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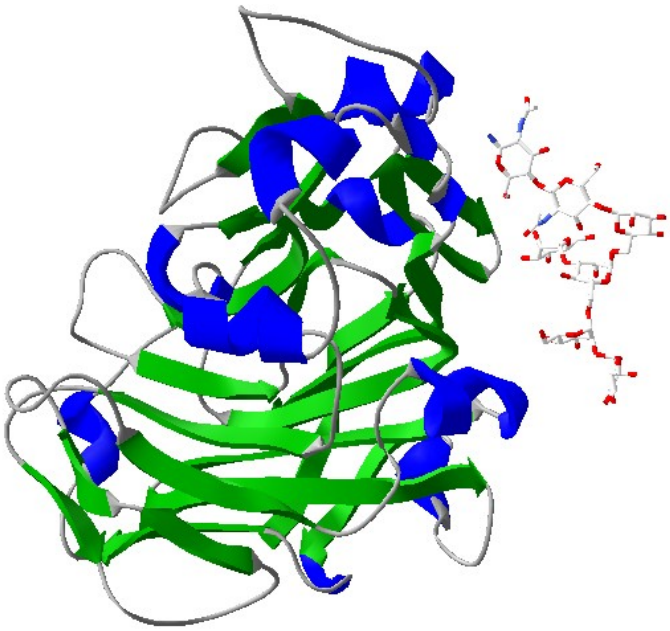
Hydrolysis and Glycosynthase Activity Studies of Laminarinase, Lam16A, Wildtype and its Catalytic Deficient Mutants E115G, E115S and E120A from *Phanerochaete chrysosporium*



Zarah Forsberg

Department of Molecular Biology
Swedish University of Agricultural Sciences
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MicroDrivE
Microbially Derived Energy

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Hydrolys- och glykosyntasaktivitetsstudier av vildtyps-laminarinas, Lam16A, och dess katalytiskt inaktiva mutanter E115G, E115S och E120A från *Phanerochaete chrysosporium*

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Zarah Forsberg

Supervisors: Jerry Ståhlberg, Henrik Hansson, Dept Molecular Biology, SLU

Examiner: Stefan Knight, Dept Molecular Biology, SLU

Institutionen för Molekylärbiologi
SLU, Sveriges Lantbruksuniversitet
Box 590
751 24 Uppsala

Examensarbete för Masterutbildning i Bioteknologi vid SLU

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ABSTRACT

Laminarinase 16A from *Phanerochaete chrysosporium* is a 36 kDa enzyme with typical endo-β-1,3(4)-glucanase activity, belonging to family 16 of glycoside hydrolases. The catalytic amino acids in the active site are identified as nucleophile Glu 115 and acid/base Glu 120. Prior to this project, these residues had been mutated to make catalytic deficient mutants E115G, E115S and E120A in order to obtain structures in complex with natural substrates. In the present work, the nucleophile mutant E115G has been expressed and successfully purified and shown to possess glycosynthase activity when using an α-fluoride derivative of laminariheptaose as substrate by the detection of circular laminariheptaose using HPLC. Activity measurements revealed that the E115G mutant had substantial activity in presence of acetate and formiate, and the other nucleophile mutant, E115S, in the presence of formiate, which indicates that those molecules can act as external nucleophiles. Circular β-glucans from two different nitrogen-fixing bacterial species were tested as potential substrates for wildtype Lam16A and the E115G mutant in presence of acetate. HPLC analysis revealed that there was no effect on the preparation with circular β-1,2-glucan, whereas one component of the other β-1,3/1,6-glucan preparation was consumed and new peaks appeared in the chromatogram, although further studies are needed to identify these components. The findings from this project reveals that this enzyme has many interesting capacities that hopefully in the future may be used in the synthesis of interesting oligosaccharides that can be applied in the fields of environmental protection, pre-biotics and medicine.

Keywords
Laminarinase 16A (Lam16A), β-glucans, glycoside hydrolases, catalytic deficient mutants, glycosynthases activity, circular oligosaccharides, HPLC, external nucleophiles

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ABBREVIATIONS

αL2F– alpha laminaribiosyl fluoride
αL7F - alpha laminariheptosyl fluoride
AOX1 – alcohol oxidase 1
CAD – charged aerosol detection
cDNA – complementary deoxyribonucleic acid
cv – column volume
GH – glycoside hydrolase
Glc – glucose
HPAE – high performance anion exchange
HPLC – high performance/pressure liquid chromatography
IEC – ion exchange chromatography
kDa – kilo Dalton
L2 – laminaribiose
L3 – laminaritriose
L4 – laminaritetraose
L5 – laminaripentaose
L6 – laminarihexaose
L7 – laminariheptaose
LMW – low molecular weight
MPa – mega Pascal
MWCO – molecular weight cut off
PAD – pulsed amperometric detection
PHBAH – parahydroxybenzoic acid hydrazide
pNP-L2 – para-nitrophenyl laminaribioside
rpm – rotations per minute
RT – room temperature
SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC – size exclusion chromatography
TLC – thin layer chromatography
wt - wildtype
YTG – yeast tryptone glycerol
YTM – yeast tryptone methanol

1. Introduction

Daily, we are constantly in contact with polysaccharides via food, textiles, and different kinds of materials, since they are the major constituents of biomass on earth. Polysaccharides belong to a structurally varied group of macromolecules, polymers and monosaccharides connected by glycosidic linkages. In combination with proteins and lipids, these molecules represents the key building blocks to all living things. Due to their high diversity and versatile properties, polysaccharides have many essential roles in nature; in fact, polysaccharides have the greatest potential of carrying biological information since they can interact in a number of different ways (Wasser, 2002). For instance, polysaccharides function as structural constituents in plant cell walls, they can form glycoconjugates with proteins or lipids which are crucial factors in cell signalling and recognition, and in seeds, polysaccharides are stored as energy reserves. For many centuries, humans have taken advantage of using polysaccharides for a number of purposes, although, over the years of development, many of these applications have been replaced with synthetic substitutes. However, with the reduction of oil supplies and increase in environmental awareness, an interest to find new polysaccharide materials has been renewed in areas such as food technology, drug development, and material science and research.

In nature, enzymes in the groups of glycosyl transferases, glycosidases, and phosphorylases are responsible for the synthesis of polysaccharides (Hancock *et al.*, 2006). The natural products are often heterogeneous, in terms of branching patterns, derivatization or mixed linkages. It is therefore of interest to synthesise more organised structures, but to do that by organic chemistry is very cumbersome, if not impossible. Therefore, enzymatic methods have emerged as an attractive alternative for the purpose of synthesising new, homogenous structures of poly- and oligosaccharides by genetically modifying the natural enzymes (Mackenzie *et al.*, 1998). Glycosynthases are enzymes that are engineered from naturally occurring retaining glycoside hydrolases, so that their hydrolytic activity is lost, and instead they enable the formation of glycosidic bonds when provided with oligosaccharyl fluoride donor substrates of opposite anomeric configuration compared to the natural substrate. Most commonly they are engineered such that the catalytic nucleophile is replaced by a smaller, inert amino acid that is unable to serve as catalytic nucleophile. Such mutants are of interest for synthesis of substrates and inhibitors for carbohydrate active enzymes, for sub-site mapping of the active site, and also for studies of substrate binding and specificity (Gullfot *et al.*, 2009).

1.1 Laminarinase 16A from *Phanerochaete chrysosporium*

1.1.1 Phanerochaete chrysosporium

The basidiomycete *Phanerochaete chrysosporium* is one of the most intensively studied wood-decaying fungi. It is present on forest litter and logs on the ground and it is classified as a white-rot fungus, which attacks the lignin part of wood in an early stage and causes bleaching of the wood tissue. In addition to lignin, the fungus degrades cellulose, hemicellulose and other polymers present in wood (Vasur *et al.*, 2006). In 2004, as the first basidiomycete genome, the *P. chrysosporium* complete genome was sequenced, and two years later the gene models were updated (Wymelenberg *et al.*, 2006), which revealed an impressive repertoire of at least 87 putative glycoside hydrolase (GH) encoding genes.

1.1.2 Laminarinase 16A

When *P. chrysosporium* was grown on laminarin, a β-1,3/1,6-glucan, as the sole carbon source, one major extracellular protein was obtained with a molecular weight of 36 kDa. The corresponding

cDNA encoding this protein was cloned and recombinantly expressed in the methylotrophic yeast *Pichia pastoris* for further enzymatic characterization (Kawai *et al.*, 2005). The 298 amino acid protein sequence revealed that this enzyme belonged to the glycoside hydrolase family 16, and since this was the first enzyme detected in the GH 16 family from this organism, it was named Laminarinase 16A, Lam16A. GH 16 contains representatives from all of the three kingdoms (archea, bacteria, and eukarya) and there are at least 5 subfamilies; (i) κ-carrageenases/1,4-β-galactosidases, (ii) agarases/1,4-β-galactosidases, (iii) nonspecific 1,3(4)-β-glucanases, (iv) lichenases/1,3/1,4-β-D-glucan endohydrolases, and (v) xyloglucan transglucosylases/hydrolases (XTHs), where Lam16A belongs to subfamily (iii). The enzyme has typical endo-1,3(4)β-glucanase activity with broad substrate specificity, and randomly hydrolyses linear β-1,3-glucan, branched β-1,3/1,6-glucan and β-1,3/1,4-glucan (Kawai, 2006). Its physiological role in *P. chrysosporium* is still not known, it may degrade glucans in wood for feeding or it may be mainly involved in the metabolism of endogenous glucans. In 2006, Vasur *et al.,* published the structure of Lam16A as the first structure to be solved in subfamily (iii) in the GH 16 family. Thereby, structures were available in all of the subfamilies and comparison showed that within all subgroups the structures and folds were very similar, but with differences in length and sequence of beta-turn-loops. These differences result in wider or more closed active sites and influence differences in substrate specificities between the subfamilies of the GH 16 enzymes. In the family of GH 16, the catalytic amino acids and other active site surrounding residues are conserved and these are evolutionary related to the cellulases in GH family 7. The fold is similar and the catalytic machinery consists of three conserved carboxyl residues; nucleophile Glu, assisting Asp, and acid/base Glu, in close proximity on the same beta-strand. Subfamilies (i)-(iii) in GH 16 has the same sequence motif, Glu-X-Asp-X-X-Glu, as the enzymes in GH 7, whereas the remaining subfamilies of GH 16, (iv) and (v), have lost one amino acid and the sequence motif is instead Glu-X-Asp-X-Glu. For Lam16A, the identified catalytic amino acids are Glu 115 (E115) as nucleophile, assisting Asp 117 (D117), and Glu 120 (E120) as acid/base.

1.1.3 Reaction mechanisms; hydrolysis and transglycosylation

In family GH 16, the enzymes possess a retaining mechanism, meaning that the anomeric configuration at the glycosidic bond that is cleaved is retained after hydrolysis. Hydrolysis of the beta-glycosidic bond gives a new reducing end where the new hydroxyl group is in beta-position on the anomeric carbon. The reaction occurs in two (inverting) steps (Figure 1). In the first step, glycosylation, the catalytic acid/base (E120) protonates the glycosidic oxygen, and simultaneously, the catalytic nucleophile (E115) provides a free electron pair to the anomeric carbon. This results in cleavage of the glycosidic bond and formation of a glycosyl-enzyme intermediate where "half" of the sugar is covalently bound with an alpha-linkage to the carboxyl group of the nucleophile E115. In the second step, E120 acts as a base that abstracts a proton from a water molecule so that the water molecule becomes activated and can perform a nucleophile attack on the anomeric carbon, leading to hydrolysis of the glycosyl-enzyme intermediate (Kawai, 2005).

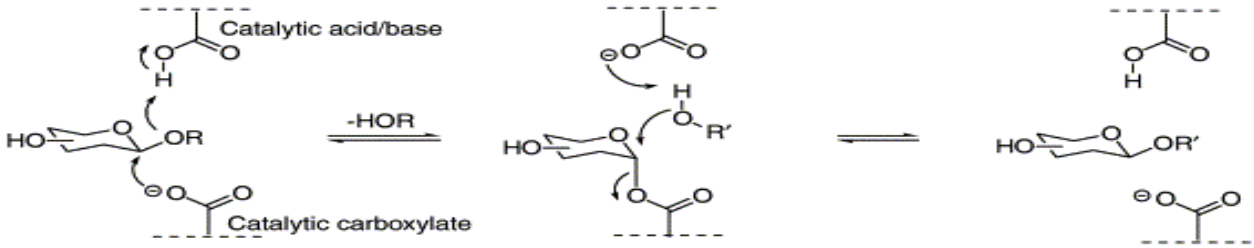

Figure 1. Hydrolysis and transglycosylation catalysed by a retaining glycoside hydrolase such as Lam16A, where HOR´ in the hydrolysis reaction corresponds to a water molecule, and the HOR´ in the transglycosylation reaction corresponds to another glycosyl residue (Picture reference: Hult & Berglund, 2003).

Like other β-1,3(4)-glucanases, Lam16A possesses transglycosylation activity that is of interest to study, since this may be used to provide new β-glucooligosaccharides and artificial β-1,3-glucanase substrates and branched/non-branched polyglucans for instance in the use as immunostimulators in pharmacology and medicine (Neustroev *et al.*, 2005). The transglycosylation mechanism is similar to hydrolysis, although, instead of an activated water molecule, another glycosyl residue is present that leads to the formation of a new glycosidic bond (Kawai, 2005).

1.2 Catalytic deficient mutants of Lam 16A

By the use of site-directed mutagenesis, three catalytically deficient mutants, see **Figure 2**, had been created in order to obtain structures of Lam16A in complex with natural substrates without the risk of hydrolysis. In two of those mutants, the catalytic nucleophile E115, was replaced with smaller non-nucleophilic residues; glycine (E115G) and serine (E115S). In the third mutant, E120A, the acid/base catalytic glutamate has been replaced with an alanine that is unable to serve in this capacity, and this mutant may be a potential thioglycoligase.

