*Botanik*

# Molecular biological and biochemical studies of proteolytic enzymes of the cereal pathogen *Fusarium graminearum*

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*groovy, groovy, funky fungi*

*- anonymous 2003 -*

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# Abbreviations

| | |
|---|---|
| AGP | arabino galactane protein |
| A. dest | *Aqua destillata* |
| A. bidest | *Aqua bidestillata* |
| ATP | adenosine triphosphate |
| BLAST | basic local alignment search tool |
| bp | base pair(s) |
| cDNA | complementary DNA |
| CDS | coding sequence |
| CM | complete medium |
| CWDE | cell wall degrading enzyme(s) |
| dpi | days past inoculation |
| dNTP | deoxyribonucleotide mix (dATP, dCTP, dGTP, dTTP) |
| E-64 | trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane |
| EBI | european bioinformatics insitute |
| EDTA | ethylene diamine tetraacetic acid |
| EMBL | european molecular biological laboratory |
| et al. | et altera (lat: and others) |
| Fw | fresh weight |
| gCM | gelatine-containing complete medium |
| gDNA | genomic DNA |
| GRP | glycine-rich protein |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hpi | hours past infection |
| HR | hypersensitive response |
| HRGP | hydroxyproline-rich protein |
| IPTG | isopropyl-beta-D-thiogalactopyranoside |
| kb | kilo bases (1000 base pairs) |
| kDa | kilo dalton |
| M | molar (mol / litre) |
| MES | 2-(N-morpholino)-ethanesulfonic acid |
| min | minute(s) |
| mRNA | messenger RNA |
| MW | molecular weight |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PCR | polymerase chain reaction |
| pI | isoelectric point |
| PI | protease induction (medium) |
| PMSF | phenyl methyl sulfonyl fluoride |
| pPI | predigested protease induction (medium) |
| PRP | proline-rich protein |
| PR-protein | pathogenesis-related protein |
| RNA | ribonucleic acid |
| rpm | rotations per minute |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SDS | sodium dodecyl sulfate |
| Soy.Trp.Inh. | soybean trypsin inhibitor |
| TAIL PCR | thermal assymetric interlaced polymerase chain reaction |
| TBE | tris borate EDTA buffer |
| TRIS | tris(hydroxymethyl)aminomethane |
| U | unit |
| UV | ultra violet |
| v/v | volume per volume |
| w/v | weight per volume |
| X-Gal | 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside |

# 1 Introduction

Fungi – like animals – are heterotrophic organisms that depend on exogenous sources of organic material for surviving. By far the majority of fungi are saprophytes which make use of dead material and play a leading role in the recycling of nutrients. Others form mutualistic symbioses with other organisms, the most common examples being the mycorrhizal fungi – which provide enhanced mineral uptake to the plant host and profit from supplied carbon compounds [Cairney, 2000] – and lichens, which are formed together with algae or cyanobacteria [Honegger, 1993]. In these associations, both partners benefit from each other.

The interaction of pathogens with their hosts potentially causes damage to the attacked organism. This ranges from the exploitation of the resources of the target – thus limiting its viability – to killing the host and feeding on the remains. Biotrophic pathogens like the rust fungi have developed sophisticated methods to colonize the target without being noticed and make use of the metabolism of the living tissue [Mendgen et al., 2000]. Perthotrophs on the other hand destroy the colonized tissue at some point in time during the infection process and absorb nutrients from the dead cells. Necrotrophs kill the tissue before colonizing the host [Schlösser, 1997].

However, plants are not defenseless. In fact, it is estimated that only about 2% of the known fungal species are able to colonize plants and cause disease [Buchanan et al., 2000]. Even though plants are in permanent contact with potential pathogens (e.g. fungi, bacteria, viruses) successful infection is rarely established.

If a phytopathogen succeeds in invading the plant, the interaction is called compatible, the host is susceptible and the pathogen is virulent. In most cases however the plant is resistant to potential pathogens, the interaction is termed incompatible and the pathogen is avirulent to this target. The general ability of a pathogen to cause disease symtoms in a given host is called pathogenicity. Virulence describes the relative capacity of pathogenicity or agressiveness against the target [Hoffmann et al., 1994; Schlösser, 1997].

