Structure-Function Study on the Activation Mechanism of Endo T

Crystallization and biochemical characterization of the intact Endo T from Trichoderma reesei (Hypocrea jecorina)

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List of abbreviations

AP ................................................................................................................................................... acid protease
Endo T ............................................................................................................................................. endo-N-acetyl-β-D-glucosaminidase from T.reesei.
ENGase .............................................................................................................................................. endo-N-acetyl-β-D-glucosaminidase
GH ................................................................................................................................................... Glycoside hydrolase family
Man .................................................................................................................................................... mannose
NAG ................................................................................................................................................... N-acetyl-β-D-glucosamine
PDB .................................................................................................................................................. Protein Data Bank
SP .................................................................................................................................................... serine protease
Abstract

Endo T is a deglycosylating enzyme secreted by the filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*). The active Endo T protein is both deglycosylated and C-terminal processed. Since the intact protein was never observed in the *T. reesei* culture medium, a construct of the intact Endo T was expressed in *P. pastoris*. Here, the glycosylated, intact and inactive protein was found in the medium but this form is slowly converted to the deglycosylated and proteolytic cleaved form. The structure of the active Endo T was already solved in previous work. We tried to solve the structure of the intact inactive Endo T to compare it with the active structure. Several structures of Endo T were solved, but none of them were structures of the intact Endo T. Different pH and T parameters were screened to find a condition where the C-terminal peptide was stable and the enzyme kept intact. A protease inhibitor cocktail was also added to limit the proteolytic process. We were able to control the proteolysis with the help of the protease inhibitor cocktail at pH 5 and higher, but we were not able to limit the proteolysis at pH 3, nor in any of the crystallization samples.

Keywords

Endo T, glycoside hydrolase family 18, EC 3.2.1.96, deglycosylation, proteolysis, C-terminus, protein structure.
1 Introduction

Endo T is an endo-N-acetyl-β-D-glucosaminidase (ENGase) secreted by the filamentous fungus *Trichoderma reesei*. It is an enzyme that hydrolyzes the β1–4 bond between two acetyl glucosamine residues in high-mannose N-glycans. Glycosylation is an important post-translational modification involved in protein folding mechanisms and important for protein stability. ENGases are present in eukaryotes as part of the ERAD degradation pathway of misfolded proteins. Especially with prokaryotes ENGases are often involved in parasitism (Karamanos 1997). Endo T from *T. reesei* is responsible for the extensive deglycosylation observed for its cellulases and other secreted proteins, but its biological role is still unclear.

Endo T has been expressed both homologously in *Trichoderma reesei* and heterologously in *Pichia pastoris*. The *P. pastoris* expressed protein is secreted as an intact protein, fully glycosylated but inactive form, while the *T. reesei* expressed protein has lost 9 and 49 amino acids at its N- and C-terminus respectively and contains single N-acetyl glucosamine attached to Asn 70 and Asn 240 of the protein. This lower molecular weight enzyme form is catalytically active and hydrolyzes the β1-4 bond in the chitobiose core of high mannose N-glycans. These can be bound on a glycoprotein or a glycopeptide, but the enzyme also acts on free N-glycans.

Endo T has EC classification 3.2.1.96 and is classified as a glycoside hydrolase (GH) family 18 enzyme. GH18 enzymes are retaining glycoside hydrolases using neighboring group participation with Asp and Glu as the catalytic residues. These two catalytic residues are conserved throughout the entire GH 18 family.

Previously the structure of the active Endo T expressed in *T. reesei*, both with and without ligand has been solved to a resolution of 1.3 Å. The aim for this master project was to solve the structure of the intact and presumably inactive form of Endo T. This new structural knowledge about Endo T will help us to better understand how and why Endo T is activated. Solving the structure of the intact Endo T might be problematic. First off all it is possible that the C-terminal peptide and/or the N-glycans are too flexible to be visible in the electron density map for the protein. The second problem, originates from previous observations that the protein is very hard to keep intact over an extended period of time. The proteolytic cleavage of Endo T is difficult to prevent even with a purified protein sample. From the moment a little amount of active protein is formed, the deglycosylation process starts to proceed very fast. The reason why the C-terminus of the protein is removed so fast is not clear. The fast deglycosylation is due to Endo T that is deglycosylating itself.

2 Literature

2.1 Expression organisms

2.1.1 *Trichoderma reesei*

*Trichoderma reesei* is a mesophile filamentous fungus from the division of the ascomycota. The subdivision is pezizomycotina with sordariomycetes as class and hypocreaceae as family. Trichoderma is the genus name whereas *T. reesei* is the species name. *T. reesei* is the species name. *T. reesei* is an anamorphous or asexual reproduction form of *Hypocrea jecorina* (Taylor, Spatafora and Berbee 2006, Harman n.d.).

*T. reesei* is known to produce high amounts of cellulose-degrading enzymes. This is why modified strains of this fungus are commercially used for production of cellulases and other enzymes that degrade complex plant cell wall polysaccharides (Harman n.d., Sharma, et al. 2009). *T. reesei* is also of interest for its high production of extracellular heterologous proteins and is often used as a host organism (Maras, et al. 1997, Dienes, et al. 2007, Sharma, et al. 2009).
The *T. reesei* (hemi)-cellulases are partially of interest for 2nd generation bio-ethanol production. Instead of using starch or sucrose for the production of bio-ethanol it is possible now to use cellulose rich plant material as feedstock for the production of bio-ethanol (European biofuels TECHNOLOGY PLATFORM n.d.). Proteolytic degradation is a known cause of cellulase degradation, as reported by several groups (Dienes, et al. 2007, Hagspiel, Haab and Kubicek 1989).

### 2.1.2 Pichia pastoris

*Pichia pastoris* is a methylotrophic yeast from the division of the ascomycota. The subdivision is saccharomycetes with sacharomycetales as class and Saccharomyctaceae as family. *Pichia* is the genus name whereas *P. pastoris* is the species name (Taylor, Spatafora and Berbee 2006).

*P. pastoris* is commonly used as an eukaryotic expression host in research for heterologous protein expression due to different advantages. *P. pastoris* grows on an inexpensive and simple mineral medium. It is capable of generating post-translational modifications such as N- and O-glycosylation and disulfide bonds (Cregg, et al. 2000). It has a strong, inducible promoter and secretes few endogenous proteins, which makes the downstream processing of heterologous secreted proteins much easier (Dosanjh 1996, Cereghino and Cregg 2000, Cregg, et al. 2000).

In most researches where *P. pastoris* or other yeast organisms are used as expression organism, proteolytic degradation has been observed (Cregg, et al. 2000, Sinha, et al. 2005).

### 2.2 Protease

#### 2.2.1 Proteases: an introduction

Proteases, which have EC-classification 3.4, hydrolyze the peptide bond. Differentiation of the subclass are documented, there are two sets of subclass peptidases, the exopeptidases (EC 3.4.11-19 and the endopeptidases (EC 3.4.21-24 and EC 3.4.99). The exopeptidases act only near the ends of polypeptide chains, while the endopeptidases act in-between the polypeptide chains. Both sub-subclasses can be differentiated on the basis of catalytic mechanism or substrate specificity. Table 1 gives an overview of different proteases and their classification by mechanism; the substrate specificity is not shown (Moss, E.C. 3.4 acting on peptide bonds n.d.)

Trypsin and chymotrypsin are serine endopeptidases, Trypsin is synthesized as trypsinogen, the zymogen from trypsin, is the inactive protein that is initially transcribed. It is important for a protease to be inactive after synthesis. This way it can only act on the peptide bond where necessary and won’t digest the tissues where it’s synthesized (McDowall sd).

<table>
<thead>
<tr>
<th>Subclasses</th>
<th>Mechanism</th>
<th>Example</th>
<th>EC-classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>carboxypeptidase</td>
<td>amino</td>
<td>aminopeptidase I</td>
<td>3.4.11.22</td>
</tr>
<tr>
<td></td>
<td>serine</td>
<td>carboxypeptidase C</td>
<td>3.4.16.5</td>
</tr>
<tr>
<td></td>
<td>cysteine</td>
<td>cathepsin X</td>
<td>3.4.18.1</td>
</tr>
<tr>
<td></td>
<td>metallo</td>
<td>carboxypeptidase A</td>
<td>3.4.17.1</td>
</tr>
<tr>
<td>endopeptidase</td>
<td>threonine</td>
<td>HsI-HsIV peptidase</td>
<td>3.4.25.2</td>
</tr>
<tr>
<td></td>
<td>serine</td>
<td>trypsin/ chymotrypsin</td>
<td>3.4.21.1</td>
</tr>
<tr>
<td></td>
<td>cysteine</td>
<td>cathepsin B</td>
<td>3.4.22.1</td>
</tr>
<tr>
<td></td>
<td>metallo</td>
<td>coccolysin</td>
<td>3.4.24.30</td>
</tr>
</tbody>
</table>
Trypsinogen activation is autocatalytic, once a small fraction of trypsinogen is activated, with the help of enteropeptidase to trypsin, trypsin will activate the rest of the trypsinogen (McDowall sd). Trypsin and chymotrypsin are synthesized in the pancreas, where most of the secreting proteins are synthesized (McDowall sd). Chymotrypsin is activated by trypsin-catalyzed cleavage (Voet, Voet and Pratt 2006, Berg, Tymoczko and Stryer 2006).

2.2.2 Protease activity in T. reesei

Proteolytic degradation of T. reesei proteins has been described in several researches. Hagspiel et al. detected proteolytic degradation of Cel7A, Cel7B and an α-galactosidase, which were produced by the modified T. reesei strainQM9414 (Hagspiel, Haab and Kubicek 1989).

Eneyskaya et al. showed the presence acid protease activity in T. reesei medium on α-galactosidase, β-glycosidase, Cel7A, Cel7B and with a fluorogenic peptide, but the protease itself was not identified (Eneyskaya, et al. 1999). Selinheimo et al. observed a C-terminal processed tyrosinase in T. reesei (Selinheimo, et al. 2006). In a more recent study by Stals et al. a C-terminal processed endo-N-acetyl-β-D-glucosaminidase, secreted by T. reesei was also detected (Stals, Samyn, et al. 2009).

Contrary, only few studies have been published on the biochemical characterization of T. reesei proteases. Dienes et al. identified in 2007 a trypsin-like serine protease from this T. reesei strainQM9414 (Dienes, et al. 2007). The protease activity was shown with zymogram analysis. The protease itself was identified by mass spectrometry after trypsin digestion. Other proteolytic experiments were carried out on BSA and Hypocrea jecorina Cel7B. The identified protease showed high homology with fungal serine protease belonging to peptidase family S1 (Dienes, et al. 2007).