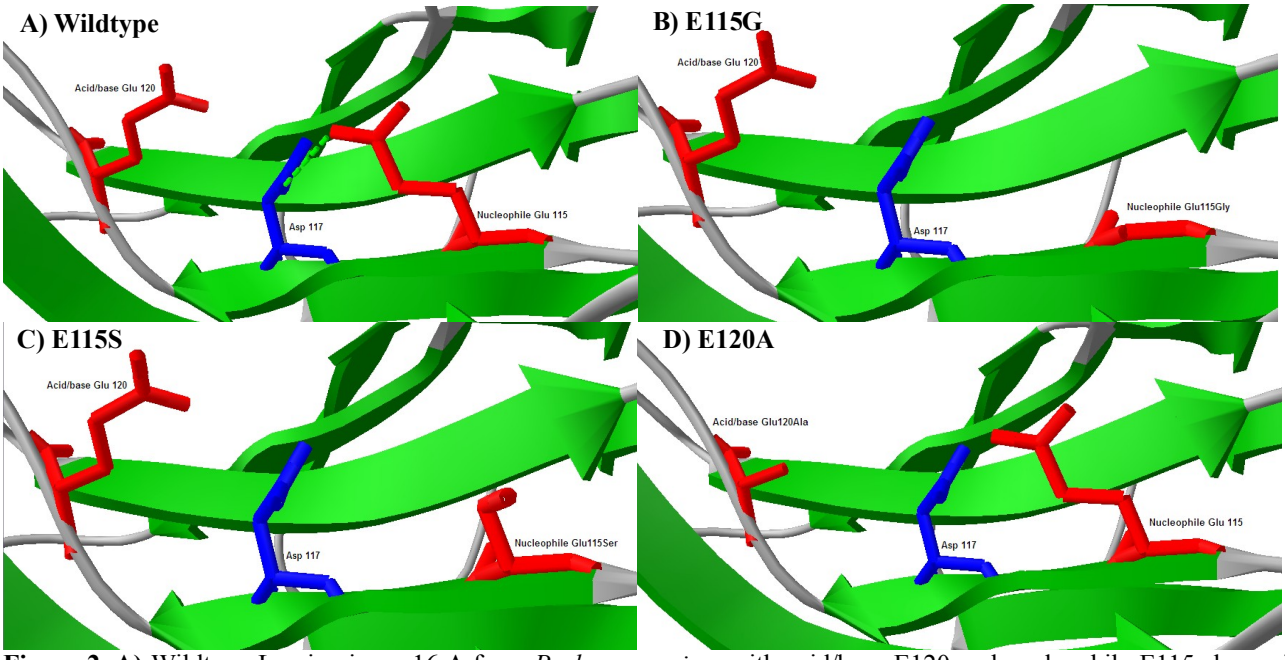

Figure 2. A) Wildtype Laminarinase 16 A from *P. chrysosporium* with acid/base E120 and nucleophile E115 shown in red and the assisting D117 shown in blue. Predicted structure of the mutants **B)** where the nucleophile glutamate has been replaced with the small glycine residue, **C)** where the nucleophile glutamate has been replaced with a serine amino acid, and **D)** where the acid/base glutamate has been replaced with an alanine. (Picture reference: the program Swiss PDB Viewer was used for viewing the Lam16A apo-structure, pdb-code 2CL2, mutating the residues *in silico*, and for making the images).

Prior to this project, these three mutants had been successfully expressed and purified, and activity measurements demonstrated that these amino acids are essential for the activity of wildtype Lam16A. In addition, mutants had been crystallized in complex with laminarioligosaccharides L2 to L7 and the alpha fluoride derivates of laminaribiose and laminariheptaose. The E120A mutant was shown to have considerable residual activity against para-nitrophenyl laminaribioside (pNP-L2) and studies on the E115S mutant showed that it has glycosynthase activity. The glycosynthase mechanism, see **Figure 3**, is similar to the hydrolysis mechanism of retaining glycosidases except that no intermediate is formed, since it is prevented by the replacement of a non-nucleophile in the active site. Required for this reaction is an activated glycosyl donor with an anomeric leaving group, which often is a fluoride. This leaving group is then displaced by an alcohol of the acceptor sugar aided by

the active site general base amino acid of the enzyme (Gullfot, 2009). An interesting observation is that the E115S mutant can produce circular laminariheptaose when αL7F is used as substrate. The formation of circular oligosaccharides are of great interest as potential pharmacophores analogous to the 6–8 residues long α-1,4-cyclodextrins. These circular oligosaccharides have the ability to transfer hydrophobic drugs on the inside of the molecule through hydrophilic surroundings. They can also be used in other applications such as environmental protection, where they can immobilize toxic compounds or heavy metals inside the ring, and this might be true for circular oligosaccharides produced by Lam16A glycosynthase mutants as well.

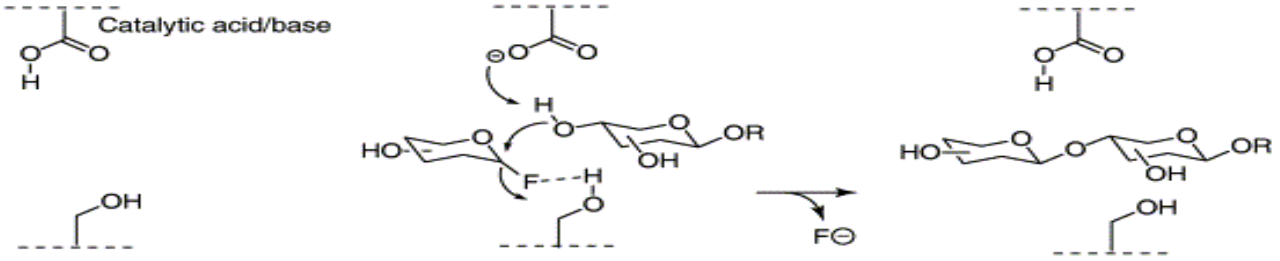

Figure 3. Glycosynthase mechanism by a genetically modified glycosidase enzyme, where the catalytic nucleophile has been replaced by a non-nucleophilic amino acid, in this case serine as in the E115S mutant (Picture reference: Hult & Berglund, 2003).

β-glucans that has shown to be substrates for Lam16A makes the enzyme interesting to study since the glucans have many similar properties as other fungal β-glucans with immunostimulating and anti-tumour activity. Many bioactive agents in forms of poly- and oligosaccharides are present in the fungal cell wall, and these polysaccharides varies a lot in chemical composition and structure. Studies have revealed that there are connections between the tumour activity and the water solubility, the form and the size of the polysaccharide molecule. Structures with β-1,3 linkages in the main chain and β-1,6 branches has shown to be essential for the anti-tumour activity, which is similar to laminarin and other Lam16A substrates. Glucans which only possesses β-1,6 linkages have less activity against cancer cells. The glucans do not attack the cancer cells directly, instead, they stimulate different components of the immune system in the host organism such as NK cells, T-cells, B-cells and macrophages. However, the biochemical mechanism behind polysaccharides activity against tumours are not fully understood yet (Wasser, 2002).

1.3 Aim of project

This 20 weeks master degree project is part of an ongoing project where the long-term goal is to extend the knowledge of the physical and enzymatic properties of Lam16A and to evaluate if this enzyme could be used for large scale production of β-glucans with interesting properties as mentioned in the sections above.

The first goal of this study was to express the Lam16A E115G mutant in the *P. pastoris* yeast and successfully purify it using different methods of protein chromatography. The E115G mutant had been produced previously, but had shown much higher residual activity than expected for a nucleophile-crippled mutant. It was suspected that there might be a risk that the previous preparation had been contaminated with wildtype Lam16A, why it seemed necessary to repeat the preparation of the mutant under conditions where utmost caution was taken to avoid such a risk.

One major purpose was to investigate whether the E115G mutant possesses glycosynthase activity, as the other nucleophile mutant E115S, and can produce longer oligosaccharides and/or circular laminarioligosaccharides when α-fluoride laminarioligosaccharides are used as substrates. In addition, the residual hydrolytic activity of the mutants compared to wildtype Lam16A should be

determined using the natural substrate laminarin and also compared with the mutants in presence of different potential external nucleophiles and how the concentration of these external nucleophiles influences the relative activity. Furthermore, the hydrolysis pattern of laminarin should be compared between the wildtype enzyme and the E115G mutant in presence of external nucleophile and also the hydrolysis pattern between possible substrates from two different circular bacterial β-glucans from the species *A. caulinodans* and *M. amorphae*. The main analysis method used is high performance liquid chromatography, HPLC.

Finally, crystallisation attempts are performed with the nucleophile mutants, aiming at obtaining a structure with the circular laminariheptaoside glycosynthase product bound at the active site. In previous crystallisation studies of mutant E115S, structures have been obtained with either laminariheptaose or αL7F bound in the active site. In both cases the sugar was forming an arch where the ends met at the catalytic centre of the enzyme. These structures indicate that the enzyme should be able to bind the circular heptasaccharide.

2. Material and methods

2.1 Cultivation and expression of Lam16A E115G mutant using *Pichia pastoris*

From a glycerol stock of a *Pichia pastoris* strain containing the insert of the Lam16A mutant E115G cDNA, constructed prior to this project, 100 µl was spread onto a YTG plate (10 mg/ml yeast extract, 20 mg/ml tryptone, 20 mg/ml agarose, and ~ 15 g/l glycerol) and incubated in 28 °C for two days. A pre-culture consisting of one single colony suspended in 20 ml of freshly made YTG medium (10 mg/ml yeast extract, 20 mg/ml tryptone, and ~ 15 g/l glycerol) was thereafter prepared and incubated on a shaker at 90 rpm in 28 °C for ~ 48 hours. This culture was equally divided to three 2.8 litre side-baffled Fernbach cultivation flasks (Bellco Glass Inc., USA) containing 1 L of YTG medium each, and incubated on a shaker at 80 rpm in 28 °C for additionally 48 hours. To induce expression of the protein, the glycerol was removed and replaced by methanol which induces the AOX1 promoter inserted into the *P. pastoris* genome to over-express the Lam16A E115G mutant enzyme. This was carried out by centrifugation of the 3 L culture under sterile conditions using a Sorvall RC 3C Plus centrifuge and the H6000A Rotor at 3500 rpm for 15 minutes. The supernatant was discarded and the pellet was suspended in autoclaved water by vigorous shaking and centrifuged in the same manner as before. Washing with autoclaved water was performed twice to ensure that all glycerol was removed before suspending the pellet in 600 ml YTM medium (10 mg/ml yeast extract, 20 mg/ml tryptone, and ~ 1 % methanol). The induction phase was carried out for four days, and each day, 6 ml of methanol was added to the culture that was incubated at 85 rpm in 26 °C. Simultaneously with the addition of methanol, one sample of approximately 1.5 ml was taken from the culture for SDS-PAGE analysis. Those samples were spun down at 13,300 rpm for 10 minutes in an Eppendorf-centrifuge, and the protein containing supernatant was sterile filtered using a 20 µm filter.

2.1.1 SDS-PAGE analysis

To 2 µl solution of already prepared 5×SB including β-mercaptoethanol, 8 µl of the sterile filtered supernatant was added. The tubes were heated for about 1 minute in a 95 °C heating block before they were loaded onto a 8-25 % gradient Phast gel (GE Healthcare) together with a low molecular weight ladder, LMW. The gel was stained by heating 20 seconds in Coomassie blue staining solution and destained in destaining solution (40 % ethanol and 10 % acetic acid) for 30 minutes at RT.

2.2 Protein harvest and diafiltration

In the *Pichia* expression system used, the target gene is expressed extracellularly. Taken that into advantage, harvest was carried out by collecting the supernatant by centrifugation using the Sorvall RC 5C centrifuge and the GSA Rotor at 3500 rpm for 30 minutes. The supernatant was poured into new centrifuge bottles and centrifuged an additional time of 40 minutes to get rid of cell debris. The 600 ml received protein solution was circulated through a diafiltration system using a Vivaflow 50 tangential flow filtration cassette with a membrane pore size of 10,000 Da MWCO pre-washed with 0.1 M sodium hydroxide, MilliQ water and 25 mM Bis-tris buffer pH 7.0 (start buffer in the first purification step). The protein solution was diluted 100 times with the 25 mM Bis-tris buffer by repeated concentration and dilution to a final volume of 250 ml. From this volume a sample of 8 µl was taken for another SDS-PAGE analysis.

2.3 Protein purification

The purification procedure of Lam16A E115G was divided into two different steps; ion exchange chromatography (IEC) and size exclusion chromatography (SEC). During the purification process, analysis of the peak fractions were performed using 8-25 % precast gradient SDS-PAGE gels on a Phast system (GE Healthcare) earlier described in section *2.1.1*.