In the following chapter the basic concepts of resistance mechanisms available to plants will be outlined. Further focus will then be placed on the plant cell wall which represents a major barrier to the pathogen during the infection and colonization of plant tissue. Cell wall degrading enzymes including proteases as a means of the pathogen to breach this barrier will be described and the classification system of proteases will be listed. Finally, *Fusarium graminearum,* a phytopathogenic fungus that is the subject of research in the present work will be introduced.

## 1.1 Introduction to plant disease resistance mechanisms

The general resistance of plants against most pathogens has been named 'horizontal resistance' [VAN DER PLANK, 1968] or '**non-host-resistance**' [HEATH, 1981]. This reflects the fact that the plant is not a suitable target for infection by a specific pathogen due to preformed, **passive resistance mechanisms** resulting in 'basic incompatibility'. These resistance mechanisms comprise structural barriers and toxic compounds that are present in the unaffected, healthy plant and limit successful infection to specialized pathogens that have the ability to overcome these factors and therefore exhibit 'basic compatibility'. Examples of structural barriers are the epidermis with the cuticle and the rhizodermis. Pathogens have to either breach these barriers with cutinases and / or cell wall degrading enzymes or gain entrance through natural openings (stomata) or wounds [MOERSCHBACHER AND MENDGEN, 2000]. After successfully establishing contact with the plant tissue, pathogens are often confronted with preformed chemical components named phytoanticipins [VAN ETTEN, 1994]. This term comprises a variety of compounds produced by different biosynthetic pathways which possess antimicrobial properties. These low-molecular weight secondary metabolites are mainly stored in inactive form in the vacuoles or organelles and are released upon destruction of the cells. Since destroying the integrity of the plant tissue is part of the colonization strategy by pertotrophic and netrotrophic fungi, phytoanticipins represent an important resistance mechanism against these pathogens. The compatible interaction requires the ability of the fungus to inactivate or tolerate these components [MANSFIELD, 2000].

In addition to constitutive resistance mechanisms plants have the ability to react to potentially harmful external influences. These influences elicit **induced resistance mechanisms**, hence they are termed 'elicitors'. Elicitors are grouped into 'abiotic elicitors' like heavy metals, extreme temperatures or UV irradiation and 'biotic elicitors' that are derived from living organisms [EBEL, 1986; DIXON AND LAMB, 1990]. The latter are further divided into 'exogeneous elicitors' originating from the pathogen and 'endogenous elicitors' released from the host by action of the pathogen. Presently the role of oligo- and polysaccharides that are released from the fungal or plant cell wall by hydrolytic enzymes are best understood. However, representatives of other substance classes like peptides, proteins, glycoproteins and fatty acids are also known to elicit defense reactions [DIXON AND LAMB, 1990; ELSTNER ET AL., 1996].

The resistance of a specific cultivar of a plant species against a specific race of a pathogen is called '**cultivar resistance**' [HEATH, 1981] or 'vertical resistance' [VAN DER PLANK, 1968]. A prerequisite for the cultivar resistance is the recognition of the pathogen by the host. According to the 'gene-for-gene concept' [FLOR, 1971] this is mediated by

corresponding combinations of plant resistance genes (R genes) and pathogen avirulence genes and their products (Avr proteins) which are also termed 'specific elicitors' in contrast to the 'unspecific elicitors' which elicit defense reactions in the interaction with a broad range of pathogens. Some plant resistance genes have been shown to be part of signal transduction pathways leading to complex defense responses [Martin, 1999; Boller and Keen, 2000].

Induced resistance mechanisms include the production of reactive oxygen species, accumulation of phytoalexins and pathogenesis related proteins (PR-proteins), rapid cell death (hypersensitive response) and cell wall modifications.

The massive production of reactive oxygen species ($H_2O_2$, $\bullet O_2^-$, •OH) is observed within minutes to hours in elicited plant cell cultures and is amongst the fastest reactions to pathogen attack [Wojtaszek, 1997]. The so called 'oxidative burst' is involved in diverse defense mechanisms like direct toxic effects on the pathogen, signal transduction within the plant, rapid cell death and the oxidative linkage of cell wall structural proteins [Otte and Barz, 1996; Lamb and Dixon, 1997; Bolwell, 1999; Dat et al., 2000].