The trypsin-like protease that Dienes et al. identified is different in substrate specificity to the dibasic endopeptidase activity detected by Calmels et al. and Goller et al. in fungi (Goller, et al. 1998, Calmels, et al. 1991). Calmels et al. found that several secreted proteins in fungi were processed after a dibasic motif RR, KK, KR and RK (Goller, et al. 1998). Several slightly different motifs were also found. Goller et al. confirmed this later, they used T. reesei Cel7A and Cel7B and two of the fungus xylanases to identify cleavage motifs in T. reesei. They identified RA, ER, RR RK and KR amino acid sequence motifs on these proteins (Goller, et al. 1998). When we compare this to the data of Dienes et al., we can see that the serine protease cleaves after and arginine or a lysine, but doesn’t need a dibasic motif. They found that when arginine or lysine was followed by a proline, the site was almost never recognized as a motif. The substrate specificity of the proteolytic cleaved tyrosinase described by Selinheim et al. and the other detected proteolytic degradation of proteins was not described. The C-terminal processing of an endo-N-acetyl-β-D-glucosaminidase secreted by T. reesei is in a region and not at one specific place in this protein’s amino acid sequence.

2.2.3 Protease activity in P. pastoris

Proteolytic degradation has been a perpetual problem when yeasts are used as expression organism for recombinant proteins (Cereghino and Cregg 2000). In comparison to other expression hosts, the proteases from P. pastoris are not well characterized (Sinha, et al. 2005). One of the main reasons for the proteolytic degradation can be found in the oxidative stress and heath shock response due to several external factors, such as the change of carbon source, pH or temperature change. Oxidative stress is in this case a very probable cause, since the methanol metabolism in P. pastoris demands high oxygen levels and a form hydrogen peroxide as by-product (Sinha, et al. 2005).

Several factors influencing proteolytic degradation in the methylotropic yeast P. pastoris have been studied by Sinha et al. with recombinant ovine interferon-T (Sinha, et al.
2005). The pH is one of those factors; this can be explained given that each enzyme and protease has a pH-optimum. The use of casamino acids can reduce the proteolysis, perhaps by acting as a preferential substrate for the protease. The effect of the phosphate-level on the proteolysis has been studied by Sinha et al. (Sinha, et al. 2005). Results from these studies proved that the protease activity was inversely proportional to the phosphate level. A more important factor for the proteolytic activity is the oxidative stress when \textit{P. pastoris} is grown on methanol. Methanol, which induces the AOX1 promoter for recombinant protein expression, creates oxidative stress conditions (Cereghino and Cregg 2000).

These conditions were tested by comparing the proteolytic degradation when \textit{P. pastoris} was cultivated on methanol and when \textit{P. pastoris} X-33 recombinant cell line was cultivated on glycerol as the sole carbon source. Not only the overall protease activity was measured, but different specific protease activities were measured as well. The protease activities that were measured when methanol was used as substrate were found as well when glycerol was used as substrate, but in much more reduced levels. This proves that the oxidative stress is a very important parameter when recombinant proteins are expressed by \textit{P. pastoris}. A last factor influencing proteolytic degradation is protease inhibition. Different inhibitors belonging to several protease classes were added separately and in combination, to determine their effect on the proteolytic degradation. Sinha \textit{et al.} found that phenyl methyl sulfonyl fluoride (1mM) reduced the total protease activity by 78% and EDTA (1mM) reduced the activity by 45%. When a combination of both (1mM) was used the protease activity was reduced up to 94.2%. In increasing of the concentration of the inhibitors had no decreasing proteolytic effect (Sinha, et al. 2005). The paper by Sinha \textit{et al.} do describe several methods and options to limit proteolytic degradation when \textit{P. pastoris} is used as heterologous expression host.

### 2.3 Endo-N-acetyl-β-D-glucosaminidase

#### 2.3.1 EC classification

Endo T, expressed by \textit{T. reesei}, is a mannosylglycoprotein \textit{endo-N-acetyl-β-D-glucosaminidase} (ENGase) and has EC-classificatie 3.2.1.96. The systematic name for Endo T is: glycopeptide-D-mannosyl – \textit{N} – \{(\textit{N}-acetyl-D-glucosaminyl)\textsubscript{2} – asparagines 1,4 – \textit{N} – acetyl – \textit{β} –glucosaminohydrolase (Moss, EC 3.2.1 Glycosidases, i.e. enzymes hydrolysing O- and S-glycosyl compounds 1992, Henrissat, Bairoch and Davies, Glycoside Hydrolase family classification 1998).

#### 2.3.2 Glycoside Hydrolase family classification

The CAZy database describes the families of structurally related catalytic and carbohydrate-binding modules of enzymes that degrade, modify, or create glycosidic bonds (Henrissat, Bairoch and Davies, Glycoside Hydrolase family classification 1998). \textit{Endo-N-acetyl-β-D-glucosaminidase} (ENGase) is found in four glycoside hydrolase (GH) families, GH family 18, 20, 85 and 73. In GH family 18 there are two known activities, ENGase (EC 3.2.1.96) and chitinase (EC 3.2.1.14) and several other non-catalytic proteins, such as concanavalin B, narbonin and a xylanase inhibitor (Henrissat, Bairoch and Davies, Glycoside Hydrolase family classification 1998). GH family 85 has only one known activity, which is \textit{endo-N-acetyl-β-D-glucosaminidase} (EC 3.2.1.96) activity and several non-catalytic proteins (Henrissat, Bairoch and Davies, Glycoside Hydrolase family classification 1998). In GH family 20 two 2-domain proteins are present containing both a ENGase GH 18 module and a … GH 20 module. GH family 73 has several non-classified activities and non-catalytic proteins. Endo-N-acetyl-β-D-glucosaminidases (EC 3.2.1.96), a \textit{β}-1,4-N-acetylmuramoylhydrolase (EC 3.2.1.17) and autolysin (EC 3.5.1.28) are also classified in the GH family 73 (Henrissat, Bairoch and Davies, Glycoside Hydrolase family classification 1998).
Table 2: Endo-N-acetyl-β-D-glucosaminidase in the different GH families.

<table>
<thead>
<tr>
<th>GH family</th>
<th>Eukaryotic / Prokaryotic</th>
<th>Protein name</th>
<th>Organism name</th>
<th>Uniprot code</th>
<th>PDB code</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH 18</td>
<td>Prokaryotic</td>
<td>Endo H</td>
<td><em>Streptomyces plicatus</em></td>
<td>P04067.1</td>
<td>1EDT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endo F1</td>
<td><em>Elizabethkingia meningoseptica ATCC 33958</em></td>
<td>P36911.1</td>
<td>2EBN</td>
</tr>
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<td></td>
<td></td>
<td>Endo F2</td>
<td><em>Elizabethkingia meningoseptica ATCC 33958</em></td>
<td>P36912.1</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>O07088</td>
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<td>EndoE(*)</td>
<td><em>Enterococcus faecalis HER1044</em></td>
<td>Q6U890</td>
<td>/</td>
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<tr>
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<td></td>
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<td><em>Enterococcus faecalis V583</em></td>
<td>/</td>
<td>/</td>
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<td>Endo-Fsp</td>
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<td>P80036</td>
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<td>Q9PAG4</td>
<td>/</td>
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<tr>
<td></td>
<td></td>
<td>Eukaryotic</td>
<td>Endo FV</td>
<td>Flammulina velutipes</td>
<td>D1GA49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endo T</td>
<td><em>Hypocrea jecorina</em></td>
<td>C4RA8</td>
<td>(**)</td>
</tr>
<tr>
<td>GH 20</td>
<td>Prokaryotic</td>
<td>EndoE(*)</td>
<td><em>Enterococcus faecalis HER1044</em></td>
<td>Q6U890</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EF0114(*)</td>
<td><em>Enterococcus faecalis V583</em></td>
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<td>/</td>
</tr>
<tr>
<td>GH 73</td>
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<td>Mur2</td>
<td><em>Enterococcus hirae ATCC 9790</em></td>
<td>P39046</td>
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</tr>
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<td></td>
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<td><em>Lactococcus lactis subsp. cremoris MG1363</em></td>
<td>A2RHZ5</td>
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</tr>
<tr>
<td></td>
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<tr>
<td>GH 85</td>
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<td><em>Artrobacter protophormiae AKU 0647</em></td>
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<td>Q93HW0</td>
<td>/</td>
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<tr>
<td></td>
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<td><em>Streptococcus pneumoniae TIGR4</em></td>
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<td><em>Homo sapiens</em></td>
<td>Q8NF13</td>
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<td>Endo-LE</td>
<td><em>Solanum lycopersicum</em></td>
<td>/</td>
<td>/</td>
</tr>
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</table>

(*) Both EndoE and EF0114 enzymes are classified in both GH family 18 and GH family 20 according to the CAZy classification of carbohydrate enzymes (Henrissat, Bairoch and Davies, Glycoside Hydrolase family classification 1998) The reason for this dual classification of these enzymes is due to that EndoE and EF0114 have 2 domains, one belonging to GH family 18 and the second to GH family 20 (personal communication by B. Henrissat).

(**) The structure of Endo T has not been published yet.
Table 1 gives an overview of all the different endo-N-acetyl-β-D-glucosaminidase found within various glycoside hydrolase families, with their PDB-code, Uniprot code and organism listed. In that table

2.3.3 Glycoside hydrolase family 18

The enzymes classified into GH family 18 were initially only coming from prokaryotes. Endo H from *Streptomyces plicatus* (Tarentino, Plummer and Maley 1974), Endo F1, F2, F3 from *Elizabethkingia meningoseptica* (Trimble and Tarentine 1991) and other GH family 18 ENGases are shown in table 2. Eukaryotic ENGases belonging to GH family 18 have only been discovered recently. Endo T, from *Trichoderma reesei* (Stals, Samyn, et al. 2009), and Endo FV, from *Flammulina velutipes* (Hamaguchi, et al. 2010), are the first two eukaryotic ENGase found to be classified into in the GH family 18. All ENGases from GH family 18 are also shown in table 2. Only for four of them, the 3D-structure has been determined. With Endo T as the only fungal ENGase. Typical for them and for all GH family 18 proteins, is their fold. They all have a (β/α)₈ TIM barrel structure. This structure is shown in figure 1 and has the form of a cask, where eight β-sheets are placed internally in the barrel anti-parallel with 8 external α-helices. The 8 β-sheets form an inner circle, around which the 8 α-helices form an outer circle. The cavity in the inner circle is apolar due to the apolar side chains of both the α-helices and β-sheets. The side chains between the α-helices and β-sheets are dominated by Val, Ile and Leu. The polar amino acids are localized on the surface exposed parts of the barrel, on the top or the bottom, where they contribute to the solubility or the catalytic activity of the enzyme (Branden and Tooze 1999, Voet, Voet. and Pratt 2008). A structural comparison between the characterized GH 18 ENGases is shown in appendix 4.