2.3.1 Ion exchange chromatography

A 4.7 ml Capto Q column (GE Healthcare) connected to a ÄKTA™ Explorer (GE Healthcare) was equilibrated with Buffer A (25 mM Bis-tris pH 7.0) and Buffer B (25 mM Bis-tris pH 7.0 + 0.5 M NaCl) before the 250 ml sample was loaded onto the column with a flow rate of 2 ml/min. When the sample had entered the column, it was washed with 5 column volumes of Buffer A whereupon a 50 minutes salt gradient was set up to 100 % Buffer B, and the fraction volume was set to 2 ml. After adding approximately 100 ml of the sample, the column became saturated with protein. Therefore the collected flow-through fraction with unbound protein was divided in two portions and loaded onto the column and separated in the same way, except that the column was coupled to an ÄKTA™ Purifier system. The fractions were analysed using SDS-PAGE. A large amount of the Lam16A protein was present in the flow-through fractions, which were pooled and the 500 ml sample was applied to a 56 ml Source 30Q (GE Healthcare) column. Using the same buffers, the column was loaded with the protein sample with a flow rate of 4 ml/min. The column was washed with Buffer A before setting a linear salt gradient of 100 % Buffer B for 50 minutes and the faction collector to 2 ml. Again, the fractions were analysed by SDS-PAGE. The fractions containing Lam16A E115G were pooled and diluted in Buffer A to get rid of salt by concentrating and diluting about ×50 using Viva spin 20, 10,000 MWCO (GE Healthcare) concentration tubes at 4500 rpm. To get a higher resolution and purity, a smaller Source 30Q column with a column volume of 10 ml was set up and the 40 ml sample was loaded with a flow rate of 1 ml/min. The column was thereafter washed with one column volume of Buffer A before a salt gradient of Buffer B was set up as follows; 0-50 % for 100 minutes, 50-100 % for 20 minutes. Due to previous experience the Lam16A protein elutes when one third of Buffer B is present, and since the total concentration of NaCl is 0.5 M, 0.15 M should be the most suitable elution concentration ($0.3 \times 0.5 = 0.15$). Once again, the peak fractions were analysed using SDS-PAGE.

2.3.2 Size exclusion chromatography

From the SDS-PAGE it could be seen that a rather big protein was present in the same peak as the Lam16A E115G protein. For that reason, a gel filtration method was chosen to separate the two proteins, using a Superdex 200 16/60 column. The protein containing fractions were pooled and

concentrated at 4500 rpm to an obtained volume of 2 ml using a Viva spin 6, 10,000 MWCO concentration tube. The column was attached to the ÄKTA™ Purifier system and equilibrated with one column volume, 130 ml, Buffer A (10 mM ammonium acetate pH 5.0) at a flow rate of 1 ml/min. A 10 ml sample loop with a pressure of 0.15 MPa was used to introduce the 2 ml sample, with a measured protein concentration of 3.27 mg/ml, to the column. The flow rate was set to 0.15 ml/min when loading the sample and after 30 ml the fraction collector was started to collect fractions of 2 ml, leading to 90 fractions, with a flow rate of 0.5 ml/min. To verify the separation from this purification step, SDS-PAGE was carried out.

2.4 Residual activity measurements

In order to investigate whether the peak shoulder from the SEC really was functional Lam16A E115G, PHBAH reducing sugar assay was used to measure the activity against the substrate laminarin. This assay makes it possible to measure the amount of reducing ends present in the reaction since parahydroxybenzoic acid hydrazide (PHBAH) reacts with the reducing ends of carbohydrates. By using a standard curve consisting of glucose in different concentrations, it becomes possible to calculate the change in glucose equivalents per enzyme molecule per second. This value can then be used to determine a relative activity in comparison with the wildtype Lam 16A. 25 µl of all peak fractions from the SEC and the wildtype Lam16A was incubated at RT for 1 hour and 20 hours, respectively with 0.25 mg/ml laminarin in 0.1 M sodium citrate pH 5.0 buffer at a final reaction volume of 50 µl. The concentration of the wildtype enzyme was 1.25 nM whereas the mutant enzyme concentration was unknown, though most likely in the µM range. To stop the reaction, 50 µl of 1 M sodium hydroxide was added and immediately thereafter 200 µl PHBAH solution (0.1 M PHBAH, 0.2 M NaK-tartrate, and 0.5 M NaOH). The samples were boiled for 15 minutes in a 100 °C heating block and then cooled on ice for at least 10 minutes, before they were transferred to a 96-well microtiter plate. The absorbance at 410 nm was measured in a microtitre plate reader, and the absorbance at 280 nm was also measured in a UV-1800 spectrophotometer for all the peak fractions. The absorbances after 1 hour, 20 hours and the protein absorbance, 280 nm, was plotted in a graph to ensure that the residual activity belonged to no other than the Lam16A E115G enzyme. Selected fractions from the SEC peak were then pooled and the absorbance at 280 nm was measured to calculate the protein concentration in molar using Lambert Beer's law; $A = \varepsilon lc$, and the concentration in mg/ml by using the estimated molar extinction coefficient 62 910 M^{-1} cm^{-1} (ExPASy ProtParam tool) and multiplying the molar concentration with the molecular weight of the protein, 33,762 Da (ExPASy ProtParam tool). The sample was concentrated using Viva spin 6, 10,000 MWCO to a total volume of 0.75 ml and sterile filtrated through a 20 µm filter. Again the concentration was measured in triplicates using a Nano Drop spectrophotometer.

2.4.1 Residual activity of the catalytic deficient mutants; E115G, E115S, and E120A

In previous experiments, the mutant E115G showed much higher residual activity than expected for a nucleophile-crippled enzyme. It was suspected that this may be due to the presence of acetate, which possibly can act as an external nucleophile. Thus, in this experiment two buffers were tested; 1) 0.1 M sodium citrate pH 5.0, and 2) 0.1 M ammonium acetate pH 5.0. All mutants including the wildtype enzyme was incubated in 30 °C water bath with 0.25 mg/ml laminarin and 0.1 M citrate buffer, and the mutant E115G and the wildtype was also incubated separately with laminarin in 0.1 M acetate buffer. The concentrations of the mutants were 1 µM and for the wildtype 0.25 nM, and the reaction volume was 50 µl. The reactions were performed in triplicates for 1 hour and 24 hours, and for the wildtype 0.33, 1, 2, 4, and 6 h in citrate buffer, and 1 hour in acetate buffer. Glucose standards with the following concentrations: 0, 10, 50, 200, and 500 µM were also set up in citrate

buffer and immediately stopped with 50 µl 1 M NaOH, whereupon PHBAH was added and the absorbance at 410 nm was measured in a microtitre plate reader.

2.5 Possible external nucleophiles and concentration dependence

Since acetate can act as an external nucleophile for the Lam16A E115G mutant, and the protein was present in ammonium acetate pH 5.0 buffer, the buffer was changed using a Biorad 10-DG desalting column to sodium citrate pH 5.0 buffer before testing other possible external nucleophiles. The 10-DG column was equilibrated with citrate buffer before the mutant E115G was added. When 2.7 ml protein sample had entered the column, elution was performed by adding 3.3 ml MilliQ water. The absorbance of the collected sample was measured at 280 nm using a Nano Drop spectrophotometer. Thereafter, the sample was concentrated to 0.5 ml, and again the absorbance was measured to estimate the concentration.

2.5.1 Influence of acetate concentration with the E115G mutant

E115G mutant and Lam16A wildtype enzyme, respectively, were incubated in 30 °C water bath with 0.25 mg/ml laminarin in acetate buffer at concentrations of 10, 40, 200, and 450 mM for 1 hour and the wildtype for 0.33, 1, 2 and 4 hours. The wildtype enzyme was also incubated in 0.1 mM acetate buffer for one hour. The reaction volume was 50 µl and the mutant concentration was 1 µM and 0.2 µM and the wildtype concentration was 0.25 nM. A glucose standard was prepared as before, in citrate buffer, with concentrations of 0, 10, 50, 200, 500, and 700 µM. The reaction was stopped with 1 M NaOH and the detection method used was PHBAH reducing sugar assay.

2.5.2 Formiate and azide as external nucleophiles

In addition to acetate, formiate ($CHNaO_2$) and azide (NaN_3) were tested as potential external nucleophiles with all of the catalytic deficient mutants; E115G, E115S and E120A, and compared with the wildtype Lam16A. The same enzyme concentrations were used as before, 1 µM for the mutants and 0.25 nM for the wildtype. The mutants were incubated at RT together with 0.25 mg/ml laminarin and 0.1 M formiate buffer pH 5.0 or 0.1 M azide/0.1 M citrate buffer pH 5.0 for 1 hour and 24 hour. The wildtype enzyme was incubated with laminarin and formiate or azide for 0.33, 1, 2, and 4 hours. Also for this experiment, the PHBAH detection method was used.

2.5.3 Influence of formiate concentration with the E115G and the E115S mutants

From the previous experiment, section *2.5.2*, formiate ions seemed to be able to act as external nucleophile for both the E115G and E115S mutants. Thus, the activity of these mutants were measured at different concentrations of formiate, similar to the experiment with acetate, with the only difference of the enzyme concentrations were 0.2 µM for E115G and 2 µM for E115S.

2.6 Hydrolysis of laminarin with wildtype Lam16A and mutant E115G in presence of acetate

One objective in this project was to monitor hydrolysis of polymeric substrates such as laminarin. Three different HPLC columns were tested for this experiment, first a PA10 column was used on the Dionex ICS-3000 HPLC system, although the acetate needed for hydrolysis activity of the E115G mutant affected the separation of the standards too much, therefore this method was rejected. Another column, Reprosil-Pur C18-AQ.5um 100×4.6 mm (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany), using the Agilent 1100 HPLC system was tested, but in this system both the acetate and the citrate gave large peaks in the chromatograms that covered the peaks of glucose and laminaribiose. The third column used was Dionex PA200 3x250 mm on the Dionex system which

gave a good separation, although the peaks were still difficult to identify. Anyway, wildtype Lam16A (0.25 nM) or mutant E115G (1 μM) with 0.5 mg/ml laminarin in 10 mM acetate buffer pH 5.0, were incubated in the auto-sampler chamber at 30 °C. 20 μl was injected to the column after 10 min, 1 h, 4 h and 16 h. Eluent A was MilliQ water, eluent B was 200 mM NaOH and eluent C was 200 mM NaOH + 1.2 M sodium acetate and the flow rate was set to 0.5 ml/min. After injection, elution was performed with a 58 min gradient program where the concentration of eluent A (MilliQ water) was kept constant at 50 %, as follows: 20 min 44.2-31.4% B and 5.8-18.6% C, 12 min 31.4-0% B and 18.6-50% C, 1 min 0-44.2% B and 50-5.8% C, and 25 min re-equilibration with 44.2% and 5.8% C. Eluted compounds were detected with a pulsed amperometric detector (PAD) and Chromeleon software. As standards, Glc+L2-L7 (glucose and laminarin oligosaccharides with 2 to 7 Glc residues), and P4 (6-O-β-glucosyl-laminaritrioside) at the concentrations of 0.1 mM and 0.5 mg/ml laminarin were used.

2.7 Glycosynthase experiment with the Lam16A E115G mutant

The mutant Lam16A E115G is considered as a potential glycosynthase enzyme, although in previous studies no such results had been obtained, neither by thin layer chromatography, TLC, nor with HPLC. The lack of evidence for glycosynthase activity was probably due to the fact that during those experiments, the E115G mutant had acetate ions present from the purification procedure, and with this external nucleophile, the enzyme starts to hydrolyze the oligosaccharides instead of putting them together. Therefore the mutant E115G was transferred to citrate buffer to avoid this problem and to investigate whether this mutant, as the E115S mutant, had glycosynthase activity. The E115G mutant was incubated at 30 °C at a concentration of 1 μM together with a 10 mM α-laminariheptaosyl fluoride, αL7F (synthesized and provided by Hanna Jonsson and Prof. Göran Widmalm, Dept. Organic Chemistry, Stockholm University). The reaction was performed in potassium phosphate at pH 6.0 and before the enzyme was added, 10 μl was analysed on an Agilent 1100 HPLC system with Clarity control and acquisition software, using a Reprosil-Pur C18-AQ 5 μm column 150 × 4.6 mm with a 10 × 4.6 mm pre-column cartridge, thermostated at 25 °C. Eluent A was MilliQ water and eluent B was 100 % acetonitrile (ACN) and the flow rate was set at 1 ml/min. 10 μl sample was injected with a syringe at the time points of 3.5 and 17 hours and manual injector at start of a 30 min gradient program: 24 min 0-20 % ACN, 1 min to 95 % ACN, 1 min at 95 % ACN, 4 min at 0 % ACN. Eluted compounds were detected with a Corona charged aerosol detector (CAD) set at 500 pA FSD (full scale deflection). Standard samples used were 10 mM αL7F and 0.5 mM each of Glc+L2-L7.

2.8 Hydrolysis of circular β-glucans from *Mesorhizium amorphae* and *Azorhizobium caulinodans*

From Dr Adam Choma, (Dept. General Microbiology, Maria Curie-Sklodowska University, Lublin, Poland), cyclic β-glucans from two different nitrogen fixing bacterial species were provided. These β-glucans were of interest since one of them, from *Azorhizobium caulinodans* is built up of β-1,3/1,6 glycosidic bonds which is similar to laminarin. The other cyclic β-glucan though, is linked with β-1,2 glycosidic bonds which has not been recognised as substrate for Lam16A. However, the hydrolysis pattern of mutant E115G may differ from the wildtype Lam16A, and for that reason 0.5 mg/ml cyclic β-glucans from both species were incubated in RT with wildtype Lam16A, 0.25 nM, and the mutant E115G, 1 μM, in presence of 10 mM ammonium acetate pH 5.0. The cyclic β-glucans were analyzed on the Agilent 1100 HPLC system using a 50 minutes gradient; 5 min at 1% ACN, 10 min at 1-5.1% ACN, 21 min at 5.1-10% ACN, 1 min at 10-50% ACN, 1 min at 50-95% ACN, 5 min at 95% ACN, and 8 min at 1% ACN. Samples of 10 μl were manually injected after 20 minutes and

24 hours, respectively. In addition the *A. caulinodans* and the wildtype enzyme was incubated 48 hours. Unfortunately, the sample with E115G and the *M. amorphae* glucan got lost in the HPLC system.