Phytoalexins are comparable to the phytoanticipins listed above. In contrast to these preformed antimicrobial components they are synthesized *de novo* after elicitation [Barz, 1997; Mansfield, 2000].

PR-proteins are proteins that are synthesized in response to elicitation. They are either secreted into the apoplast or stored in the vacuole and are mainly found at the primary site of infection. Accumulation in more remote parts of the plant has also been described. PR-proteins include chitinases and β-1,3-glucanases which are capable of attacking the fungal cell wall as well as peroxidases, proteases and protease inhibitors all of which are discussed as having antimicrobial effects. For some PR-proteins however, it was not yet possible to assign a function [Bol et al., 1990; Stintzi et al., 1993; Broekaert et al., 2000].

The hypersensitive response (HR) is an active, genetically determined process that is characterized by the rapid death of cells at the site of pathogen contact and the accumulation of phenolic compounds [Moerschbacher and Reisener, 1997]. It is regarded as a means to withdraw the basis for nutrition of the pathogen as well as to form a barrier to prevent it from colonizing the plant tissue. However the exact nature of the HR with respect to resistance against pathogens is subject to controverse discussion [Heath, 2000; Jabs and Slusarenko, 2000]

Cell wall modifications enhance the resistance of the plant cell wall to pathogen attack. Reactive oxygen species produced during the oxidative burst mediate the cross-linking of pre-formed structural proteins and phenolic compounds with the aid of peroxidases localized in the cell wall [Fry, 1986; Bradley et al., 1992; Otte and Barz, 1996; Wojtaszek, 1997; Otte and Barz, 2000]. Dimerization of tyrosine residues within structural proteins to isodityrosine has been proposed as an important mechanism of the peroxidative formation of complex protein networks in the cell wall [Brady, 1996]. Furthermore, pectin or arabinoxylan molecules can be cross-linked in a similar fashion via attached ferulic acids that form diferulate bridges and the polymerization of monolignols to lignin is also dependent on peroxidative radicalization [Moerschbacher and Mendgen, 2000]. These rapid reactions change the physical properties of the cell wall and make it less vulnerable to penetration by the pathogen.

Another defense reaction of the plant that is related to the mechanisms of cell wall modification is the rapid formation of papillae – localized appositions of dense material between the plasmalemma and the cell wall at the penetration site of the pathogen. They are composed of cross-linked proteins, phenolic compounds and callose (β-1,3-glucan) [Heitefuss, 1997].

Both, cell wall strengthening and the formation of papillae, are not regarded as defense reactions that can completely stop the pathogen on their own. Instead, they are believed to slow it down to gain valuable time for the initiation of defense reactions that require gene activation and transcription of proteins (hypersensitive reaction, synthesis of phytoalexins and PR-proteins) [Lamb and Dixon, 1997]. Hence, the ability of the pathogen to quickly degrade the cell wall is crucial for pathogenicity. To demonstrate the complexity of this task, the structure of the cell walls of dicot plants and grasses will be presented in the next chapter. Emphasis will be placed on the structural proteins and the differences between dicot and grass cell walls.

## 1.2 The structure of plant cell walls

Plant cells are surrounded by a complex network that is mainly composed of cellulose microfibrils which are embedded in a complex matrix of pectins, hemicelluloses and proteins [Carpita and Gibeaut, 1993]. The cellulose fibers form an extended network that is responsible for the stability of the cell wall while the hemicelluloses and pectins are arranged in a less strict order. The hemicelluloses are closely attached to cellulose by hydrogen bonds. Pectin polymers are associated with water molecules and $Ca^{2+}$ ions to form a gel like matrix. The different polymers are thought to form separate networks that

are only loosely intertwined with each other. Hence the term 'multi network gel' was applied [SCHINDLER, 1993; ALBERSHEIM ET AL., 1994; BRAAM, 1999]. Depending on the specialization of the different cell types, the cell wall can also be strengthened and impregnated by lignin, suberin, cutin and waxes.