2.4 N-Glycosylation

2.4.1 N-glycosylation an introduction

Glycosylation, a post-translational modification, is involved in immunogenicity, protein folding and protein stability (Stanley, Schachter and Taniguchi 2009 2nd edition). N-glycosylation is the transfer of a specific sugar chain to a asparagine residue. The transfer of the N-glycan by the ER-oligosaccharyltransferase only occurs in consensus regions consisting of the Asn-x-Ser/Thr motif. X can be any amino acid except a Pro (Karamanos 1997). Different N-glycans are found between organisms and in different cells or tissues, but they can all be classified into three types; high-mannose, complex and hybrid N-glycans (Stanley, Schachter and Taniguchi 2009 2nd edition).

![Figure 1: (β/α)₈ TIM barrel structure from Endo T (Boel 2010)](image)

Figure 1: Different N-glycosylation forms (Stanley, Schachter and Taniguchi 2009 2nd edition).
High-mannose glycans consist predominantly of mannoses and N-acetyl glucosamines. Complex type N-glycans are built of mannose, N-acetyl glucosamine, galactose, sialic acid and are sub classified according to the number of branching points. Hybrid type N-glycans typically contains two branches; one of the high-mannose type and one of the complex type. The different N-glycans are shown in figure 2.

### 2.4.2 N-glycosylation in filamentous fungi

Filamentous fungi are often used as expression organism due to their capability of post-translational modifications, such as glycosylation (Deshpande, et al. 2008, Sharma, et al. 2009). In filamentous fungi N–glycosylation is important in secretion and localization of proteins. Only high-mannose glycans are found in filamentous fungi. They can though still vary between different organisms. The degree of mannosylation can vary; from 5-7 mannose residues in e.g. \( T. \) reesei (Stals, Samyn, et al. 2009) upto 8-9 mannose in e.g. \( A. \) niger and extra other modifications (e.g. phosphate, sulphate and phosphordiester) can be present (Tsutomu Takayanagia 1994, Deshpande, et al. 2008). Yeast N-glycans can contain more then 100 mannoses on one N-glycan (Lehle 1992).

### 2.4.3 N-glycosylation in Trichoderma reesei

The glycosylation patterns of protein expressed by \( T. \) reesei can change depending on the cultivation conditions. Other hydrolytic enzymes are expressed and secreted in the cultivation medium and can be active in the correct pH conditions. These exo- and/or endoglycosidases can act on the O- and N-glycans present on the glycoproteins (predominantly cellulases). These post-secretorial modifications of both O- and N-glycosylations have been studied extensively on Cel7A in \( T. \) reesei (Stals, Sandra and Geysens, et al. 2004, Stals, Sandra and Devreese, et al. 2004). And the activity of a α-mannosidase, phosphatase and endo-N-acetyl-β-D-glucosaminidase activity was observed (Stals, Samyn, et al. 2009). Two genes for endo-N-acetyl-β-D-glucosaminidas, one intracellular and a second extracellular ENGase (Endo T) are present in the \( T. \) reesei genome (Martinez D 2008). Endo T was in a later study purified from the culture broth an partially characterized (Stals, Samyn, et al. 2009).

### 2.5 Endo T

#### 2.5.1 Substrate

\( T. \) reesei Endo T hydrolyzes the β-1,4 glycosydic bound between the two N-acetyl glucosamine (NAG) of high-mannose N-glycans as described above. Therefore one NAG stays bound to the protein after the removal of the N-glycan by Endo T. The second NAG remains bound to the removed high-mannose N-glycan (Karamanos 1997). The presence of the enzyme in the medium explains for the occurrence of single NAG residues on \( T. \) reesei proteins as reported by several groups (Stals, Sandra and Geysens, et al. 2004).

#### 2.5.2 Mechanism

Endo T and all ENGases are retaining glycoside hydrolases using neighboring group participation for the catalytic mechanism. The distance between the catalytic residues Asp 129 and Glu 131 in the retaining mechanism is approximately 10 Å. The mechanism is shown in figure3. There has to be 1 acetic residue being an Asp or Glu and one stabilizing group. For all ENGases in GH family 18 the catalytic residues are Asp and Glu with only one residue between them. The acetic residue interacts with the substrate and forms an oxazoline intermediate, this is formed after a transition state. A second acetic group stabilizes the intermediate and brings the active site back to its original state (Davies and Henrissat 1995).
2.5.3 Structure

The structure of the active form of the endo-N-acetyl-β-D-glucosaminidase secreted by T. reesei, i.e. Endo T, has been solved by X-ray crystallographic methods and the structure was refined to 1.3 Å. The protein was crystallized using a protein concentration of 8.3 mg/ml, in 0.1 M citric acid buffer, with a pH between 2.4-3.0 and using PEG 3350 or PEG1500/PEG8000 as a precipitant, with both PEG concentrations varying from 4 to 14 % and by incubating the experiments at 20° C (Digre 2010). The tertiary structure of Endo T is an (β/α)8 TIM barrel and the structure has overall dimensions of approximately 45 Å x 34 Å x 57 Å A cartoon representation of the Endo T structure is shown in figure 4.

Figure 3: Retaining mechanism with a N-acetyl or N-glycol group on position 2 (Davies and Henrissat 1995).

2.6 X-ray crystallography

2.6.1 Protein crystallography

Protein crystallography has been thought to be more an art then a science for a very long time (Chayen, Turning protein crystallization from an art into a science 2004). A lot of patience, perseverance, intuition and luck is needed to grow high-quality protein crystals (Navarro, Wu and Wang 2009). Nowadays more and more proteins are routinely crystallized. This is mainly due to the high-throughput crystallization methods that have been developed over the past decade (Chayen, Protein crystallization strategies for structural genomics 2007).

Figure 4: A cartoon representation of the Endo T structure with a ligand bound in the active site of the enzyme, on top of the B-barrel. The bound ligand is colored yellow, the catalytic residues are colored blue and the two bound NAG residues red

Figure 5: Protein crystallization phase diagram. The different paths are show for the different crystallization methods; (1) batch method, (2) vapor diffusion, (3) dialysis and (4) free interface diffusion (Chayen, Turning protein crystallization from an art into a science 2004).
Proteins molecules in supersaturated state can precipitate, nucleate or form crystals. Figure 5 shows the phase diagram for all these different states of a protein. Only after nucleation, protein crystals start to be formed and grow. Crystal growth occurs in the meta-stable zone, shown on Figure 5. It is the level of super-saturation, which is the main drive for a protein to crystallize. For X-ray crystallography big high-quality crystals are needed to obtain a good x-ray diffraction pattern when the protein crystals are exposed to x-ray beam. Massive nucleation and precipitation should thus be limited (Chayen, Turning protein crystallization from an art into a science 2004). Different techniques can help to overcome this problem when normal methods fail to crystallize proteins (Chayen, Methods for seperating nucleation and growth in protein crystallization 2005). Examples of these methods are e.g. adding nucleation seeds (both macro and micro seeding) to a crystallization drop and dilution of the protein solution used for the crystallization experiment. The difference between macro and micro seeding is that in the former one introduce single crystals to the crystallization drop while in the later case one try to ad controlled amounts of pre-grown nucleation sites to the crystallization drop. In both cases with the hope that these seeding strategies will give raise to bigger and better quality crystals of the target protein (Chayen, Methods for seperating nucleation and growth in protein crystallization 2005).

Different parameters influence a crystallization experiment, these can be categorized in ether bio-physical phenomena or crystallization methods (Bergfors 1999). Bio-physical parameters are for instance protein concentration, temperature, pH, the precipitant and its concentration, the salt and its concentration and so on. Different thermodynamic and kinetic parameters for a crystallization experiment could in theory be calculated, but would create a lot of work and these parameters will be different for each new protein (Navarro, Wu and Wang 2009).

The importance of the different crystallization methods should not be underestimated. A different path to get to the meta-stable zone and to grow high-quality crystals characterizes each method. These different paths are shown in Figure 5 and all of these methods will pass the nucleation phase, before dropping to the meta-stable zone and crystal growth (Chayen, Turning protein crystallization from an art into a science 2004). The batch method was the first method that was developed for protein crystallization. It is an easy and simple method, still in use but not so common any longer (Bergfors 1999). Various types of so called vapor diffusion methods are now more commonly used. In the vapor diffusion crystallization method different setups are available. These different setups are hanging drop, sitting drop and sandwich drop and are shown in Figure 6. The hanging drop vapor diffusion method is commonly used to set up manually crystallization experiments. The sitting drop method is most often used to setup crystallization plates with various types of crystallization robots. For the hanging drop method a volume of 500 µl is usually used in the crystallization experiment reservoir containing the crystallization solution. This crystallization solution contains buffer (concentration often varying between 0.1 and 0.2 M), metal ions and crystallization precipitant, all in different concentrations depending on the crystallization condition. One of the most commonly used precipitants in a protein crystallization experiment are different types of poly ethylene glycol (PEG). Their average molecular weight can vary.

![Figure 6: Different vapour diffusion methods. Hanging drop (1), sitting drop (2) and sandwich drop (3) method (Bergfors 1999).](image-url)
There are two steps to get high-quality crystals. Screening to identify initial crystallization conditions is the first step. Different companies sell crystallization screens that are used to identify the initial crystallization condition for the protein of interest. An example of a crystallization robot is the Oryx robot from Douglas instruments, Hungerford (Bergfors, Terese M. 2009). Two examples of commercially available crystallization screens are; the JCSG or the Core 96 screen and the PEG ION screen (HAMPTON 2003, QIAGEN 2003). Crystallization robots are very useful tool to save time and reproduces crystals in a higher ratio than manual crystallization setups do.

