlm.nih.gov/11607483/ (Accessed: 15 April 2024).

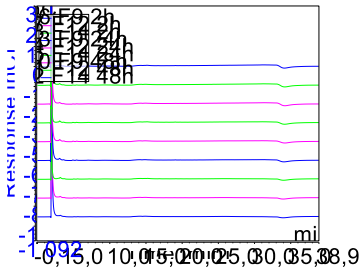
Plantz, B. and Andersen, J. (2003) Detection of non-host viable contaminants in Pichia pastoris cultures and fermentation broths, Journal of industrial microbiology & biotechnology. Available at: https://pubmed.ncbi.nlm.nih.gov/14605910/ (Accessed: 15 May 2024).

Østby, H. et al. (2023) Functional characterization of a lytic polysaccharide monooxygenase from Schizophyllum commune that degrades non-crystalline substrates, Scientific reports. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10575960/ (Accessed: 15 April 2024).

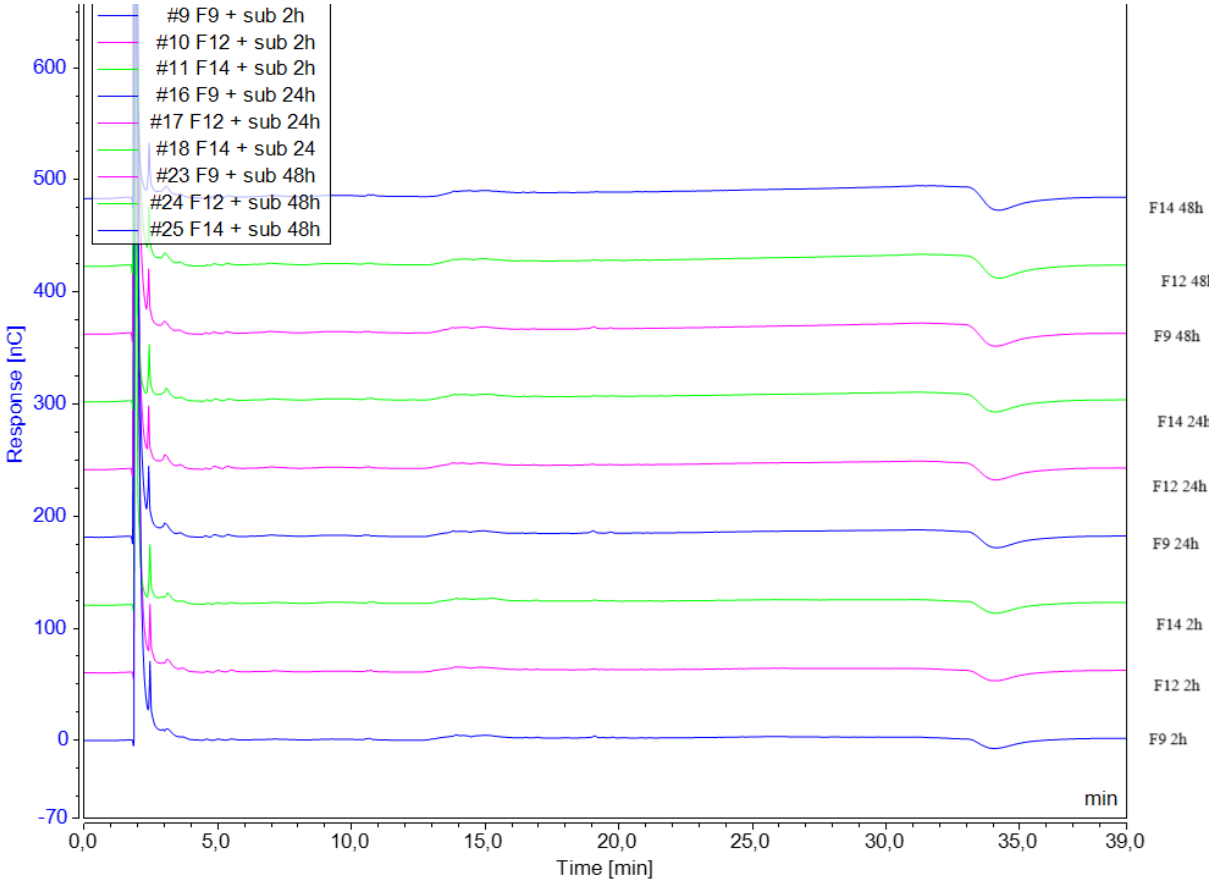
Appendix



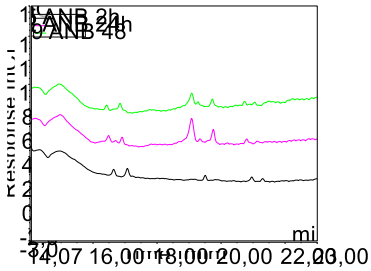
Appendix 1. Chromatographic Analysis of Enzymatic Activity on KGM and KGM+PASC on with protein F14. The graph displays peaks on F14+KGM+PASC and F14+KGM around the 8.6m mark.



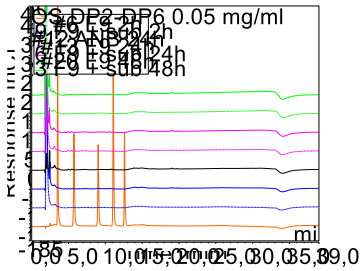
Appendix 2. Chromatographic Analysis of the proteins. Comparison of the proteins two hours, 24 hours and 48 hours after treatment.



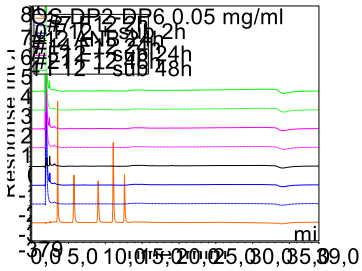
Appendix 3. Chromatographic Analysis of Enzymatic Activity on ANB. The graph displays behaviour of ANB under treatment at different time intervals 2 hours, 24 hoursand 48 hours.



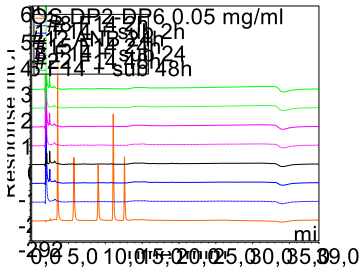
Appendix 4. Chromatographic Analysis of ANB at different time intervals. The graph displays one more peaks after 24 and 48 hours of treatment at around 19 minutes.