In addition, the cell wall contains many different proteins and glycoproteins which fulfil structural and enzymatic functions. The cell wall associated enzymes can be roughly devided into three groups. Enzymes which modify polysaccharides of the cell wall include endoglucanases, xylosidases, pectinases, pectin methylesterases and xyloglucan endotransglycosylases. Enzymes that act on other substrates present in the cell wall include invertase, peroxidase, phosphatase and various dehydrogenases. Enzymes with potential activity against fungal pathogens include chitinases and β-1,3-glucanases [COSGROVE, 1997]. Thionins, small proteins that interact with lipid membranes and are toxic to bacteria and fungi, have also been located in the cell wall [FLORACK AND STIEKEMA, 1994].

Structural proteins account for the major amount of protein in the cell wall. They can be divided into four groups based on structural motifs and distinct differences in their predominant amino acid composition. With the exception of the glycine-rich proteins they all contain considerable amounts of hydroxyproline (post-translationally modified proline) and are glycosylated [SHOWALTER, 1993; CASSAB, 1998].

The hydroxyprolin-rich glycoproteins (HRGPs) of dicot plants are characterized by the high content in hydroxyproline and a conserved pentapeptid motif (ser-hyp-hyp-hyp-hyp; ser = serine, hyp = hydroxyproline). Their molecular structure is dominated by the so called 'polyproline helix' which – together with arabinose and galactose sidechains – gives them a rod-like structure. The cell walls of grasses contain a different form of HRGPs. The grass homologues are threonine-hydroxyproline-rich glycoproteins which have a higher threonine content and contain reduced amounts of hydroxyproline [CARPITA, 1996].

Proline-rich proteins (PRPs) also contain hydroxyproline, but to a lesser extent than HRGPs. Furthermore, they are lacking the characteristic Ser-$Hyp_4$ motif. PRPs have been discussed as being part of the lignification process of the cells in vascular bundles [RYSER ET AL., 1997].

Arabinogalactan proteins (AGPs) are discussed as being involved in the signal exchange mechanisms between the cytoplasm and the cell wall or between adjacent cells. They are also thought to play a role in wound healing, cell adhesion and morphogenesis. AGPs apparently are not covalently linked to the cell wall. Highly branched arabinogalactan side chains account for up to 98% of their molecular weight [COSGROVE, 1997; SHOWALTER, 2001].

Glycine-rich proteins (GRPs) are a diverse group of proteins which share an extremely high content of glycine (up to 70%) arranged in short repetitive units [Showalter, 1993]. In contrast to the other groups of cell wall structural proteins listed above, they are not glycosylated. GRPs apparently are not limited to the occurrence in the cell wall, but are also found in the cytoplasm. Some GRPs contain domains that interact with nucleic acids. The expression of GRP-encoding genes in response to a multitude of exogeneous and endogeneous factors like circadian rhythms, phytohormones, wounding, water and temperature stress as well as lighting conditions has been described [Sachetto-Martins, 2000]. GPRs are also expressed during the early stages of nodulation in legumes and in response to pathogen infection [Cornels et al., 2000; Kevei et al., 2002]. Amongst the diverse functions that are proposed for GRPs are cell wall repair mechanisms, involvement in lignin anchoring, cell adhesion, gene regulation and RNA processing [Sachetto-Martins, 2000].

PRPs, GRPs and particularly HRGPs are partially bound in the cell wall in maturing cells. A rapid insolubilization by oxidative cross-linking has been shown in response to elicitation and pathogen attack (see above). This suggests that despite the fact that the prevailing components of the cell wall are carbohydrate polymers, structural proteins are an important part of the defensive potential of the cell wall. Figure 1.1 shows a schematic overview of the basic composition of the cell wall of dicots as well as some cell wall modifications in response to elicitation.

The cell walls of dicot plants (type I cell walls) and grasses (type II cell walls) differ in the nature of their components, though the underlying principles of cell wall architecture are similar [Carpita and Gibeaut, 1993]. Both contain a fundamental network of cellulose fibers, but they vary in the relative amounts and the chemical structure of their hemicelluloses and pectins. The major hemicellulose in dicot cell walls is xyloglucan while the cell wall of grasses contains mainly glucuronoarabinoxylan and only small amounts of xyloglucan. Furthermore, type II cell walls contain considerably less pectin than type I cell walls [Carpita and Gibeaut, 1993; Wiethölter et al., 2003]. Differences in the types of hydroxyproline-rich proteins have already been mentioned above. The amount of structural proteins is reduced in the cell walls of grasses with a protein content of about 2 – 10% compared to 10 – 20% in dicot cell walls. An overview of these differences between the cell walls of dicots and grasses is given in table 1.1.