When initial crystal conditions are found, these crystals have to be tested to determine whether these crystals are protein or salt crystals. If the initial crystals are found to be protein crystals the crystallization condition is further refined to produce bigger crystals. This is the second step in a crystallization process and is often called optimization of the crystallization condition. Different ways of identifying initial crystallization conditions and improving this further to produce high quality protein crystals are well described in the book: “Protein crystallization second edition, edited by Terese M. Bergfors”. Other techniques that have not been mentioned, can also be found in this handbook (Bergfors, Terese M. 2009). When protein crystals are big enough to be used for an X-ray data collection, these are picked out the crystallization drop. The crystal is moved to a cryo solution and frozen in liquid nitrogen. The function of keeping the crystal frozen at a cryogenic condition is to prevent that the crystal is thermally destroyed in the strong X-ray beams in use today, often strong synchrotron X-ray sources. To identify a good cryo-solution can be very tides and difficult. Therefore several conditions should be tried (Bergfors, Terese M. 2009). After freezing the crystals, these can be stored in liquid nitrogen before being transported to the synchrotron, where the crystals will be exposed in the X-ray beam.

2.6.2 X-ray diffraction

The longest and most difficult part to get a good 3D model, is getting high-quality crystals. After subjecting the crystal to X-ray’s, the diffraction pattern has to be translated in to a 3D structure model. Depending on the size of the protein and the quality of the diffraction pattern obtained when exposing the crystal in the X-ray beam, solving the structure and refining the structure model it is the second biggest task. At a synchrotron the protein crystals are exposed in an X-ray beam with a wavelength of approximately 1 Å. A X-ray data collection can consist of anything between hundred up to many hundreds images, where the oscillation of the crystal in the x-ray beam and the exposure time can be adapted to achieve as god diffraction as possible from the exposed crystal.

2.6.3 Electron density map

The first task after a X-ray data collection is to translate the diffraction pattern into an electron density map. Crystals, which are subjected to X-rays, will give a specific diffraction pattern. This diffraction is the result of enhanced X-ray radiation in some directions and extinguished X-ray radiation in other directions. Each electron of the atoms building up the molecules of the crystal will influence the final diffraction pattern obtained, since each reflection will change the intensities of other reflections. A crystal is a symmetric repetition of the molecule building up the crystal, (Lattman and Loll 2008).

Each reflection is characterized by its position, amplitude and its phase. Both the amplitude of all diffraction spot of a X-ray diffraction dataset and the phases information for each of these diffraction spots are necessary to know to be able solve the three dimensional structure of a protein using the diffraction dataset obtained. The amplitudes can directly be derived from the intensity of the reflection recorded on the diffraction image collected when exposing the crystal in the x-ray beam. The phases on the other hand cannot be derived directly.
Several techniques are available to determine the phases for the recorded reflections. Determination of the phases by isomorphous replacement, and phasing with a highly similar known structures (molecular replacement) are two techniques which are commonly used to derive the phase information for the collected reflections (Lattman and Loll 2008). Once the phase information for the diffractions spots are known the diffraction pattern will be translated into an electron density maps. This translation is done by computer software.

### 2.6.4 Protein structures

A structure model of the protein that is studied is modeled into the electron density map calculated from the diffraction pattern obtained. Refining a protein model is the modeling in of the individual amino acids of a protein into the calculated electron density map. Usual during refinement of a structure two different electron density maps are calculated from a diffraction pattern, the (2Fo –Fc) and the (Fo-Fc) map. The quality of the built structure model depends a lot on the quality of the calculated electron density map and by the refinement and fitting of the model in the electron density map. The higher resolution of the diffraction pattern on can collect, the better the electron density map gets. An electron density map with a resolution of 3 Å will show the main structure elements of the protein, an electron density map of approximately 1 Å resolution will show the protein studied at atomic resolution, including the hydrogen atoms of the protein. The higher the resolution of a collected diffraction pattern gets, the more reflections the collected dataset contains (Wlodawer, et al. 2007). A second parameter, which is a control for the quality of the protein model are the R and R$_{free}$ factors. These factors measure the difference between the observed and calculated amplitudes of the electron density. The lower these R-factors are the better the quality is. There is though one general remark, the value of the R-factors cannot keep dropping, they are correlated to the quality of the electron density map. The lower the difference between the R and R$_{free}$ the less difference there is between the observed and calculated amplitude and the better the model is. The angles of the phases are also a parameter, which should be checked regularly (Wlodawer, et al. 2007).

The amino acids of a protein are modeled into the electron density map calculated from the collected diffraction pattern. Not all geometric conformations of the peptide bond between two connected amino acid in the poly-peptide chain of a protein are allowed. Depending on the quality of the electron density map, the restrains for the geometric parameters for the individual amino acids building up a protein and the geometric for the angles between two connected amino acids in the poly-peptide chain can be loosened up gradually, and the dependents between the structure model and the electron density map can be increased. The refinement of a structure model can be very time consuming and will depend a lot on the resolution and the quality of the calculated electron map for the protein. The better the phases get, the better the structure model will be. The phases for the calculated electron map can be improved by adding solvent molecules to the structure model in addition to adding the amino acids for the protein part of the model. Several programs are capable of refining structures and they are the perfect tools to solve protein structures.
3 Materials and methods

3.1 Protein samples
The intact Endo T samples were heterologously expressed in *P. pastoris*. A mutated Endo T (D129A) protein expressed in *T. Reesei* was also used, but we weren’t able to confirm if it was catalytic dead.

3.2 Endo T protein analysis

3.2.1 SDS-PAGE analysis
Samples of expressed and purified Endo T proteins were checked using 4-20% (15 well) gradient gels or on 12% (15 well) homogenous poly-acrylamide gels from Bio-Rad. The gels were bought pre-casted from the Bio-Rad. 5 µl of the precision standard ladder was loaded on gel, while 12 µl of the treated protein sample was loaded. Protein samples were treated by adding 5 µl of SDS dye sample buffer to 15 µl of non-treated protein samples and heated for 5 minutes at 95°C. The gel was run for 40 minutes with a SDS containing running buffer and washed several times with water before being stained. After washing, the gels were stained for one hour with Bio-safe G-250 stain from Bio-Rad and the gels were destained overnight in water.

3.2.2 Native-PAGE analysis
Endo T proteins were treated as the SDS-PAGE analysis, with the only difference that the protein sample was loaded untreated. The gel was run for 3 hours minutes with native running buffer and washed several times with water before being stained. After washing, the gels were stained for one hour with Bio-safe G-250 stain.

3.2.3 Peptide fingerprinting by mass spectrometry
Several bands were analyzed with mass spectrometry after trypsin digestion, to identify if the detected band on the gel corresponded to the intact or processed form of Endo T. All the mass spectrometry experiments were carried out using a Ultraflex MALDI TOF/TOF, Bruker Daltonics and carried out by Åke Engström at the Department of medical biochemistry and microbiology, biomedical centre Uppsala, Sweden.

3.2.4 Western Blot
SDS-PAGE gels were run in duplicate, one was stained as described above, while the proteins of the unstained gel were transferred on a nitrocellulose membrane as described by Burnette (Burnette 1981). After the transfer, the bands containing terminal mannoses can be detected with the glycan differentiation kit from Boehringer Mannheim Biochemica as described in the DIG kit procedure (Biochemica 1996). But the transfer of the proteins to the nitrocellulose filter failed twice. The gels are shown in appendix 3.

3.2.5 Protein concentration
The absorbance of the Endo T protein sample was first measured using a Nanodrop spectrophotometer. Samples of 2 µl protein were used to determine the protein concentration using the Nanodrop 2000. The protein concentration was measured at 280 nm, and the absorbance at 260 nm was also measured to determine the concentration of DNA in the sample. The absorbance was then used to calculate the protein concentration with the specific protein extinction coefficient.

When a bad 260/280 value was measured, the absorbance couldn’t be used to determine the total protein concentration of the sample. The total protein concentration was then measured by using the Bio-Rad protein assay, which is based on the Bradford method (Bio-rad n.d.). 20 µl of a bovine serum albumin standard (from 0 to 0.8 mg/ml) was incubated with 1 ml of 5 times diluted dye reagent (diluted in de-ionized water) at room temperature for 5 minutes. The same procedure was carried out applied for the dilution series of the Endo T protein sample. The concentration of the Endo T protein samples were then calculated back from the absorbance. All measurements were conducted in triplicates and measured at $\lambda=595$ nm.
3.3 Purification of the intact Endo T for crystallization

3.3.1 Size-exclusion purification with a 3 kDa cutoff filter.
The extracellular culture medium from a 500 ml Pichia pastoris culture was first Buchner filtered to get rid of cell debris. Then the culture medium was concentrated with a 3 kDa cutoff filter to a final volume of approximately 12 ml in 20 mM pH 8 Tris buffer, protease inhibitors were added to keep Endo T intact. This Endo T protein sample was checked on SDS-PAGE. Patricia Ntarima conducted the first purification steps of Endo T at the university college Ghent.

3.3.2 Superdex 75 size-exclusion chromatography
The intact Endo T with protease inhibitors added to the protein solution was purified by size-exclusion chromatography. The column was first cleaned with water and thereafter pre-incubated with 100 mM sodium chloride 20 mM pH 5 sodium acetate buffer. 500 µl of 9.40 mg/ml Endo T sample was loaded to the column and eluted with 100 mM sodium chloride 20 mM pH 5 sodium acetate buffer after 15 minutes. The concentration of the Endo T was measured using the protein assay from Bio-Rad. The fractions from the peaks were checked on a 4-20% gradient gel SDS-PAGE after first being concentrated to approximately 750 µl with a 2 ml Vivaspin column with a molecular cutoff filter of 3kDa.

3.3.3 MonoQ anion exchange chromatography
The monoQ column was first washed with water, with 2ml 2M NaCl, 2ml 1 M NaOH, 2ml 1M HCl and 2 ml of 75 % acetic acid to get rid of all impurities before using it. The column was cleaned with water and with a strong buffer before calibrating the column. The calibration of the column was done by first running 40 ml 20 mM pH 6.5 Bis-Tris buffer and with approximately 40 ml 1 M NaCl 20 mM pH 6.5 Bis-Tris buffer. 20 ml 20 mM pH 6.5 Bis-Tris buffer was brought over the column before loading the protein sample on the column. 1 ml of Endo T in 20 mM pH 8 Tris buffer was diluted in 49 ml of 20 mM of Bis-Tris buffer to change the pH to 6.5 and to lower the conductivity of the sample. The protein was eluted with a linear gradient from 0 % to 100% (1 M NaCl 20 mM pH 6.5 Bis-Tris buffer). Changing the gradient from 0 % to 70 % gave a better separation of the different eluted peaks. The peak fractions were concentrated with Vivaspin columns with a molecular weight cut-off filter of 3kDa and then checked on 12 % homogenous SDS-PAGE. The protein concentration was determined using the Nanodrop.