2.9 Crystallization of E115G and E115S nucleophile mutants

Crystallization attempts of the mutants E115G and E115S were performed utilizing the hanging drop vapour diffusion method. The reservoir solution contained 15 – 25 % polyethylene glycol (PEG 3350) with intervals of 2.5 %, 0.2 M ammonium nitrate and 10 mM sodium citrate pH 5.0, and water up to a final well volume of 1 ml. From the reservoir solution, 1 µl was taken and placed on the lid together with 1 µl protein (E115G 2.2 mg/ml and E115S 2.5 mg/ml). After 24 and 72 hours, seeding was performed using a previous set up of E115S and E120A crystals. Each day for approximately one week, the crystallization set up was viewed under a microscope to monitor the formation of crystals.

3. Results and discussion

3.1 Expression and purification of the Lam16A E115G mutant

Each day, of the four day induction phase using methanol, one sample of approximately 1.5 ml was taken from the culture for SDS-PAGE analysis. This was performed in order to see that the Lam16A E115G mutant was successfully expressed and also to estimate the amount of enzyme produced by comparing to a Lam16A enzyme batch with known concentration, see **Figure 4**. From this gel picture it can be seen that after three days, weak bands appear and become thicker with the induction time. The bands are also of the same size as the reference Lam16A E115G mutant which is about 34 kDa. The total molecular weight of Lam16A is estimated at 36 kDa, including the sequence signal and one N-linked glycan consisting of two N-acetylglucosamine (NAG) and nine mannose (MAN) residues. By using the ExPASy ProtParam tool after identification and removal of the signal sequence (SignalP, http://www.cbs.dtu.dk/services/SignalP/), the molecular weight of the expressed protein was calculated to 33,765 Da (~ 34kDa) which is in agreement with the gel bands. However, the gel bands are very thin since they are not concentrated before loaded onto the precast Phast gel, and for that reason, a more sensitive staining method would have been preferable, for instance the silver staining method.

Using *P. pastoris* as expression host has many advantages, as an example, the cells can be reused for additional protein expression. In this experiment though, this option was not recommended since it did not give much additional protein when the E115S mutant was expressed one year ago.

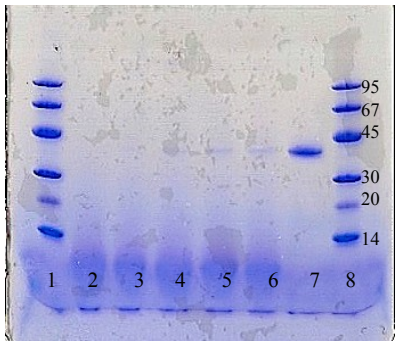

Figure 4. SDS-PAGE analysis of samples from *P. pastoris* expression of Lam16A E115G. From left to right: lane 1, low molecular weight ladder (LMW); lane 2, induction day 1; lane 3, day 2; lane 4, day 3; lane 5, day 4; lane 6, after harvest; lane 7, reference Lam16A E115G mutant (0.7 mg/ml); lane 8, LMW ladder. Weak bands are visible from induction day three even though the protein solution is not concentrated.

The first purification step was the HiScreen Capto Q IEC column, predicted to be a good method to start with for crude material as in this situation. However, this turned out to be a misjudge since the column became overloaded after only 100 ml of the 250 ml protein solution. For that reason the 300 ml flow-through fraction was stored and loaded onto the same column in two steps, see **Appendix 1 A)**. From the first run (chromatogram data not shown), fractions 9, 25, 36, 42, 48, and 54 were analyzed on a SDS-PAGE precast gradient gel, see **Figure 5 A)**, and from the other runs the fractions of interest; 7, 12, 17, 21, and 29 were pooled and analyzed on another gel, see **Figure 5 B)**. From this gels, it can be seen that the Lam16A E115G mutant ended up in the flow-through fraction, which indicates that this column did not have the capacity needed to bind this protein at the chosen conditions.

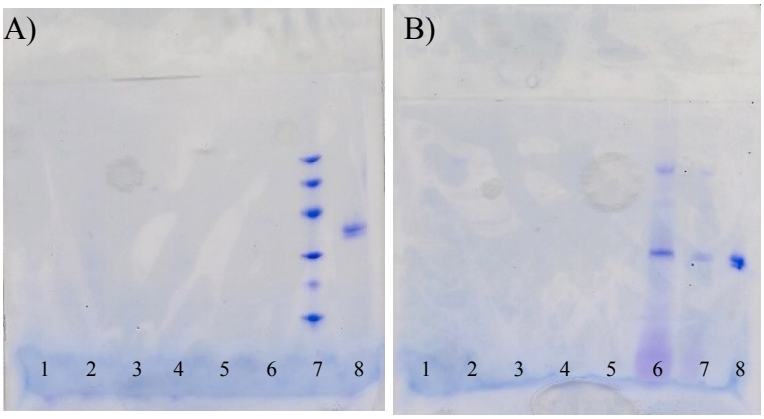

Figure 5. SDS-PAGE analysis of the peak fractions from the three Capto Q purifications. First purification **A)** , from left to right; lane 1 fraction 9, lane 2 fraction 25, lane 3 fraction 36, lane 4 fraction 42, lane 5 fraction 48, lane 6 fraction 54, lane 7 LMW ladder, lane 8 reference Lam16A E115G mutant. **B)** Pooled fractions from the second and the third purification, from left to right; lane 1 fraction 7, lane 2 fraction 12, lane 3 fraction 17, lane 4 fraction 21, lane 5 fraction 29, lane 6 concentrated start material, lane 7 unbound waste fraction, and lane 8 reference Lam16A E115G mutant.

Since the protein could not bind to the Capto Q column, another type of IEC, a 56 ml Source 30Q column, was applied and loaded with the big Capto Q flow-through fraction of 500 ml. This resulted in a chromatogram with several peaks, see **Appendix 1 B)**. Selected peak fractions, 30, 35, 40, 44, 48, 52, and 59, were analyzed on a SDS-PAGE gradient Phast gel, see **Figure 6 A)**. In addition, a sample from the Capto Q flow-through fraction was concentrated ×10 and compared to a sample from the Source 30Q flow-through fraction concentrated in the same way, and also to the reference Lam16A E115G mutant (0.7 mg/ml), see **Figure 6 B)**. From these gels it can be seen that the protein do not end up in the flow-through fraction, and that fraction 48 has a clear band in the same size as the reference Lam16A, and also some weak bands in fraction 44 and 52. In those fractions, a big protein is visible as well, thus further purification is needed to get rid of this contaminating protein.

To improve the separation, a smaller Source 30 Q column with a column volume of 10 ml was used, see **Appendix 1 C)**. The chromatogram indicates that another protein with similar charge as the Lam16A enzyme still is present, thus fractions 22, 25, 28, 30, 34, and 37 were analyzed on a gel, see **Figure 7**. This gel analysis strengthens that the protein is not completely pure, and that a big protein still is present which may correspond to a *P. pastoris* protein with similar net charge as the Lam16A E115G mutant protein.

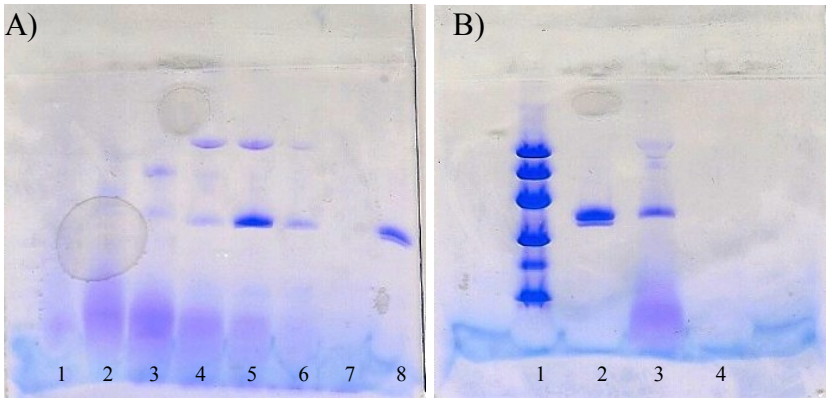

Figure 6. Gel pictures from the 56 ml Source 30Q IEC purification. **A)** From left to right; lane 1 fraction 30, lane 2 fraction 35, lane 3 fraction 40, lane 4 fraction 44, lane 5 fraction 48, lane 6 fraction 52, lane 7 fraction 59, and lane 8 reference Lam16A E115G mutant. **B)** From left to right; lane 1 LMW ladder, lane 2 reference Lam16A E115G mutant, lane 3 Capto Q waste fraction concentrated ×10 before loaded onto the Source 30Q, and lane 4 waste fraction concentrated ×10 after the Source 30Q purification.

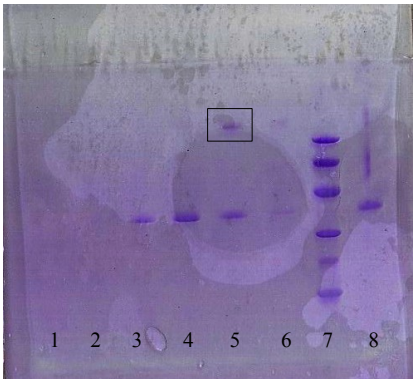

Figure 7. Selected fractions from the second Source 30Q purification with a smaller column volume. From left to right: lane 1, fraction 22; lane 2, fraction 25; lane 3, fraction 28; lane 4, fraction 30; lane 5, fraction 34; lane 6, fraction 37; lane 7, LMW ladder; lane 8, reference Lam16A E115G mutant. The black box marks the big contaminating protein with similar charge as Lam16A E115G nucleophile mutant.

To get rid of this big protein, selected fractions were pooled (fractions 27-38) and concentrated to a final volume of 2 ml. The absorbance at 280 nm was measured to 6.1, corresponding to a final amount of 6.6 mg protein, before the sample was loaded onto a Superdex 200 SEC column, see **Appendix 1 D)**. Fractions; C6, C9, C10, C11, C12, and C13 were analyzed on a SDS-PAGE gradient gel, see **Figure 8**. The chromatogram indicates good separation of the two proteins, where the small peak that is first eluted corresponds to the big contaminating protein and the large, second eluted peak corresponds to the protein of interest. Only a single band was seen in each fraction, indicating that the protein is pure and that the purification was successful. However, in the chromatogram, there was a shoulder at the right side of the Lam16A E115G peak, which could belong to an additional contaminant. To follow up on that, residual activity of all the SEC peak fractions was measured as described in the following section.

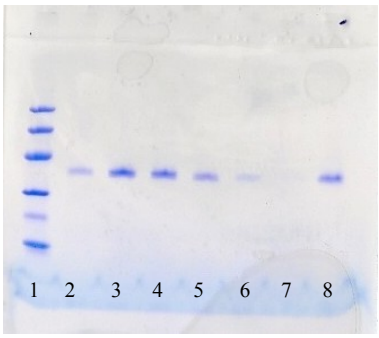

Figure 8. Fractions of interest from the SEC purification. From left to right; lane 1 LMW ladder, lane 2 fraction C6, lane 3 fraction C9, lane 4 fraction C10, lane 5 fraction C11, lane 6 fraction C12, lane 7 fraction C13, and lane 8 reference Lam16A E115G mutant.

One conclusion from these purification steps, is that the Capto Q anion exchange column can be excluded when the protein amount is as high as in this experiment since the binding capacity is highly affected. Therefore, a recommended procedure for purifying this mutant is to first use a Source 30 Q IEC column and thereafter a gel filtration column to remove the *P. pastoris* protein with similar net charge as the Lam16A E115G mutant. However, it can not be determined weather or not both of the Source 30Q columns are necessary or if there would have been enough with the second column, with the smaller cv of 10 ml. It was necessary in this experiment since the flow-through volume from the Capto Q column was as large as 500 ml.

3.2 Residual activity measurements

25 µl samples from all of the SEC peak fractions (C1-C15) were incubated with laminarin for 1 and 20 hours, respectively. The reactions were stopped with NaOH and the PHBAH reducing sugar assay was used as detection method by measuring the absorbance at 410 nm. In addition, the protein absorbance for all fractions were measured at 280 nm before the three different graphs were plotted in the same diagram, see **Figure 9**. The diagram indicates that the observed activity comes from the Lam16A E115G enzyme and not from any contaminating protein, since the absorbance plots correlate well with each other. Fractions 4-12 were pooled and concentrated to a volume of 0.75 ml. A280 was measured at 5.5 corresponding to a molar concentration of 87 µM, that is equivalent to 2.9 mg/ml.

Previous experiments with Lam16A E115G mutant indicated that acetate ions may act as external nucleophile in the hydrolysis of laminarin. To confirm this, experiments were carried out using two different buffers for the E115G mutant, citrate which is too big to be able to interfere in the reaction, and acetate with the hypothesis that it will act as an external nucleophile and thereby provide hydrolysis activity to the mutant enzyme. After incubating all of the mutants (E115G, E115S, and E120A) as well as the wildtype enzyme with laminarin for 1 hour and 24 hours, respectively, the absorbance at 410 nm was measured by using the PHBAH reducing sugar assay. As described earlier, a glucose standard, see **Appendix 2**, was used which makes it possible to calculate the change in glucose equivalents per enzyme molecule per second. This value was then used to determine a relative activity in comparison with wildtype Lam 16A, see **Table 1**.