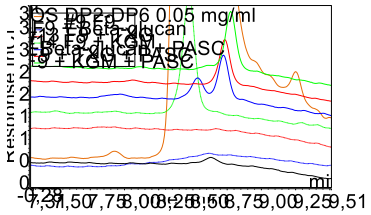
Appendix 5. Chromatographic Analysis of Enzymatic Activity on ANB. The graph displays behaviour of ANB under treatment at the different time intervals 2 hours, 24 hoursand 48 hours with protein F9 and protein alone. ANB after 24 hours of treatment was used as a baseline. Standard used was COS DP2-DP6.



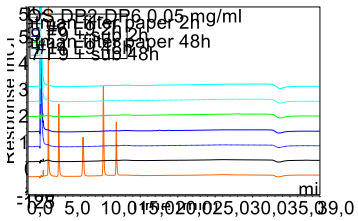
Appendix 6. Chromatographic Analysis of Enzymatic Activity on ANB. The graph displays behaviour of ANB under treatment at the different time intervals 2 hours, 24 hoursand 48 hours with protein F12 and protein alone. ANB after 24 hours of treatment was used as a baseline. Standard used was COS DP2-DP6.



Appendix 7. Chromatographic Analysis of Enzymatic Activity on ANB. The graph displays behaviour of ANB under treatment at the different time intervals 2 hours, 24 hoursand 48 hours with protein F14 and protein alone. ANB after 24 hours of treatment was used as a baseline. Standard used was COS DP2-DP6.



Appendix 8. Chromatographic analysis of Enzymatic Activity with protein F9 on the different polysaccharides with and without PASC with the known standard COS DP2-DP6.



Appendix 9. Chromatographic Analysis of Enzymatic Activity Whatman filter paper. The graph displays behaviour of Whatman filter paper under treatment after 2 and 48 hours with the presence of F9 only, F9 alone and the substrate alone. Standard applied was COS DP2-DP6.

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