3.3.4 Deglycosylation of intact Endo T for crystallization
A sample of intact Endo T, in 20 mM pH 8 Tris buffer with protease inhibitors, was buffer exchanged against 50 mM pH 5 sodium acetate buffer. The sample was then incubated at 37°C for approximately couple of days to let the active fraction of Endo T present in the sample deglycosylated all N-glycans bound to the inactive form of Endo T. The results of the deglycosylation experiments were checked on 4-20% gradient SDS-PAGE. The deglycosylated fractions could then be used for crystallization experiments.

3.4 Proteolytic stability of the intact Endo T
The protease inhibitor cocktail used is the Halt™ Protease Inhibitor Single-Use Cocktail EDTA-Free from Thermo Scientific. 10µl of the cocktail was added to 1ml of the protein sample as described in the protocol for the used protease inhibitors.

3.4.1 pH-stability
150 µl of Endo T samples with a concentration of 9.40 mg/ml, both with and without protease inhibitors, were incubated at 21°C in 100 mM citric acid buffer pH 3, 100 mM acetic acid buffer pH 5, 100 mM TRIS pH 8 and 100 mM pH 11.61 citric acid phosphate buffer to check their proteolytic pH stability. Six different
samples of all pH conditions were taken in a period between 5 hours and 14 days after incubation and checked on 12% homogenous SDS-PAGE.

### 3.4.2 Temperature stability

150 µl of 100 mM pH 5 sodium acetate buffer Endo T samples with a concentration of 9.40 mg/ml, both with and without protease inhibitors, were incubated at 4, 21 and 37 °C to check their proteolytic temperature stability. Six different samples of all conditions were taken in a period of 14 days and checked on 12% homogenous SDS-PAGE.

### 3.4.3 Proteolytic degradation

Both the wild type Endo T and the mutant Endo T (D129A) were incubated for 1 day at 20°C in a 0.1M pH 3 citric acid buffer. Protein samples were taken at time 0, 2 hours, 6 hours, and 1 day. A mixture of 1/1 was also incubated in the same conditions. The different protein samples were then checked on a 12% homogenous SDS-P.

### 3.4.1 Proteolytic activity

Both the wild type Endo T and the mutant Endo T (D129A) were incubated for 1 day at 20°C in 0.1M pH3 citric acid buffer, and thereby lose the C-terminus of the protein. These samples were then incubated with T. reesei Cel7A for 2 hours at 20°C in 0.1M pH3 citric acid buffer. The incubation was performed at different Endo T/Cel7A weight ratios of 1/1, 1/10, 1/20, 1/40 and 1/100. The non-cleaved and cleaved form of Cel7A, by papaine digestion were taken as control samples. A 1/1 ratio of both the wild type Endo T and the mutant Endo T (D129A) were incubated with Cel7A for 2 hours at 20°C in 0.1M pH3 citric acid buffer, without losing their C-terminus.

### 3.5 X-ray crystallography

#### 3.5.1 Protein crystallization

Crystallizations were setup up at 20°C, both for the hanging drop and sitting drop plates. The Oryx robot, from Douglas instruments, was used to set up crystallizations with the JSCG and the PEG ION crystallization screens (QIAGEN 2003, HAMPTON 2003). The well volume of the crystallization experiments was 70 µl. The total drop volume of the crystallization experiment was 0.70 µl. The drop consisted of 0.35 µl well solution, and of 0.35 µl protein sample. When hanging drop crystallization plates were setup the well volume contained 500 µl and the crystallization drop had a final volume of 4µl. The drop consisted of 2µl well solution and 2 µl protein sample. The well liquid consisted of a 0.100 M buffer, the pH and buffer was varied and PEG 3350, where the concentration was varied. In both setups, the protein concentration was varied.

#### 3.5.2 X-ray diffraction

The Endo T crystals were transported to the synchrotron (MAX-Lab, Lund or ESRF, Grenoble) where they were tested in the X-ray beam at the synchrotron. The oscillation range for the data collection was 1°, and the number of images collected depended on the strategy used for the data collection.

#### 3.5.3 Protein structure and refinement

The different protein structures were solved by processing all images using the program imosflm (Powell 2010). When the collected diffraction images were processed, the space group of the diffraction pattern had to be determined. When this was done. The processed diffraction data was then scaled with the scala program within the ccp4 packages (International Union of Crystallography 2011). After scaling the obtained dataset an already available Endo T model was refined against this new dataset. Waters and other components were built in to the structure model to further improve the phases of the electron density map. The R and Rfree values, the bond distance and bond angles were checked after each refinement run to obtain a good structure model.
4 Results

4.1 Endo T protein analysis

4.1.1 Endo T transport control

The protein samples with expressed Endo T were transported from the University College Ghent to SLU, Uppsala; there they were analyzed using a 4-20% (15 well) gradient SDS-PAGE gel to check if the Endo T protein in these were still intact. Both the sample with inhibitors and the sample without inhibitors did still contain intact Endo T protein after transport. If lower bands appear proteolytic degradation or deglycosylation takes place. The short processed Endo T was also loaded on gel as a reference. These results are not shown.

Lane 2 to 4: Crude processed Endo T sample

Lane 5 to 7: Crude intact Endo T sample – inhibitor

Lane 8 to 10: Crude intact Endo T sample + inhibitor

4.1.2 Protein concentration

The total concentration of the crude Endo T sample with protease inhibitors was approximately 9.40 mg/ml, the sample without protease inhibitors had a concentration of 2.84 mg/ml. These results are shown in appendix 1.

4.2 Purification of the intact Endo T for crystallization

4.2.1 Size exclusion chromatography

Three batches of 0.500 ml Endo T with protease inhibitors were purified with a superdex75 column. The purification by size exclusion failed. These results are shown in Figure 7. No further purification with this method was used. In the first trial no salt was added and the protein was lost, in the second trial the protein purification failed and in the third trial Endo T got degraded.

Figure 7: Size exclusion chromatography of Endo T with protease inhibitors. The first purification (A) without any salt in the elution buffer failed and the protein was lost. The two other attempts (B and C) with salt, failed or the protein got degraded.

4.2.2 Anion exchange chromatography

A batch of 1 ml Endo T with protease inhibitors was purified with anion exchange chromatography using a MonoQ column. Figure 10 shows the chromatogram from the separation with a linear gradient of 1 M NaCl from 0 to 70%. Different fractions corresponding with the different peaks were then checked by SDS-PAGE, to identify the Endo T fractions containing Endo T. These results are shown in Figure 11. The separation was very good and was even better with a gradient from 0 to 70%. The concentrated protein samples are shown in appendix 2.

Figure 10: Protein status of Endo T (undiluted; 10 times and 100 times diluted) was checked after transport. Lane 2 to 4 contain the dilution series of the active Endo T, lane 5 to 7 the Endo T sample without inhibitors and lane 8 to 10 contain the Endo T sample with inhibitors.

Figure 11: Anion exchange chromatography of Endo T with protease inhibitors. The peak fractions containing Endo T were then checked by SDS-PAGE, to identify the Endo T fractions containing Endo T. These results are shown in Figure 11. The separation was very good and was even better with a gradient from 0 to 70%. The concentrated protein samples are shown in appendix 2.
4.2.3 Deglycosylation of intact Endo T for crystallization

Deglycosylation of the intact Endo T, by the active fraction of Endo T, in the unpurified protein sample, is shown in Figure 9. During deglycosylation at 37 °C a pull down of protein was noticed. The sample was centrifuged and the supernatant was removed. The pull down was resuspended in a 50 mM pH 5 sodium acetate buffer and also checked on SDS-PAGE gel. The pull down was identified as Endo T. This is though the first time that a pull down of Endo T is noticed.

Figure 11: Different fractions of the first MonoQ purification. Lane 2, 3 and 5, 6 contain unpurified Endo T as control. Samples 56 to 63 correspond with the first small peak. Fraction 68 with peak 1 around 340 ml in figure 3 and fractions 71 and 72 with peak 2 around 355 ml in figure 3. Fraction 86 is from the last peak. These fractions were concentrated after purification.

Figure 10: Chromatogram of anion exchange chromatography with a MonoQ column and a 1 M NaCl gradient going from 0 to 70 %. The gradient consists of 20 mM Bis-Tris pH 6.5 and 1M NaCl 20 mM Bis-Tris pH 6.5. Peak 1 around 340 ml is the first fraction; peak 2 around 355 ml is the second fraction containing a different form of the intact Endo T.
The deglycosylated sample was incubated with protease inhibitors at 37°C in 0.1 M pH 5 sodium acetate buffer for over a week and was kept intact. After loading the different deglycosylation samples on gel, new prominent bands were detected. These bands around 75 kDa, 150 kDa and higher than 250 kDa were cut out and were subjected to mass spectrometry for peptide identification. The bands were identified as Endo T and could’ve been oligomers or highly glycosylated forms of Endo T. A native gel was run and no oligomers were visible. The concentration used in the Native PAGE is lower then the detected Endo T bands around 75 kDa, 150 kDa and higher than 250 kDa. Because of this we cannot conclude whether these higher bands are oligomerizations or not.

4.3 Proteolytic stability of the intact Endo T

4.3.1 T-dependent degradation pattern of Endo T

Proteolytic degradation of intact Endo T by protease activity was observed in protein samples incubated at different temperatures. In lane 13 of figure 13 there are 5 bands, proteolytic degradation is confirmed when band 4 and/or band 5 is observed. At 4°C proteolytic degradation was observed after 8 days. At 21°C degradation was observed after 6 days and at 37°C already after 3 days. Figure 13 to 15 show the degradation pattern at the three different temperatures. The negative control, the Endo T sample with protease inhibitors didn’t work.

Degradation was detected in samples of Endo T with and without protease inhibitors present. The reason for this is that the protein sample with protease inhibitors was kept in the freezer and it was not until a later stage we realized that the protease inhibitors lose their effect when kept frozen at -20°C for a longer period of time. No new inhibitors were added before setting up the experiment. For this reason the negative control failed. In previous experiments the protease inhibitors had been tested and had worked.

Figure 13: SDS-PAGE showing the degradation pattern of Endo T incubated at 4°C in 100 mM pH 5 sodium acetate buffer. Samples were taken after 5 hours; 1 day; 3 days; 6 days; 8 days and 14 days. Lane 1 is the molecular ladder, lane 2 to 7 contain the Endo T protein sample at the different time points without the protease inhibitor cocktail, lane 8 to 13 contains the Endo T protein sample at the different time points with the protease inhibitor cocktail.

Figure 14: SDS-PAGE showing the degradation pattern of Endo T incubated at 21°C in 100 mM pH 5 sodium acetate buffer. Samples were taken after 5 hours; 1 day; 3 days; 6 days; 8 days and 14 days. Lane 1 is the molecular ladder, lane 2 to 7 contain the Endo T protein sample at the different time points without the protease inhibitor cocktail, lane 8 to 13 contains the Endo T protein sample at the different time points with the protease inhibitor cocktail.