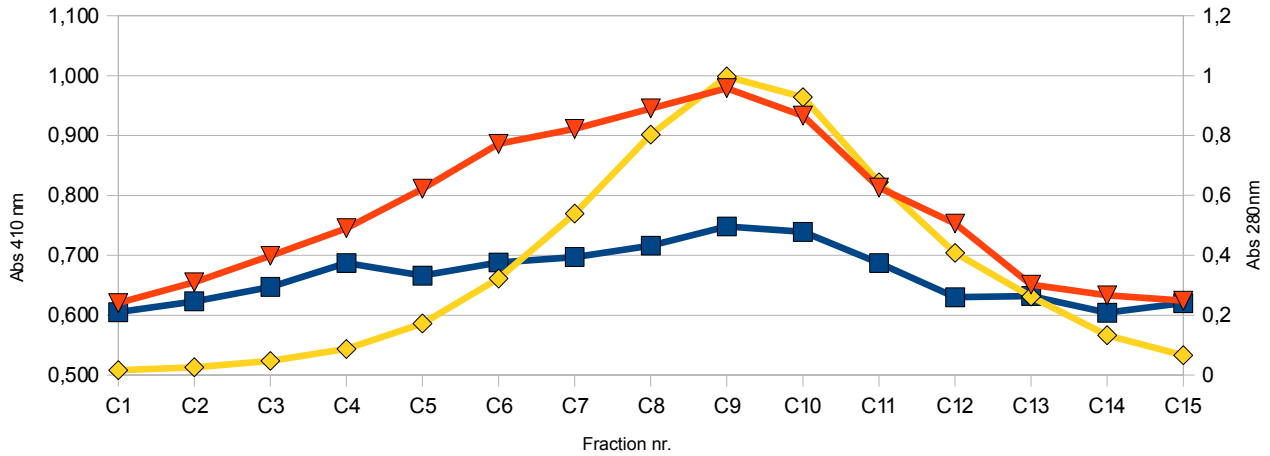

Figure 9. Measured absorbances for residual activity and protein content in the SEC peak fractions. The blue line corresponds to the residual activity measurements after 1 hour, the red line after 24 hours (both 410 nm; plotted on the left y-axis), and the yellow line to the protein absorbance at 280 nm on the right y-axis.

Table 1. Estimated relative activity on laminarin for the different mutants; E115G, E115S, and E120A in citrate buffer compared to the wildtype enzyme, and E115G in acetate buffer compared to the wildtype Lam16A enzyme. Total reducing sugar was measured using the PHBAH reagent and compared with glucose of known concentrations. Activity is expressed as increase in molar concentration of glucose-equivalents per second per molar of enzyme (dP/dt/[E]).

Buffer	Enzyme	Time (h)	[E] (µM)	△A 410 nm	dP/dt/[E] (s^{-1})	Relative activity	
Citrate	Wild type	0,3	0,00025	0,100	2,93E+02	1	1
		1	0,00025	0,314	3,06E+02		
		2	0,00025	0,486	2,37E+02		
		4	0,00025	0,517	1,26E+02		
		6	0,00025	0,532	8,65E+01		
	E115G	1	1,00000	0,037	9,02E-03		
		24	1,00000	0,087	8,84E-04	3,02E-06	<1/330000
	E115S	1	1,00000	0,019	4,63E-03		
		24	1,00000	0,044	4,47E-04	1,53E-06	<1/670000
	E120A	1	1,00000	0,012	2,93E-03		
		24	1,00000	0,070	7,11E-04	2,43E-06	<1/420000
Acetate	E115G	1	1,00000	0,324	7,90E-02	2,09E-04	1/4800
		24	1,00000	0,930	9,45E-03		
	Wild type	1	0,00025	0,388	3,78E+02		

As can be seen from this experiment, the mutants incubated with laminarin in citrate buffer have very limited hydrolytic activity compared to the wildtype Lam16A enzyme, but in acetate buffer the E115G mutant had an activity of ~1/5000 compared to the wildtype. It may thus be concluded that acetate indeed acts as an external nucleophile with the E115G mutant. To investigate this further, experiments were performed to see how the increase in acetate concentration influences the hydrolysis of laminarin with E115G. Another interest was to detect other potential external nucleophiles. However, before such experiments could be done, the buffer in the enzyme solution had to be changed since it contained small amounts of acetate that would give rise to false results if still present. By using a DG10 desalting column, the buffer for the E115G mutant was changed to citrate buffer pH 5.0, and the absorbance was thereafter measured again to ensure that all protein eluted from the column. The mean value of the absorbance was measured at 1.2 corresponding to a concentration of 65 µM, equivalent to 2.2 mg/ml.

3.2.1 Formiate and azide as potential external nucleophiles

Two other potential external nucleophiles, formiate and azide, were tested in the same manner as for the acetate buffer for all of the three catalytic deficient mutants; E115G, E115S and E120A. The estimated relative activity compared to the wildtype enzyme is presented in **Table 2**, where it can be seen that formiate has some influence both on the E115G and the E115S mutants, whereas azide shows no effect on any of the mutants.

Table 2. Relative activity in the presence of formiate ($CHNaO_2$) or azide (NaN_3). Formiate activates both the nucleophile mutants, E115G and E115S, whereas azide has no effect on any of the catalytic mutants.

Buffer	Enzyme	Time (h)	[E] (µM)	△A 410 nm	dP/dt/[E] (s^{-1})	Relative activity	
$CHNaO_2$	Wt	0,333	0,00025	0,193	6,8E+02		
		1,000	0,00025	0,285	3,3E+02		
		2,000	0,00025	0,447	2,6E+02	1	1
		4,000	0,00025	0,836	2,4E+02		
	E115G	3,500	1,00000	0,563	4,7E-02	1,81E-04	1/5500
	E115S	3,500	1,00000	0,114	9,5E-03	3,65E-05	< 1/27000
	E120A	3,500	1,00000	-0,015	-1,2E-06	-4,62E-09	No activity
NaN_3	Wt	0,333	0,00025	0,132	4,6E+02		
		1,000	0,00025	0,287	3,4E+02		
		2,000	0,00025	0,520	3,0E+02	1	1
		4,000	0,00025	0,892	2,6E+02		
	E115G	3,500	1,00000	0,005	4,3E-04	1,43E-06	< 1/700000
	E115S	3,500	1,00000	0,034	2,8E-03	9,33E-06	< 1/110000
	E120A	3,500	1,00000	0,015	1,2E-03	4,00E-06	< 1/250000

3.2.2 Influence of acetate and formiate concentration

The mutant E115G was incubated with laminarin in the presence of acetate or formiate, whereas the E115S mutant was incubated only in the presence of formiate. Since, the hydrolysis with E115S in presence of formiate is a rather slow process, as seen in the table above, a higher concentration was used of the this mutant and a lower concentration of the E115G mutant. The buffer concentrations used were; 10, 40, 200, and 450 mM, and the PHBAH reducing sugar assay was used as detection method. The relative activity estimated for the acetate concentration dependence is presented in **Table 3**, and the formiate concentration dependence in **Table 4**. It can clearly be seen that the relative activity increases with concentration of external nucleophile, for both of the mutants. For the E115G mutant, acetate and formiate seem to be equally efficient, and the activity approaches a plateau at the highest concentrations. The E115S mutant shows lower activity and needs higher concentration of formiate.

Table 3. The relative activity for the E115G mutant in presence of acetate in different concentrations; 10, 40, 200 and 450 mM in comparison to the wildtype Lam16A.

[Acetate] (mM)	Enzyme	Time (h)	[Enzyme] (uM)	Δ Abs 410 nm	dP/dt/[E] (s^{-1})	Relative activity	
0,1	Wild type	0,3	0,00025	0,044	1,53E+02		
0,1		1	0,00025	0,194	2,26E+02	1	1
0,1		2	0,00025	0,587	3,43E+02		
0,1		4	0,00025	0,727	2,12E+02		
10	E115G	1	0,20000	0,091	1,33E-01	5,88E-04	1/1700
40		1	0,20000	0,183	2,67E-01	1,18E-03	1/850
200		1	0,20000	0,348	5,09E-01	2,25E-03	1/450
450		1	0,20000	0,473	6,91E-01	3,05E-03	1/350

In **Appendix 3**, a predicted position for acetate in the active site of a predicted structure of Lam16A E115G is shown. This picture was created using the program PyMOL and the wildtype structure in complex with the product P3 (4-*O*-β-glycosyl laminaribioside), which is one of the major products from hydrolysis of the substrate lichenin by the wildtype enzyme (PDB-code 2W39; Vasur *et al.*, 2009). Glutamate 115 was replaced with a glycine residue *in silico*, while keeping the electron density for the E115 residue. An acetate ion was positioned in the electron density for the carboxylate group of E115 to show how the acetate ion may fit snugly into the empty space left by the glutamate. When mutating Glu 115 to a serine (data not shown) there is not space enough for the acetate to take the position and act as an external nucleophile. However, the smaller formiate ion (HCOOH vs. CH_3COOH) can in a better way fit into this space left from the glutamate, even though the rather big serine residue is filling up some of the space. This is not the case with the glycine mutant which is probably the reason why this mutant has higher relative activity than the E115S mutant.

Table 4. Estimated relative activity for the E115G and E115S mutants in presence of different formiate concentrations. It can be seen that an increase in formiate concentration increases the relative activity on both the mutants, although more with E115G than with E115S.

[$CHNaO_2$] (mM)	Enzyme	Time (h)	[E] (μM)	△A 410 nm	dP/dt/[E] (s^{-1})	Relative activity	
0,1 M	Wt	0,33	0,00025	0,169	6,27E+02		
		1	0,00025	0,130	1,61E+02		
		2	0,00025	0,342	2,12E+02	1	1
		4	0,00025	0,465	1,44E+02		
10	E115G	1	0,20000	0,140	2,17E-01	1,02E-03	(> 1/1000)
		6	0,20000	0,070	1,82E-02		
40		1	0,20000	0,193	2,98E-01	1,41E-03	1/800
		6	0,20000	0,232	5,98E-02		
200		1	0,20000	0,336	5,19E-01	2,45E-03	1/400
		6	0,20000	0,502	1,29E-01		
450		1	0,20000	0,404	6,25E-01	2,95E-03	1/350
		6	0,20000	0,586	1,51E-01		
10	E115S	1	2,00000	0,006	9,28E-04		
		6	2,00000	0,052	1,34E-03	6,32E-06	< 1/160000
40		1	2,00000	0,041	6,33E-03		
		6	2,00000	0,152	3,93E-03	1,85E-05	1/54000
200		1	2,00000	0,168	2,60E-02	1,23E-04	1/8000
		6	2,00000	0,436	1,12E-02		
450		1	2,00000	0,253	3,91E-02	1,84E-04	1/5500
		6	2,00000	0,529	1,36E-02		

With these results at hand it may appear that it was unnecessary to express and purify another batch of the E115G mutant, since the reason for the unexpectedly high activity of this mutant in previous experiments seems to be that the enzyme was stored in acetate buffer, which was therefore constantly present in activity measurements. In principal it could have been sufficient to remove the acetate by desalting the previous enzyme preparation. When this was realized, the previous batch was taken for desalting, but for unknown reasons the protein was lost in the desalting experiment. Thus, we were lucky that the new batch had been made. Furthermore, by repeating the preparation of the mutant, the possibility for contamination by wildtype enzyme could be effectively ruled out.

3.3 Hydrolysis of laminarin with Lam16A wt and E115G mutant in presence of acetate

The hydrolysis pattern of laminarin was investigated by incubating laminarin with the wildtype enzyme and the E115G mutant in presence of acetate, respectively. The experiment turned out to be a bit more complicated than expected, and three different columns were tested. First HPAE-PAD was

tried with a PA10 column on the Dionex system, but the high acetate concentrations needed for the mutant enzyme seriously affected the separation, whereupon this column was rejected. The second column tested was the C18 column on the Agilent system, but also here the acetate disturbed the outcome since the huge acetate peak co-eluted with glucose and laminaribiose. Fortunately, the third column, Dionex PA200, gave good results even though many of the peaks were unidentifiable to the standards used, see **Figure 10** for the wildtype and **Figure 11** for the E115G mutant. By comparing both chromatograms, no big difference in product formation can be observed, though more laminarin was consumed by the E115G mutant and taller product peaks were formed. The reason may be that the mutant enzyme was present in a higher concentration (4000 fold) than the wildtype enzyme, which on the other hand is 5000 times more efficient than the mutant according to earlier experiments. Another result that can be seen by this hydrolysis experiment is that Lam16A attacks laminarin internally rather than from the ends of the oligosaccharide, this means that Lam16A is an endoglucanase.

At this stage it is difficult to identify the different peaks that are formed. Firstly because a multitude of potential products may be formed, through cleavage and/or transglycosylation, linear, cyclic and branched, as transient intermediates or more resistant end-products, while the available standards were limited to linear β-1,3-oligosaccharides L2-L7 and a single mixed-link sugar, 6-*O*-β-glycosyl laminaritrioside, also referred to as P4. Secondly, the elution times seem to have shifted somewhat between some chromatograms, in particular with the wildtype samples. Anyway, peaks were observed with similar interspacing, and in the case of E115G at the same positions, as the L2-L7 standards, indicating that all these products may have formed, or at least products with similar size-distribution. One may also imagine an extension of the elution pattern to longer oligosaccharides such as laminarioctaose, -nonaose etc.

One interesting aspect is that no peak was observed for 6-*O*-β-glycosyl laminaritrioside (P4), although it is considered to be a prominent end-product in the hydrolysis of laminarin by wildtype Lam16A (Kawai *et al.*, 2006; Vasur *et al.*, 2009). A closer comparison between the wildtype and the mutant reveals that the mutant gives even more peaks than the wildtype. This may be because when using acetate as an external nucleophile, it probably forms an intermediate where the acetate is covalently bound to the remaining sugar. This acetylated sugar may dissociate from the active site of the enzyme and is expected to elute at a different position in the chromatogram than the corresponding non-acetylated oligosaccharide. There are peaks that elute a little bit later than the β-1,3-standards, which may correspond the acetylated sugars. For instance L3 elutes after four minutes, while there is a smaller peak that elutes after 4.5 minutes that may correspond to the acetylated sugar. However, to be certain the products should be collected and further analysed for identification, e.g. by mass spectrometry. Moreover, laminarin is a β-1,3-glucan with β-1,6-branches, and just as P4 (Glu-6Glu-3Glu-3Glu-OH) separates from L4, other branched products are likely to eluted differently than non-branched counterparts.