Figure 15: SDS-PAGE showing the degradation pattern of Endo T incubated at 37°C in 100 mM pH 5 sodium acetate buffer. Samples were taken after 5 hours; 1 day; 3 days; 6 days; 8 days and 14 days. Lane 1 is the molecular ladder, lane 2 to 7 contain the Endo T protein sample at the different time points without the protease inhibitor cocktail, lane 8 to 13 contains the Endo T protein sample at the different time points with the protease inhibitor cocktail.
4.3.2 pH-dependent degradation pattern of Endo T

Proteolytic degradation by protease activity was observed in all different pH conditions except the one at pH 11.63. The samples at this pH are not so visible compared to protein samples in other conditions. For that reason it is not possible to draw a direct conclusion about proteolytic cleavage of Endo T at this high pH condition. There was both degradation in the sample with and without protease inhibitors. The reason therefore is described above under Temperature-dependent degradation pattern of Endo T.

Protein degradation is accelerated when pH is decreased. Figure 16 to 19, show the degradation pattern of Endo T. At pH 3 full degradation of the protein is observed after one day. A second experiment was setup to give a better image of the degradation of Endo T at pH 3. In this experiment fresh protease inhibitors were added to the protein sample before incubation. At pH 5 degradation of Endo T occurred after 6 days. The same period is needed at pH 8, but the amount of degraded Endo T is lower than at pH 5. No direct conclusion can be drawn about the degradation of Endo T incubated at pH 11.6.

Figure 16: SDS-PAGE showing the degradation pattern of Endo T incubated at 21 °C in 100 mM citric acid buffer pH 3. Samples were taken after 5 hours; 1 day; 3 days; 6 days; 8 days and 14 days. Lane 1 is the molecular ladder, lane 2 to 7 contain the Endo T protein sample at the different time points without the protease inhibitor cocktail, lane 8 to 13 contains the Endo T protein sample at the different time points with the protease inhibitor cocktail.

Figure 17: SDS-PAGE showing the degradation pattern of Endo T incubated at 21 °C in 100 mM acetic acid buffer pH 5. Samples were taken after 5 hours; 1 day; 3 days; 6 days; 8 days and 14 days. Lane 1 is the molecular ladder, lane 2 to 7 contain the Endo T protein sample at the different time points without the protease inhibitor cocktail, lane 8 to 13 contains the Endo T protein sample at the different time points with the protease inhibitor cocktail.

Figure 18: SDS-PAGE showing the degradation pattern of Endo T incubated at 21 °C in 100 mM Tris buffer pH 8. Samples were taken after 5 hours; 1 day; 3 days; 6 days; 8 days and 14 days. Lane 1 is the molecular ladder, lane 2 to 7 contain the Endo T protein sample at the different time points without the protease inhibitor cocktail, lane 8 to 13 contains the Endo T protein sample at the different time points with the protease inhibitor cocktail.

Figure 19: SDS-PAGE showing the degradation pattern of Endo T incubated at 21 °C in 100 mM phosphate-citric acid buffer pH 11.6. Samples were taken after 5 hours; 1 day; 3 days; 6 days; 8 days and 14 days. Lane 1 is the molecular ladder, lane 2 to 7 contain the Endo T protein sample at the different time points without the protease inhibitor cocktail, lane 8 to 13 contains the Endo T protein sample at the different time points with the protease inhibitor cocktail.

Figure 20: SDS-PAGE showing the degradation pattern of Endo T incubated at 21 °C in 100 mM citric acid buffer pH 3. Lane 2 to 5 contains Endo T without protease inhibitors added, lane 6 to 9 Endo T with protease inhibitors added, lane 10 to 13 Endo T with protease inhibitors and PEG added.
Figure 20 shows a better degradation pattern at pH 3 than figure 16. Samples were taken after 15 minutes, 2 hours and 15 minutes, 9 hours 15 minutes and 1 day and 15 minutes. In all three conditions there is complete degradation of Endo T at pH 3 after only one day. The degradation is slower with protease inhibitors added, but still occurs. This shows us that the inhibitors are not stable or not working at this pH. The quality of the gel was not good and PEG also influences the quality of the bands on SDS-PAGE. This experiment was a control since, proteolytic degradation of Endo T occurred in a pH 3 crystallization condition.

4.3.3 Proteolytic degradation in crystallization

Proteolytic degradation of Endo T was observed in the initial crystal conditions found. These conditions were at pH 2.4 – 3 and as is shown in figure 20 the used protease inhibitors are not stable at this pH. Some crystals and crystal drops were loaded on SDS-PAGE gel to check if Endo T was degraded, this is shown in figure 21.

![Figure 21: SDS-PAGE showing the protein status in the crystals (Lane 6 and 7) and in the crystal drops (Lane 8 and 9) at pH 3, 100mM citric acid in 5-9% PEG 3350.](image1)

To see if the proteolytic cleavage of Endo T is due to the crystal condition or the protein sample, the protein samples were loaded on gel to see if they were processed. The protein samples with inhibitors were stored for more than 3 months in the fridge. Figure 22 shows us that all these samples are still very similar to the samples taken just after the protein purification.

4.3.4 Proteolytic activity on Cel7A.

In a previous paper an acid protease from *T. reesei*, was found to cleave Cel7A and Cel7B into their active form (Eneyekaya, et al. 1999). Since Endo T loses its C-terminus in acid conditions, Endo T was tested to see if it had a protease activity. The acid protease previously discovered has the same molecular mass as Endo T, for these reasons we thought Endo T potentially could possess protease activity in addition to its ENGases activity.

Both the wild type and mutant Endo T were added in different ratios (1/1, 1/10, 1/20, 1/40, 1/100 and 1/400) to purified intact *T. reesei* Cel7A. All conditions were incubated for 2 hours in 0.1 M pH 3 citric acid buffer. Both the unprocessed and processed Cel7A, by papain cleavage, were added as controls. Cel7A was also incubated without Endo T, as a control for the low pH. These results are not shown. The D129A mutant Endo T was not visible on SDS-PAGE because of a dilution error. There was also no visible protease activity after 2 hours incubation in 0.1 M pH 3 citric acid buffer for all the different ratios.
4.4 Protein crystallization, data collection and structure refinement

Crystallization conditions for Endo T are shown in table 3. These are the crystallization conditions, on which data were collected. The different data collections are shown in table 4. The final statistics for the refined Endo T models are shown in table 5. The final table 6, gives the Ramachandran plot for both new Endo T structures obtained.

Table 3: Different crystallization conditions on which data was collected.

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH 3</th>
<th>pH 6.5 (a)</th>
<th>pH 6.5 (b)</th>
<th>pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>citric acid</td>
<td>Bis-Tris</td>
<td>Bis-Tris</td>
<td>Hapes</td>
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<tr>
<td></td>
<td>0.1 M</td>
<td>0.1 M</td>
<td>0.1 M</td>
<td></td>
</tr>
<tr>
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<td>PEG 3350</td>
<td>PEG 3350</td>
<td>NaPO₄, PoPO₄</td>
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<tr>
<td>Protein</td>
<td>MonoQ</td>
<td>MonoQ</td>
<td>MonoQ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6-10 mg/ml</td>
<td>8 mg/ml</td>
<td>8 mg/ml</td>
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</tr>
<tr>
<td>Ion</td>
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<td>ZnCl₂</td>
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<td>0.02M</td>
</tr>
<tr>
<td>Time to appear</td>
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<td>1 month</td>
<td>1 month</td>
<td>1 month</td>
</tr>
<tr>
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<td><img src="image3.png" alt="image" /></td>
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</table>

Table 4: Data collection on the different crystals shown in table 3.

<table>
<thead>
<tr>
<th>Condition</th>
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<th>pH 6.5 (a)</th>
<th>pH 6.5 (b)</th>
<th>pH 7.5</th>
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</thead>
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<td>140</td>
<td>140</td>
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<tr>
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<td>Space group</td>
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<td>P21</td>
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<tr>
<td>Cell parameters (a,b,c)</td>
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<td>35.52,64.05,59.25</td>
<td>35.65,64.18,59.30</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
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<td>23.58-1.31</td>
<td>23.65-1.40</td>
</tr>
<tr>
<td>Average multiplicity</td>
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<td>2.8</td>
<td>2.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Completeness (%)</td>
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<td>98.1</td>
<td>98.1</td>
<td>99.6</td>
</tr>
<tr>
<td>R merge (%)</td>
<td>15.2</td>
<td>5.2</td>
<td>6.3</td>
<td>7.2</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>11.9</td>
<td>11.6</td>
<td>8.5</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Beam line at MAX-lab, Lund, Sweden

\[ R_{\text{merge}} = \Sigma_{\text{ijkl}} \Sigma_{i} | I_{ij} - \langle I \rangle | / \Sigma_{\text{ijkl}} \Sigma_{i} | I_{ij} | \]

\[ R = \Sigma | F_{o} - | F_{c} | / \Sigma | F_{o} | \]
All four crystallization conditions were solved; the pH 3 and pH 7.5 conditions were refined with their final refinement parameters in table 5. All structures were completely similar to previously solved structures, except the structure from the pH 3 condition. In this condition the N-terminus is both longer and in a different conformations in comparison to the three structures. The same protein and protein concentration was used in all 4 conditions. There was no difference between the structures at the C-terminus.
4.5 Structural comparison

4.5.1 Comparison to known proteins

A structural comparison between the active Endo T and all structural characterized endo-N-acetyl-β-D-glucosaminidases (Endo F1, F3 and Endo H) has been carried out in a previous MSc study (Digre 2010). In this study the differences in the active site of Endo T (T. reesei), Endo H (S. plicatus) and Endo F3 (E. meningoseptica) was described. The comparison of the overall structure of Endo T with other ENGases from GH family 18 has been made more recently. This comparison is shown in appendix 4. It was previously mentioned in this thesis that Endo T potentially also could have a protease activity in addition to its ENGases activity. Since Endo T loses its C-terminus very fast at acidic conditions. An acid protease from T. reesei with the same molecular mass as Endo T was described in a previous article (Eneyskaya, et al. 1999) Endo T was tested to have a protease activity and screened for a protease active site in the structural comparison shown in appendix 4.

Figure 23: The region in the protein sequence with the possible catalytic protease site in the structurally characterized ENGases. Potential catalytic amino acids in the sequence are colored yellow.