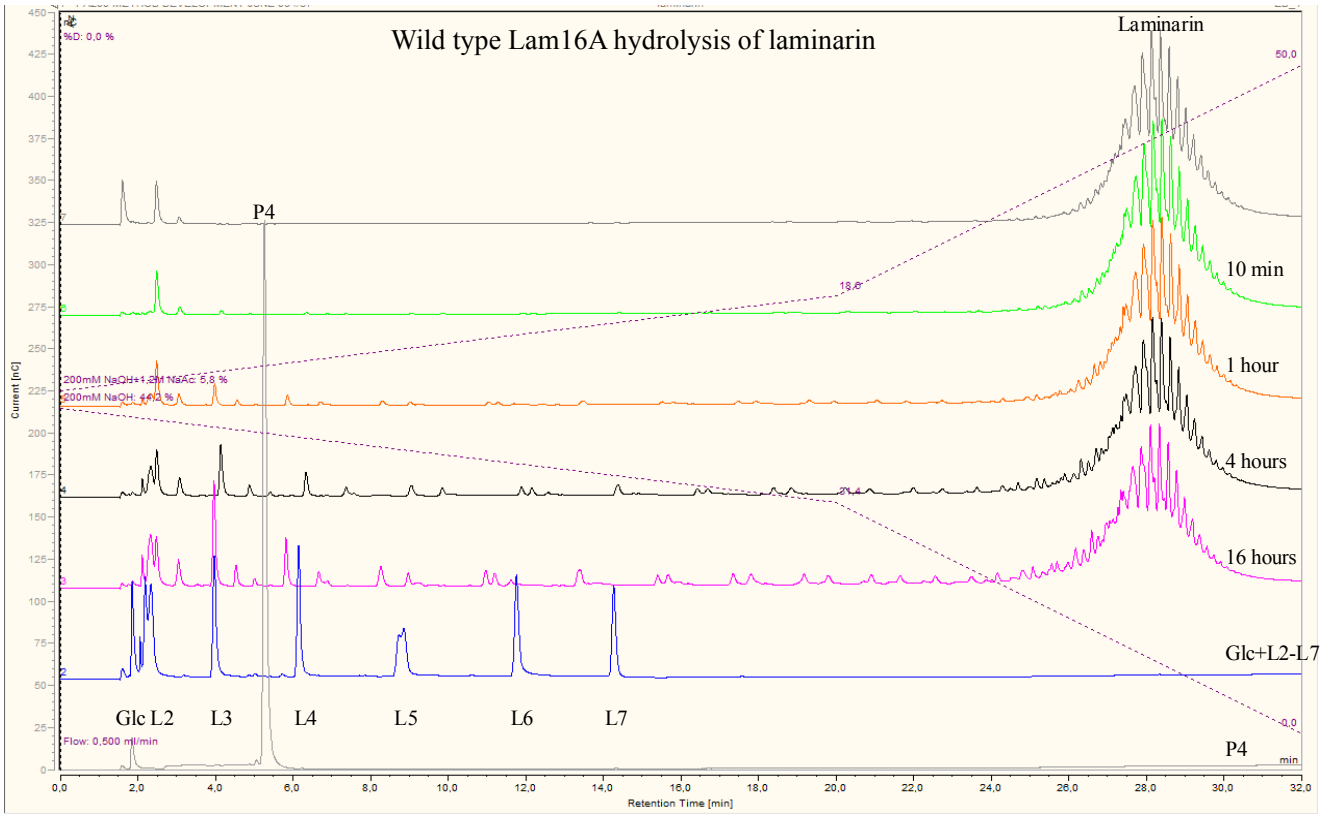

Figure 10. Wildtype Lam16A hydrolysis of laminarin at the time points of, 10 minutes, 1 hour, 4 hours, and 16 hours, analysed by HPAE-PAD on the Dionex system. Standards used for comparison are P4, Glc+L2-L7, and laminarin. 20 µl samples were separated on a PA200 column and eluted compounds were monitored on a pulsed amperometric detector.

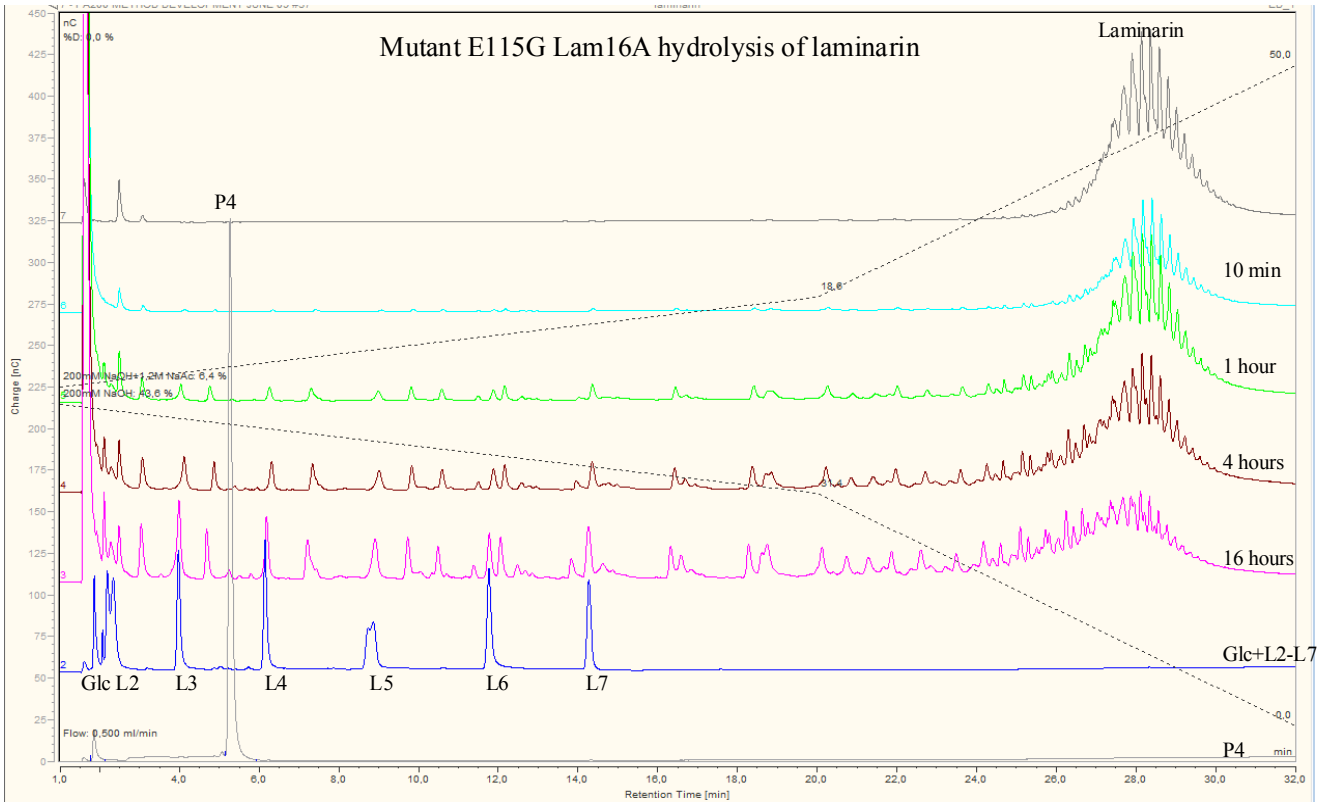

Figure 11. Mutant E115G Lam16A hydrolysis of laminarin, analysed as in Figure 10 above.

3.4 Glycosynthase experiment with Lam16A E115G mutant and αL7F

In previous studies of potential glycosynthase activity of the E115G mutant, the results were not conclusive. Since acetate was present, those experiments resulted in hydrolysis of the laminariheptaose α-fluoride derivate, see **Figure 12**. After 10 seconds of incubation a new peak started to appear at around 18 minutes elution time, which corresponds to the elution of the circular laminariheptaose oligosaccharide produced by the E115S mutant. The peak area corresponding to the circular structure increased after 4 hours of incubation. However, after 24 hours, hydrolysis had taken over and the circular oligosaccharide was hydrolysed and after 48 hours, the αL7F and L7 were completely consumed and the main hydrolysis products formed were L3 and L4.

In the present study, the same procedure was repeated with the differences that the protein was present in citrate buffer after using the DG10 desalting column, and the reaction was taken place in sodium phosphate buffer pH 6.0. The result shows that the mutant E115G, like the other nucleophile mutant E115S, has glycosynthase activity, and that the circular L7 (cL7) structure is formed, see **Figure 13**. However, the E115G mutant seems to have lower glycosynthase activity rate, since in previous studies with the E115S mutant (data not shown) all αL7F was consumed, whereas all L7 was still remaining. In this study, it seems like also L7 decreased with time, which probably is due to differences in injection volumes for the different HPLC runs, and since the experiment was not lasting for 48 hours as with the E115S mutant, conclusive comparisons between the two mutants can not be made. To do this, integration of the peak areas must be carried out to be able to measure the amount of circular L7 that is produced and preferably the auto-sampler of the HPLC should be used since the injection volume is expected to be more reproducible than with manual injection.

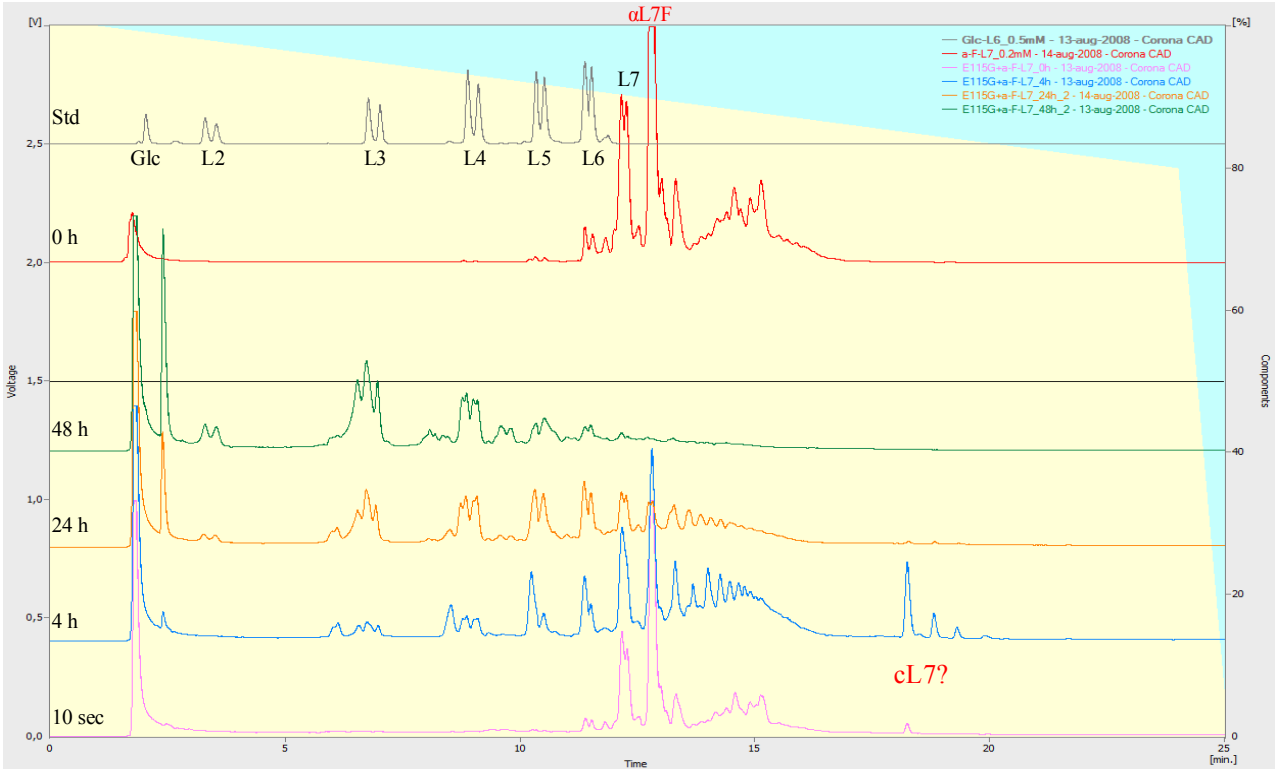

Figure 12. Previous result from glycosynthase activity with Lam16A E115G mutant with acetate present. It can be seen that directly after 10 seconds the circular L7 structure is formed and elutes after approximately 18 minutes and the peak area increases after 4 hours. After 24 and 48 hours hydrolysis has taken over and all αL7F and L7 is consumed to the main products L3 and L4, though the other laminarioligosaccharides; L2, L4, L5 and L6 are also produced.

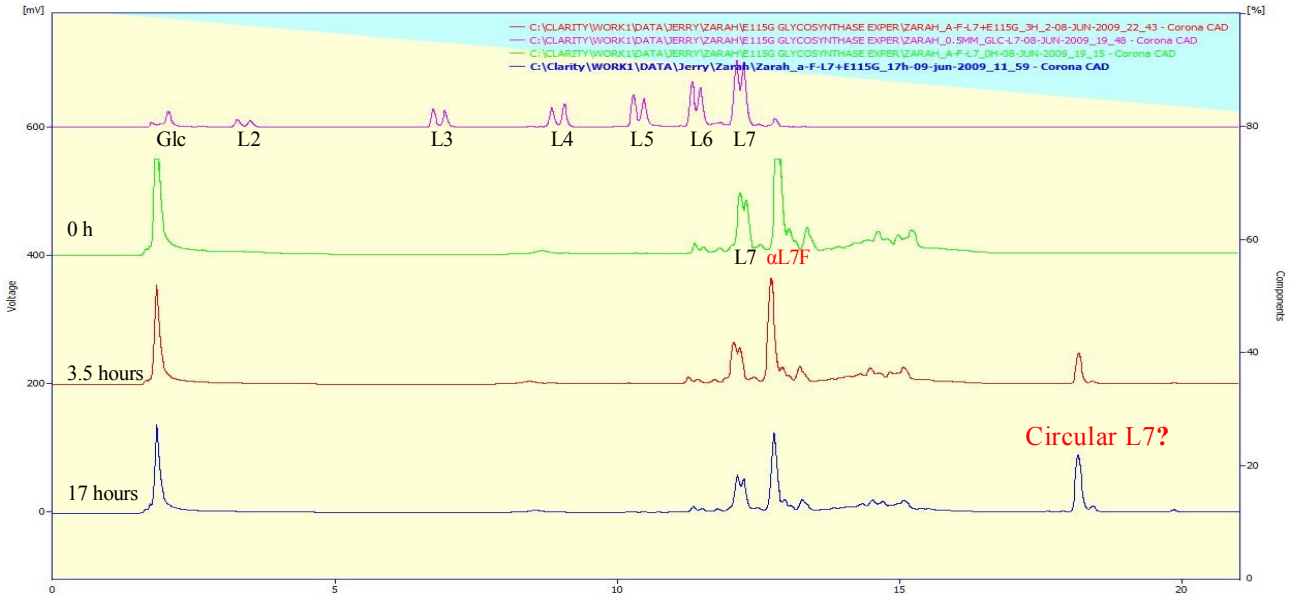

Figure 13. New glycosynthase experiment with Lam16A E115G mutant in absence of acetate. From this chromatogram it can be seen that no smaller laminarioligosaccharides are produced which indicates that no hydrolysis is taking place.

3.5 Hydrolysis of circular β-glucans from *M. amorphae* and *A. caulinodans*

From two species of symbiotic nitrogen fixing bacteria, *Mesorhizium amorphae* and *Azorhizobium caulinodans*, that live in root nodules in certain leguminose plants, circular β-glucans were received from Dr. Adam Choma, Lublin, Poland. The first mentioned glucan, from *M. amorphae*, is built up of glucose residues linked by β-1,2 glycosidic bonds (A. Choma, personal communication), which is not substrate specific for Lam16A, whereas the other from *A. caulinodans* has β-1,3/1,6 glycosidic linkages (Komaniecka *et al.*, 2003 and McIntosh *et al.*, 2004). For that reason it was of interest to see if the wildtype Lam16A could hydrolyse such β-glucans, and also if there were any difference in comparison to the mutant E115G. Thus, the two enzymes were incubated with those circular β-glucans for 20 minutes and over night, and for the β-1,3/1,6-glucan for 48 hours as well. The reactions were analysed on the Agilent 1100 HPLC system, see **Figure 14** for the *M. amorphae* β-1,2-glucan and **Figure 15** for the *A. caulinodans* β-1,3/1,6-glucan. As expected, the wildtype enzyme did not show any effect on the β-1,2-glucans and if there is any difference for the E115G mutant is not known, since the reaction got lost in the HPLC. With the *A. caulinodans* β-1,3/1,6-glucan some changes were obtained with the wildtype enzyme, because minor peaks that elute later are completely consumed by the enzyme and new peaks appear as earlier eluents in the chromatogram. It is impossible at this stage to say which products that are formed from this hydrolysis, although it can be said for sure that the wildtype enzyme has hydrolytic effect on minor components of this circular *A. caulinodans* β-1,3/1,6-glucan. However, the mutant E115G, shows no indication to hydrolyse this oligosaccharide after 24 hours incubation even though the enzyme concentration was much higher than the wildtype enzyme concentration.

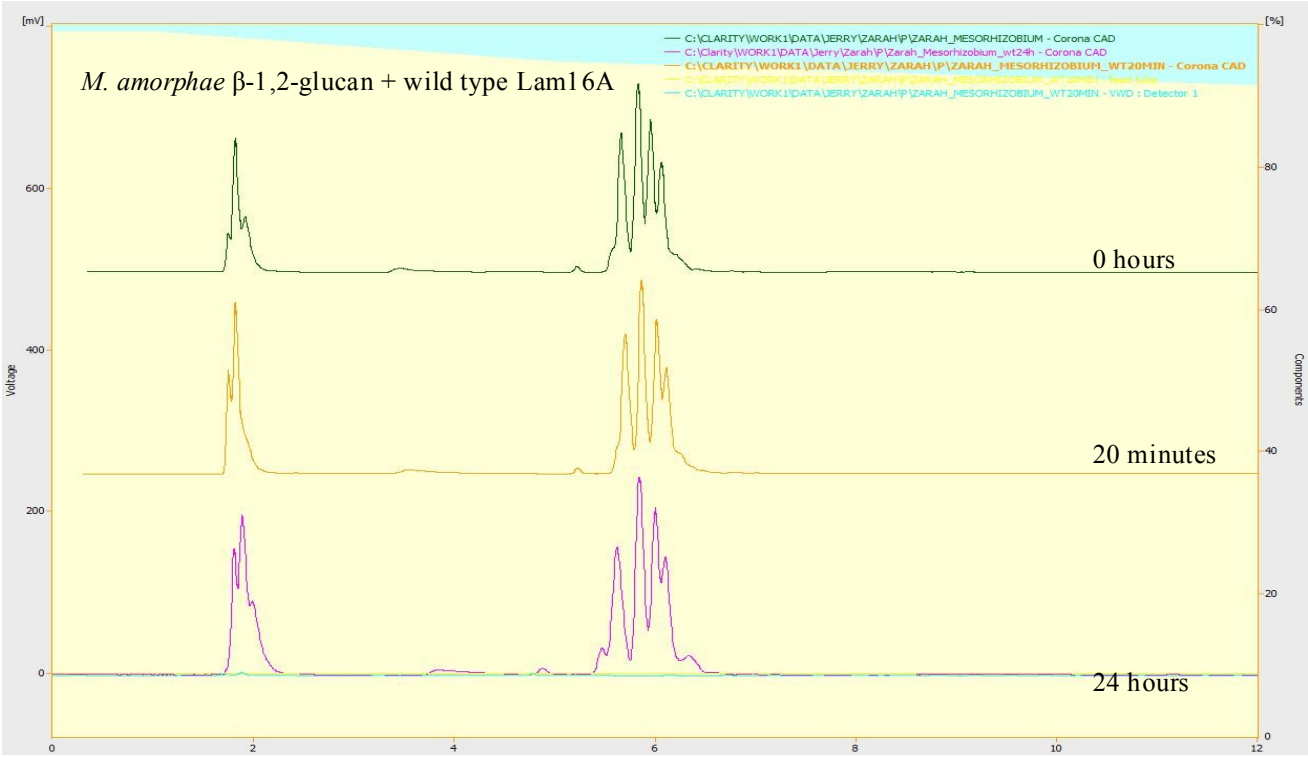

Figure 14. Circular β-1,2-glucan from *M. amorphae* incubated with wildtype Lam16A for 20 minutes and 24 hours. The enzyme shows to have no hydrolytic activity for this kind of substrate. Unfortunately, when incubating with the mutant E115G, the sample got lost in the HPLC.

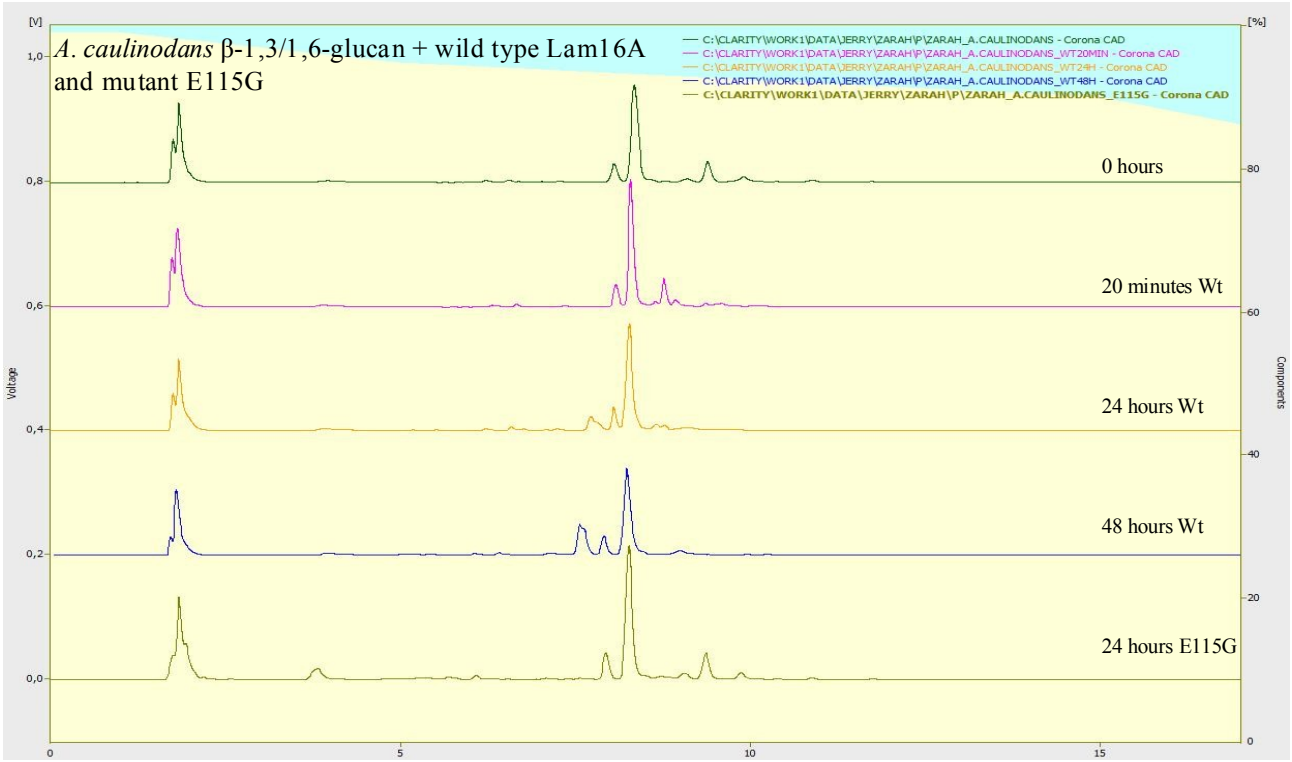

Figure 15. *A. caulinodans* circular β-1,3/1,6-glucan incubated with the wildtype enzyme at the different time points of 20 min, 24 hours and 48 hours, and the Lam16A E115G mutant for 24 hours. The wildtype enzyme hydrolysis the later peak that elutes after approximately 9 minutes and a new peak elutes at approximately 7.5 minutes. For the mutant though, no hydrolytic activity is observed.

3.6 Crystallization of E115G and E115S nucleophile mutants

A crystallization experiment was set up with the two nucleophile mutants, E115G and E115S using the hanging drop vapour diffusion method and citrate as buffer. Every day for one week the wells were inspected under a microscope to obtain potential formations of crystals or precipitation. However, no crystals were found at all even though seeding with earlier set ups of crystals was taken place twice. All the mutants E115G, E115S and E120A as well as wildtype Lam16A have been successfully crystallised previously in similar conditions, but with 10 mM sodium acetate as buffer. The acetate was replaced with citrate in the present set up to avoid hydrolysis when using the E115G mutant and this might be the reason why no crystals were formed.

4. Future prospects

At this point, after all those findings, there are many interesting follow up experiments that can be performed in order to get more knowledge about the Lam16A enzyme. It would be interesting to see if there are even more and stronger external nucleophiles that can make the enzymes even more efficient than acetate and formiate. For instance, propionate (CH_3CH_2COOH) that is more similar to glutamate in structure, or hydroxylamin that is known to be a really strong nucleophile.

By the new awareness of external nucleophiles, it would also be of interest to take a closer look at the hydrolysis patterns of laminarin or other polymeric substrates such as curdlan and analyze the eluted compounds by mass spectroscopy, and also to analyse if any circular products are produced by transglycosylation. Just because Lam16A was discovered when it was grown on laminarin, may not mean that this is the main substrate, and therefore it is of interest to see if it is more efficient on other substrates.

It would also be of interest to examine more in detail the rates of the glycosynthase reaction and compare it between the two nucleophile mutants, although, in order to do that the present α-fluoride laminarioligosaccharide derivates must be isolated since the samples contained other compounds such as L2 and L7 which may be a result of spontaneous decomposition. In addition, by using the nucleophile mutants, maybe the glycosynthase reaction could be driven by adding a high concentration of fluoride ions to the reaction. In that case there will be no need for α-fluoride oligosaccharide derivates.

For the acid/base catalytic deficient mutant E120A it would be of interest to see if it has thioglycoligase activity. So far, there has not been any substrate available at the lab to test this, however, maybe it can be performed by using β-mercaptoethanol as an acceptor substrate.

As mentioned in section 3.3 regarding the hydrolysis of laminarin, P4 should be one of the main components produced in this reaction. P4 is a Glu6-Glu3-Glu3-Glu oligosaccharide and by producing and purifying a large amount of this compound and thereafter synthesize an α-fluoride derivate of it and utilize it in the glycosynthase reactions with the nucleophile mutants, we hope to be able to see formation of a nine residue long circular β-1,3-oligosaccharide with β-1,6-branches on every third glucose residue in the ring structure that might have interesting properties.