Since the structure of the active Endo T was solved, it was scanned for different protease active site motifs. One potential protease active site was site found, which has the sequence catalytic triade for a serine protease. The distances in the structure are not optimal, but proteins are known to be flexible. The serine and histidine residue of this potential catalytic triade are colored yellow in figure 23. Two possible acid protease active sites can also be found in the structure. It is much harder to predict the existence and position of an acid protease active site since only two aspartic acid residues are needed to for the active site of an acid protease.

4.5.2 Comparison to unknown proteins

A BLAST search with the Endo T sequence (using the BLAST server at Uniprot) against eukaryotic similar enzymes was carried out to compare Endo T to non-structural characterized homologous proteins. The Blast results of the BLAST search are shown in appendix 5. After the BLAST search, the identified sequences that had the highest similarity in amino acid sequence and function (ENGases) were selected and aligned. A genealogic tree and amino acid sequence alignment were generated from this. The genealogic tree is shown in appendix 6, a part of the alignment is shown in appendix 7. This part contains both the ENGase active site and the possible serine protease active site. In the alignment only the six first sequences contain both the serine and histidine residue in the potential protease active site of these proteins, this is shown in Figure 24. Endo FV (F. velutipes) was also included in the alignment, but lacks both the serine and histidine residue of the potential serine protease active site, while most of the proteins have the histidine residue. The other proteins, which were used in the sequence alignment are shown in appendices 5 to 7.

Figure 24: BLAST results, which could, just as Endo T have a possible protease active site. Serine and threonine residues are shown in green.

The proteins, which have both the ENGase active site and the possible serine protease active site, come from Metarhizium acridum (ENGase, E9EFP8, Metarhizium robertsii (ENGase, E9F659), Trichoderma reesei
ENGase, Magnaporthe oryzae (unknown function, A4RJB3), Sordaria macrospora (unknown function, D1ZGL1) and Podospora anserine (unknown function, B2AES6). All of these organisms are filamentous fungi. The protein from Magnaporthe oryzae has a threonine residue instead of a serine residue.

4.6 Possible protease active site

Since the structure of the active Endo T was already solved, it was checked for a possible protease active site. There were no free cysteine residues, which would exclude a potential cysteine proteases. One possible serine protease site was found, close to the ENGase active site. Two possible acid protease sites were also found, but after checking the structure one was excluded. Since the proteolysis occurs without any metals in the protein sample we can also exclude a possible metallo protease activity. Other protease activities are more specific and too difficult to screen in an alignment or a known structure. There is though no direct proof yet that Endo T actually possesses both ENGase and protease activity.

4.6.1 Serine protease

The possible serine protease (SP) active site consists of Ser 44, His 46 and possibly Asp 57 and Gly 87. This possible SP (colored blue in figure 25) site is located right next to the ENGase active site (colored red in figure 25). In between these two possible activity sites of Endo T is the loop going from β-sheet to α-helix 3, which forms some sort of barrier between the ENGase active site of Endo T and the potential serine protease active site of the enzyme.

In the ligand structure of Endo T, there were interactions between His 46 and the branching Man. The suggested Asp also interacted with this Man. There were no interactions with other residues in the protein. Figure 26 is the catalytic site of the protease trypsin a serine protease from pig (PDB code 1AN1). There are differences in distance and conformation between the amino acids building up the catalytic site of the trypsin protease and the amino acids building up the possible serine protease active site of Endo T. This difference between the active site of the two enzymes might be due to crystal packing interactions causing changes in the position of the potential catalytic amino acids of the two compared structures.

4.6.2 Acid protease

Two aspartic acids are needed in the active center of an acid protease (AP). After going through the sequence alignment and structure of Endo T, only one potential active site had two Aspartate residues in a conformation where the distances between these two residues could build up an AP active site. The two amino acids that potentially could build up an AP active site are Asp 202 and Asp 205. Figure 27 shows Endo T in green with the
previously described SP active site in blue, the ENGase active site in red and the AP active site in orange compared to the structure of Endo F3 in yellow. The Asp residues are not conserved in the known structures from the GH 18 family, nor in the unknown proteins identified in the BLAST search.

Figure 27: Cartoon representation of Endo T superimposed on Endo F3 in yellow, showing the possible acid protease active site in orange with Asp 202 and Asp 205 in α-helix 6 of Endo T. The ENGase active residues are shown in red, the previously described serine protease residues in blue.

5 Discussion

5.1 Biochemical characterization

5.1.1 C-terminal stability

The proteolytic cleavage of the C-terminus is both influenced by temperature and pH. With the addition of a protease inhibitor cocktail we are able to inhibit the proteolytic cleavage, except at very low pH. The bachelor student Jason Blomme did a more extent biochemical characterization (Blomme 2011). He showed that at pH 5 and pH 8 Endo T stays intact for more than 10 days, when the protease inhibitor cocktail is added. This control failed in the first experiments which were carried out. The protein samples were stored in the freezer, where the protease inhibitor cocktail is not stable. Therefore the protease inhibitor cocktail should be added fresh, before each incubation. At pH 5 the proteolysis is enhanced when the intact Endo T is stored at a higher temperature.

When we lower de pH at 21°C the proteolysis is also enhanced. Especially at pH 3 and lower, here complete degradation happens within a day and the protease inhibitor cocktail is not working. The inhibitor might not be stable at this pH or the protease activity is enhanced in such extent that the concentration of the inhibitor is insufficient. This posed big problems for the crystallization experiments, since the only crystallization condition of the active Endo T, which gave crystals for the intact Endo T was at pH 3 and lower. The protein in this crystal was therefore the processed Endo T and not the intact Endo T. This was verified on SDS-PAGE. This pushed us to screen for more crystallization conditions especially at higher pH. Three different crystallization conditions were found, two at pH 6.5 and one at pH 7.5. The actual pH was checked with a pH-strip and was lower than pH 5. The so called higher pH crystallization condition was also the processed Endo T. This was confirmed by SDS-PAGE and the crystals had the same shape as the previous crystals from the active Endo T.

5.1.2 Deglycosylation

The intact Endo T was incubated at 37 °C with protease inhibitors to let the active fraction of the unpurified Endo T sample deglycosylate the intact fraction. Deglycosylated proteins are easier to crystallize, a second reason for performing this experiment, was to see if the deglycosylation and proteolysis are linked or if they take place separately. We can conclude that deglycosylation and proteolysis are linked in fact. After the deglycosylation we had two bands on SDS-PAGE. If they would not be linked, we would only expect one, the completely deglycosylated Endo T with his C-terminus. Since two bands are detected, it seems that only one of the two glycosylation sites was deglycosylated. This was also confirmed by the experiments, which Jason Blomme carried out (Blomme 2011). He suggested that Asn 240, which is close to the cleavage site of the C-terminus, is not as accessible as the Asn 70.
5.2 Protease activity

Endo T gets very fast processed, especially at very low pH. The proteolytic cleavage of proteins has been described both in *T. reesei* and *P. pastoris*. We knew this could pose huge problems, even after purification. Therefore it could be that Endo T could have a protease activity. In a previous article from Eneyskaya et al., an acid protease from *T. reesei* was described (Eneyskaya, et al. 1999). The protein they describe has the same molecular weight as Endo T and behaves in the same way, as problems we encounter when we try to keep Endo T intact. Their protease activity increased massively when the pH was lower than pH 3. They were able to inhibit the protease activity at this low pH, but only when equimolar quantities of pepstatin were added to the protease. Cel7A was one of the substrates Eneyskaya et al. tried for this acid protease. After incubating Endo T with Cel7A for two hours at different enzyme/substrate ratios, no proteolytic cleavage was detected. One possible serine protease active site was found when the structure was screened.

5.3 Endo T structure

We tried to crystallize the intact Endo T, but we were not able to suppress the proteolysis. Several structures were solved at different pH's. Two models were refined to compare the structure of Endo T at low and more neutral pH.

5.3.1 N-terminus

There were no big differences in the structure, except at the N-terminus. Endo T was expressed in *P. pastoris* and since we used a construct, four more amino acids (EAEA) are found at the N-terminus. As is shown in figure 30, the orientation of the N-terminus changed. The longer N-terminus goes towards the ENGase active site of another Endo T molecule in the crystal structure. This did not change in the structure of Endo T at pH 7.5, where the four extra amino acids were not detected in the electron density map. The crystal at pH 3 had a different form (tetrahedral) then the crystal in the other crystallization conditions (cubic). Therefore it is possible that due to the pH and maybe enhanced protease activity, the N-terminus is folding to the molecule next to it. We don’t know if the structure at pH 7.5 lacked these four amino acids or that the N-terminus was too floppy to be seen in the electron density map.

5.3.2 C-terminus

Since we were not able to control the proteolysis, we were not able to solve the structure of the intact Endo T. Previously was shown that Endo T becomes active when it is both deglycosylated and proteolytic cleaved. Where the proteolytic cleavage is the main drive for activation. This activation is strongly pH dependent. There are two hypotheses why the intact Endo T is inactive. The 47 amino acid long C-terminus could fold back to the top of the $(\beta/\alpha)_8$ TIM barrel. A second hypothesis is that the C-terminus induces conformational changes in the helices that disturb the ENGase active site at the top of the $(\beta/\alpha)_8$ TIM barrel. When we look at figure 31 the C-terminus is colored dark orange and could fold back towards the active site. There is more room in between the $\alpha$-helices at the C-terminus then there is between the other $\alpha$-helices. Whether the C-terminus would fold back between the dark orange and shorter helix or between the shorter and light orange helix is not clear, both options could be possible. In figure 32 we can see that there is direct access to the active
site. When we even look closer at figure 33, we can imagine that when the C-terminus folds back, the substrate might not be able to reach the active site, since it might be hindered by the C-terminus. The second hypothesis is that the C-terminus could induce conformational changes in one of the three helices shown on figures 31 to 33. The short middle helix, has a very long loop at the top. If the C-terminus would change the conformation of this middle helix, the loop could have a very big influence on the active site. Whether it would change the conformation of Asp 129 and Glu 131 or whether it would block the substrate binding is again a pure guess. The right, light orange helix in figure 31 is directly connected to the possible serine protease site. Conformational changes in this helix could alter the conformation of the Ser and His forming a serine protease active site. The helix to the right of the light orange helix in figure 31 is not parallel with the other helices. This helix is turned over approximately 45°.