5. REFERENCES

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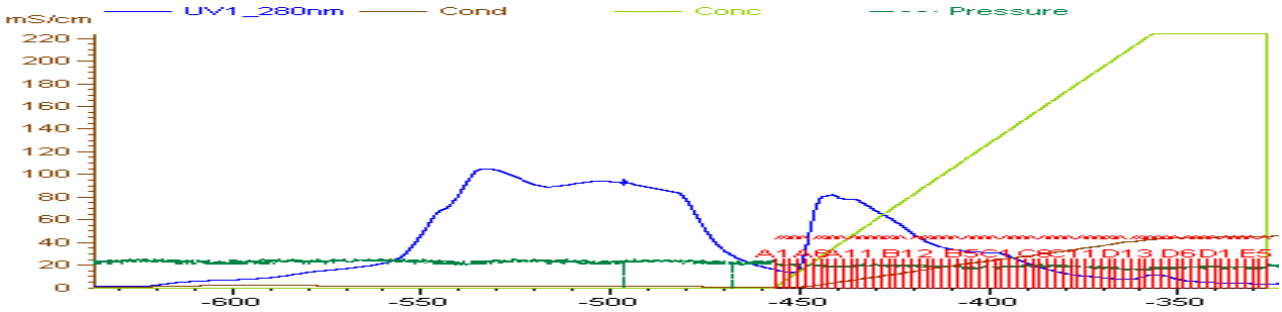
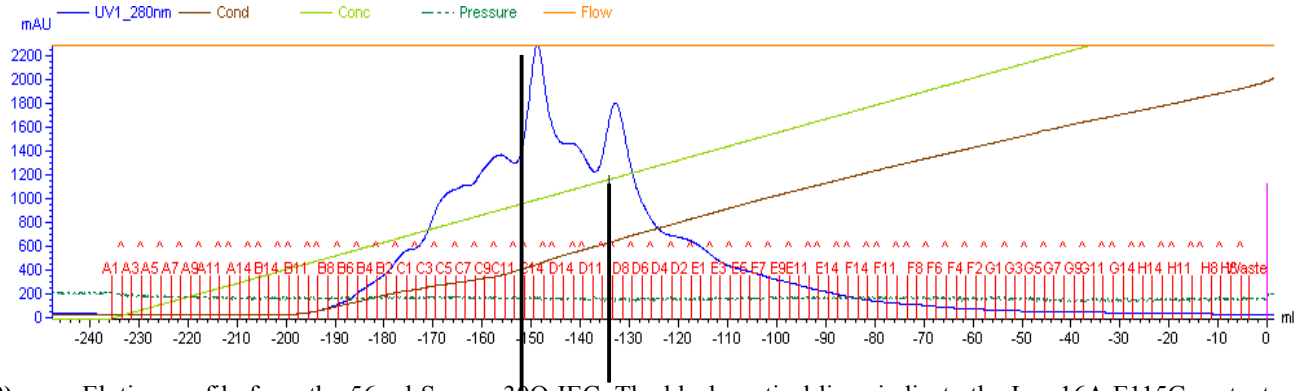
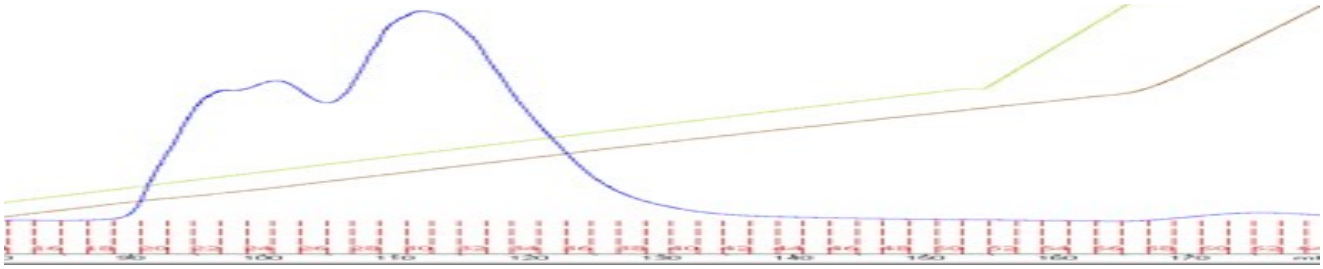
Appendix 1 – Chromatograms from purification



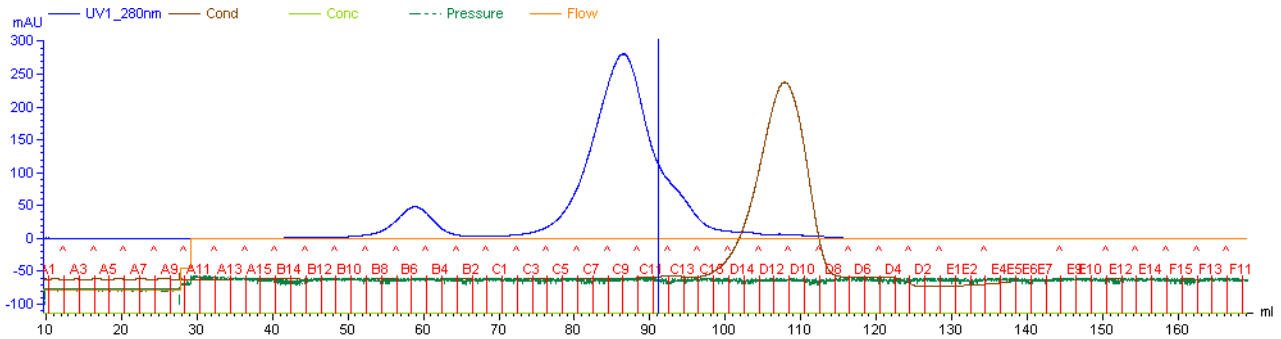
A) Chromatogram from the second HiScreen Capto Q IEC purification which appeared to not have the capacity to bind the Lam16A E115G mutant at the current conditions used.



B) Elution profile from the 56 ml Source 30Q IEC. The black vertical lines indicate the Lam16A E115G mutant containing peaks.



C) Chromatogram from the 10 ml Source 30Q IEC with a more adapted gradient, where the later eluted peak contains the Lam16A E115G mutant enzyme.

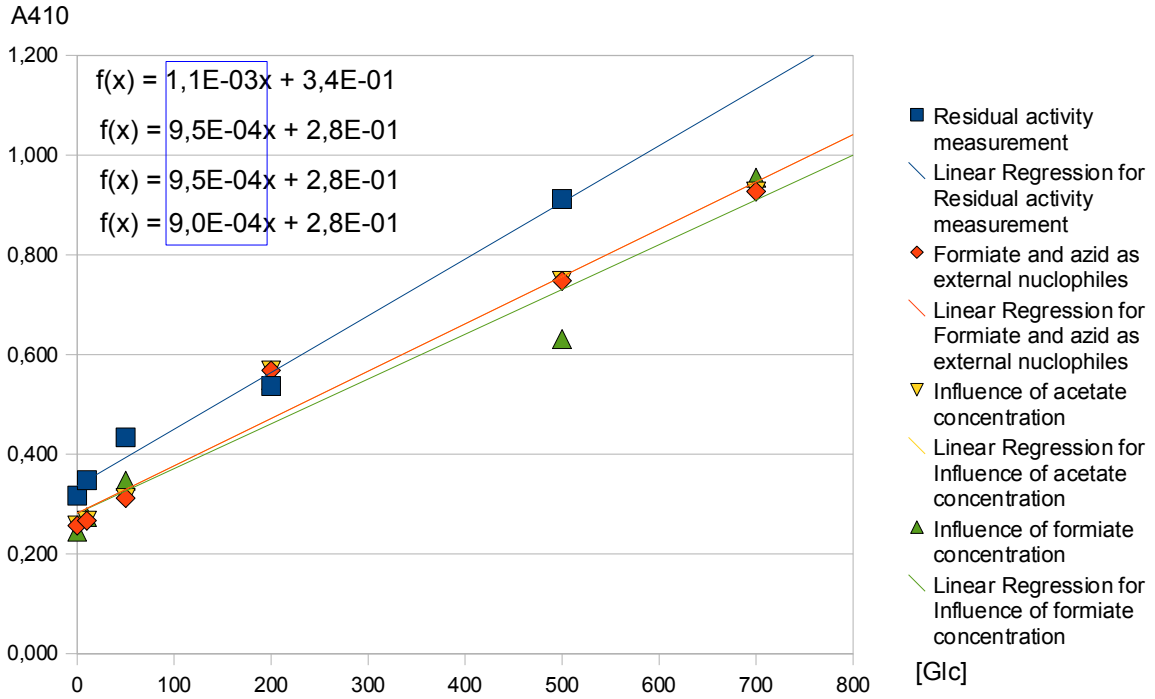


D) Elution profile from the Superdex 200 SEC column, where the bigger contaminating protein first is eluted and the large, second eluted peak with a right sided shoulder corresponds to the Lam16A E115G mutant.

Appendix 2 – Glucose standard curves

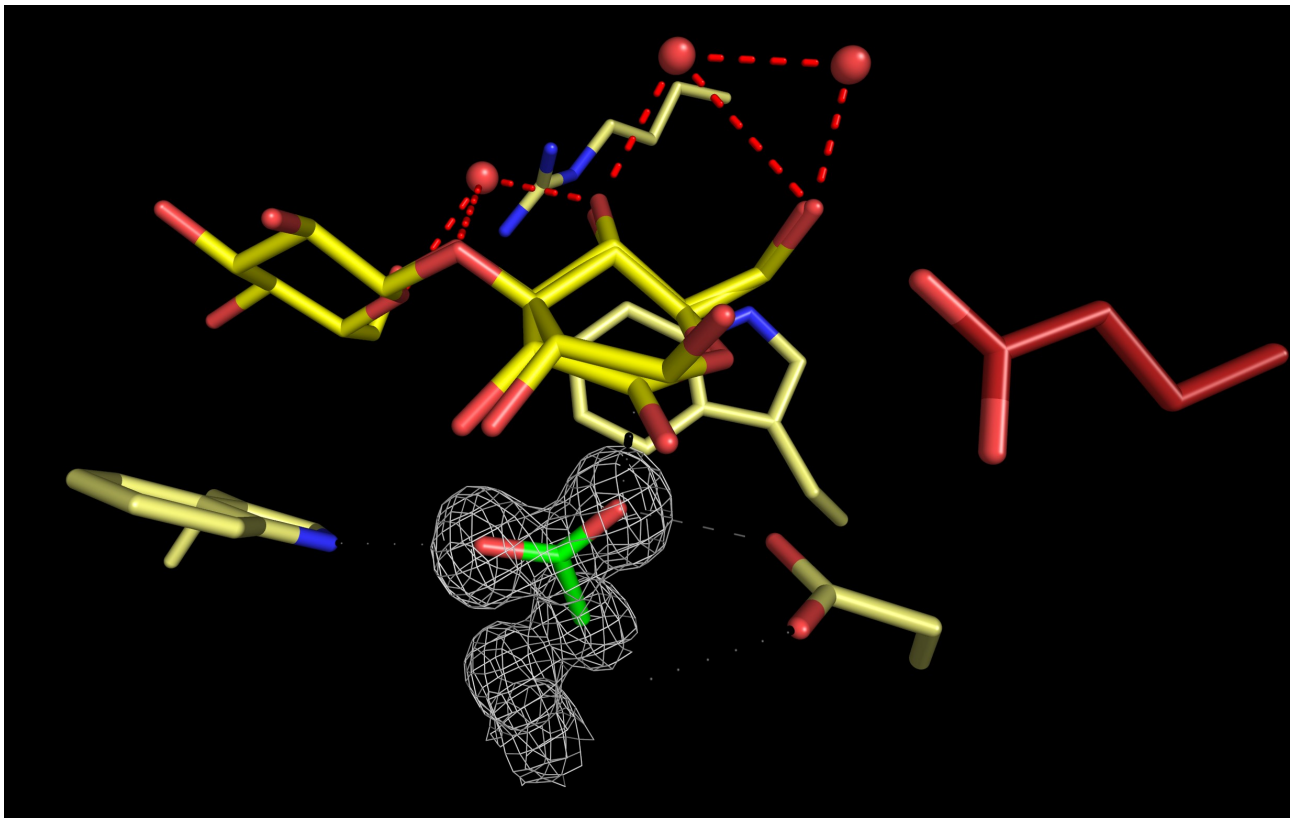
Table 5. Measured absorbances for the glucose standards in the PHBAH assay used during the residual activity measurement; ■ investigation of formiate and azide as potential nucleophiles, ■ influence of acetate, ▼ and formiate, and ■ concentration contributing to hydrolytic activity of E115G and E115S.

	Absorbance at 410 nm			
µM Glc	■	■	▼	■
0	0,317	0,257	0,257	0,245
10	0,348	0,267	0,267	0,274
50	0,434	0,312	0,312	0,347
200	0,537	0,568	0,568	0,549
500	0,912	0,748	0,748	0,631
700	No std used	0,927	0,927	0,955



Glucose standard curves with PHBAH reagent in the different assay performed. The slopes, marked in the blue box, are measured in absorbance units per µM Glc (AU/ µM Glc) which are used to calculate Δ glucose equivalents for the different measurements.

Appendix 3 – Predicted position of acetate as external nucleophile



Predicted position of acetate in the electron density of glutamate 115 from the wildtype Lam16A in complex with the P3 (4-*O*-β-glycosyl laminaribiose) substrate. This picture was created using the program PyMOL and the wildtype structure in complex with the substrate P3, which is the main wt hydrolysis product, produced from the substrate lichenin (PDB-code 2W39; Vasur *et al.*, 2009). Glutamate 115 was replaced with a glycine residue *in silico*, while keeping the electron density for the glutamate.