5.1 Concluding remarks
We can conclude that due to the fact that we could not keep the proteolytic cleavage under control, the structure of the intact Endo T was not solved. Few hypotheses could be made on how the intact Endo T might be activated, but there is no new direct proof supporting these hypotheses. The C-terminus is stable at pH 5 for 6 days, without any inhibitors. At pH 3 it gets processed within 2 hours and is completely processed within the day. This poses a perpetual problem to solve the structure. One hypothesis, is that Endo T could have protease activity. Therefore we tested Endo T for protease activity and screened its structure for a possible protease active site. A possible serine protease active site has been found close to the ENGase active site. The activity of this site might be influenced by the C-terminus itself, since it is so close to the ENGase active site. This and a previous article describing an acid protease from T. reesei, which has the same molecular weight as Endo T, suggested us that Endo T could have a protease activity.
The protease activity should be tested at lower pH and longer incubation time to determine if Endo T has a protease activity. The specific protease activity should be determined, new protease inhibitors or higher concentration of inhibitors should be tested to inhibit the protease activity. This might control the proteolysis and help to solve the structure of the intact Endo T. Specific mutations could then be made in the suggested protease active sites when the specific protease activity has been found and to determine if the proteolytic cleavage of the intact Endo T is due to itself or another protease.

6 Acknowledgments
Both my supervisors Dr. Mats Sandgren and Dr. Ingeborg Stals gave me the opportunity to perform this interesting research in Uppsala, Sweden. I couldn’t have done a fraction what I did and learned without their help and support. I thank Dr. Mats Sandgren specifically for his help with my accommodation, staying, and the scientific and personal problems we discussed in Sweden. I thank Dr. Ingeborg Stals for her help in understanding of the Endo T protein and the scientific discussions. I thank the entire group of molecular biology at the Swedish University of Agricultural Sciences for their help and support and the many coffee brakes we had together. Saeid Karkhehabadi for his help with the purification and physical characteristics of X-ray crystallography. Nils Mikkelsen for his eternal patience and time he spent answering all my questions in solving the structure of the Endo T protein. Roland Berghdahl for all the moments we spent together during experiments.
References


8 Appendix

8.1 Appendix 1: Bio-Rad assay results

![Standard BSA curve](image)

Figure 32: BSA standard curve for total protein concentration.

Table 7: Unpurified Endo T total protein concentrations.

<table>
<thead>
<tr>
<th>Endo T</th>
<th>Without inhibitors</th>
<th>Endo T</th>
<th>With inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>dilution</td>
<td>absorbance</td>
<td>concentration</td>
<td>absorbance</td>
</tr>
<tr>
<td>10</td>
<td>0.314</td>
<td>3.143</td>
<td>0.887</td>
</tr>
<tr>
<td>20</td>
<td>0.126</td>
<td>2.529</td>
<td>0.505</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>0.185</td>
<td>9.259</td>
</tr>
<tr>
<td>average</td>
<td>concentration</td>
<td>average</td>
<td>concentration</td>
</tr>
<tr>
<td></td>
<td>2.836</td>
<td></td>
<td>9.407</td>
</tr>
</tbody>
</table>

8.2 Appendix 2: Purified protein concentrations

Table 8: Purified Endo T protein concentrations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Q1F1</th>
<th>Q2F1</th>
<th>Q1F2</th>
<th>Q2F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>19.81</td>
<td>8.56</td>
<td>14.46</td>
<td>5.68</td>
</tr>
<tr>
<td>Concentration (mg/ml)</td>
<td>13.76</td>
<td>5.94</td>
<td>10.00</td>
<td>3.94</td>
</tr>
<tr>
<td>Final volume</td>
<td>&lt; 500 µl</td>
<td>± 750 µl</td>
<td>± 200 µl</td>
<td>± 750 µl</td>
</tr>
<tr>
<td>260/280 value</td>
<td>0.68</td>
<td>0.68</td>
<td>0.71</td>
<td>0.65</td>
</tr>
</tbody>
</table>

QxFy: x stands for the batch, while the y stands for the peak collected.
8.3 Appendix 3: Western blot SDS-PAGE

Figure 33: First SDS-PAGE for western blot.

Figure 34: Second SDS-PAGE for western blot.
Appendix 4: Structural comparison of known endo-β-D-glucosaminidases
### 8.5 Appendix 5: Endo T eukaryotic blast results

<table>
<thead>
<tr>
<th>Accession</th>
<th>Entry name</th>
<th>Protein names</th>
<th>Organism</th>
<th>Gene names</th>
</tr>
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<tr>
<td>E9EF8P</td>
<td>E9EF8P_METAQ</td>
<td>Endo-N-acetyl-beta-D-glucosaminidase</td>
<td>Metarhizium acridum (strain CQMa 102)</td>
<td>MAC_08696</td>
</tr>
<tr>
<td>E9F689</td>
<td>E9F689_METAR</td>
<td>Endo-N-acetyl-beta-D-glucosaminidase</td>
<td>Metarhizium robertsi (strain ARSEF 23) (Metarhizium anisopliae)</td>
<td>MAA_07758</td>
</tr>
<tr>
<td>C4RA89</td>
<td>C4RA89_TRIE</td>
<td>Endo-N-acetyl-beta-D-glucosaminidase</td>
<td>Trichoderma reesei (Hypocrea jeecina)</td>
<td>endoT</td>
</tr>
<tr>
<td>A4RJ93</td>
<td>A4RJ93_MAG07</td>
<td>Putative uncharacterized protein</td>
<td>Magnaporthe oryzae (strain 70-15 / FGSC B68) (Rice blast fungus) (Pyricularia oryzae)</td>
<td>MGG_01876</td>
</tr>
<tr>
<td>D1ZG1L</td>
<td>D1ZG1L_SORMK</td>
<td>Whole genome shotgun sequence assembly, scaff...</td>
<td>Sordaria macrospora (strain ATCC MYA-333 / DSM 957 / K(L)3346 / K-hell)</td>
<td>SMAC_05669</td>
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<tr>
<td>B2AES6</td>
<td>B2AES6_PODAN</td>
<td>Predicted CDS Pa_5_1650</td>
<td>Podospora anserina (strain S / DSM 960 / FGSC 10363) (Pleurose anserina)</td>
<td></td>
</tr>
<tr>
<td>A7EB85</td>
<td>A7EB85_SCLS1</td>
<td>Putative uncharacterized protein</td>
<td>Sclerotinia sclerotiorum (strain ATCC 19883 / 1980 / Sa-1) (White mold) (Whizeltinia sclerotiorum)</td>
<td>SSI0_01513</td>
</tr>
<tr>
<td>A6RY8P</td>
<td>A6RY8P_BOF8</td>
<td>Putative uncharacterized protein</td>
<td>Botryotinia fuckeliana (strain B5.10) (Noble rot fungus) (Botrytis cinerea)</td>
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<tr>
<td>A4R801</td>
<td>A4R801_MAG07</td>
<td>Putative uncharacterized protein</td>
<td>Magnaporthe oryzae (strain 70-15 / FGSC B68) (Rice blast fungus) (Pyricularia oryzae)</td>
<td>MGG_04073</td>
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<tr>
<td>E9F229</td>
<td>E9F229_METAR</td>
<td>Alkaline phosphatase</td>
<td>Metarhizium robusti (strain ARSEF 23) (Metarhizium anisopliae)</td>
<td>MAA_06227</td>
</tr>
<tr>
<td>E3ED1C</td>
<td>E3ED1C_METAQ</td>
<td>Alkaline phosphatase</td>
<td>Metarhizium acridum (strain CQMa 102)</td>
<td>MAC_07696</td>
</tr>
<tr>
<td>C7YP6</td>
<td>C7YP6_NEC0H</td>
<td>Glycoside hydrolase family 10</td>
<td>Nectria haematococca (strain 77-12-4 / FGSC 9596 / MPVI (Fusarium solani subsp. pis))</td>
<td>NECHADRAFT_49120</td>
</tr>
<tr>
<td>Q871X2</td>
<td>Q871X2_NEUCS</td>
<td>Related to chitinase</td>
<td>Neurospora crassa</td>
<td>B2017_040</td>
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<td>F5HF13_NEUCR</td>
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<td>Whole genome shotgun sequence assembly, scaff...</td>
<td>Sordaria macrospora (strain ATCC MYA-333 / DSM 997 / K(L)3346 / K-hell)</td>
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<td>B26W7B</td>
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<td>Predicted CDS Pa_2_9140</td>
<td>Podospora anserina (strain S / DSM 960 / FGSC 10363) (Pleurose anserina)</td>
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<tr>
<td>E3Q263</td>
<td>E3Q263_COLGC</td>
<td>Chitinase</td>
<td>Colletotrichum graminicola (strain M1.001 / M2 / FGSC 10212) (Maize anthracnose fungus) (Glomerella graminicola)</td>
<td>QRG0_11296</td>
</tr>
<tr>
<td>C9SNX0</td>
<td>C9SNX0_VER1</td>
<td>Chitinase</td>
<td>Verticillium albo-atrum (strain VaMs 102) (Verticillium albidum)</td>
<td>VDBG_06395</td>
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<tr>
<td>CSFI04</td>
<td>CSFI04_ARTGC3</td>
<td>Chitinase 3</td>
<td>Anthrdera ate (strain CBS 113480) (Microsporum canis)</td>
<td>MYCG_01803</td>
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<td>E4U03E</td>
<td>E4U03E_ARTGC3</td>
<td>Chitinase 3</td>
<td>Anthrdera gypsea (strain ATCC MYA-4064 / CBS 118933) (Microsporum gypseum)</td>
<td>MYGG_04265</td>
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<tr>
<td>D5G33S35</td>
<td>D5G33S3_TDBDMM</td>
<td>Whole genome shotgun sequence assembly, scaff...</td>
<td>Tuber melanosporum (strain Me209) (Pungent black truffle)</td>
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<tr>
<td>DBOCH6</td>
<td>DBOCH6_SUCHM</td>
<td>Glycoside hydrolase family 16 protein</td>
<td>Schizophyllum commune (strain H4-8 / FGSC 9210) (Split gill fungus)</td>
<td>SCHOCOPOST_0664</td>
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<tr>
<td>D1G498</td>
<td>D1G498_FLAYE</td>
<td>Endo-beta-N-acetylglucosaminidase</td>
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<tr>
<td>B0HY47</td>
<td>B0HY47_LAC85</td>
<td>Glycoside hydrolase family 18 protein</td>
<td>Laccaria bicolor (strain S239N-H82) (Bicoloured deceiver) (Laccaria laccata var. bicolor)</td>
<td>LACBODRAFT_185397</td>
</tr>
</tbody>
</table>

Figure 36: Endo T eukaryotic blast results.
Figure 37: Genealogic tree from Endo T BLAST results.

Figure 38: Sequence alignment from Endo T BLAST results.