**Table 1.1: Some aspects of the primary cell walls of grasses compared to primary cell walls of dicot plants.** (MOERSCHBACHER AND MENDGEN, 2000; modified)

| | Grass cell walls | Dicot cell walls |
|---|---|---|
| **Microfibrils** (cellulose) | ~20% | ~20% |
| **Major hemicellulose** | Glucoronoarabinoxylan, ~50% | Xyloglucan, ~20% |
| **Pectins** | ~10% | ~30% |
| **Structural proteins** | THRGP, PRP, GRP, AGP<br>~2 – 10% | HRGP, PRP, GRP, AGP<br>~10 – 20% |

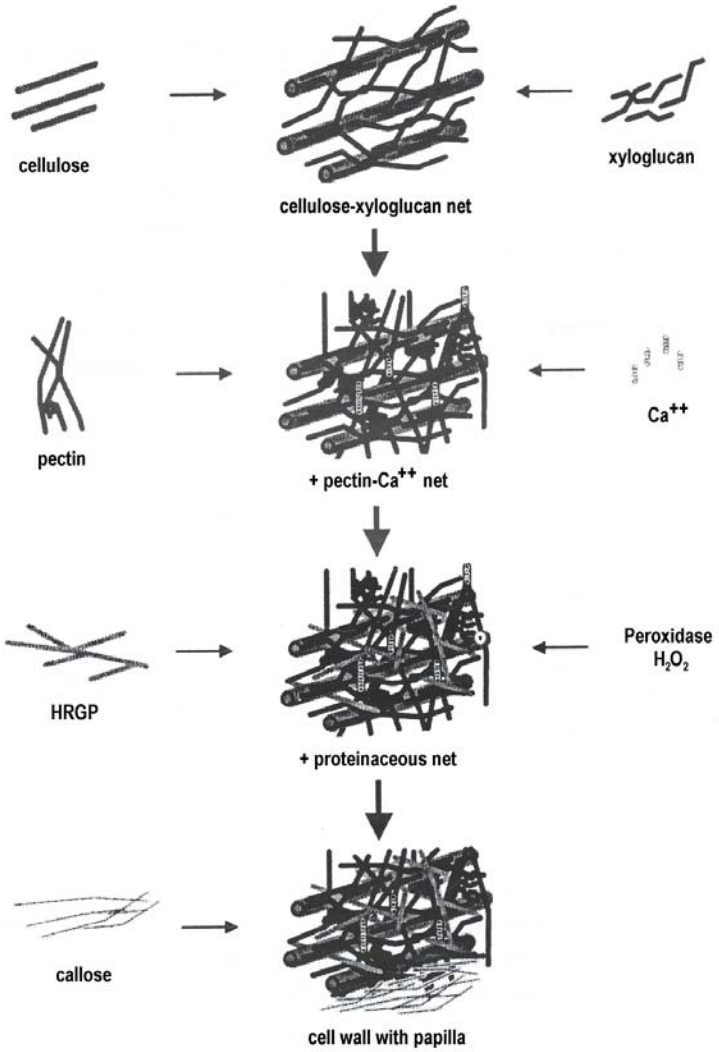


**Figure 1.1: Schematic diagram of the composition of the dicot cell wall (type I cell wall).** Shown modifications of the protein components of the cell wall in response to elicitation are the peroxidative cross-linking of structural proteins (particularly hydroxyproline-rich glycoproteins, HRGPs) and the apposition of callose (MOERSCHBACHER AND MENDGEN, 2000; modified)

## 1.3 Cell wall degrading enzymes

The structural complexity of the cell wall indicates that pathogens with the ability to penetrate the cell wall must possess elaborate tools to degrade a multitude of different components. Hence, the expression of cell wall degrading enzymes during pathogenicity is subject to substantial research effort [WALTON, 1994; HAHN ET AL., 1997].

Many saprophytic and plant pathogenic fungi secrete a large spectrum of enzymes that can degrade cutin and cell wall components. However, it is difficult to differentiate between enzymes that serve primarily nutritional purposes and those that are used to penetrate the cell wall during pathogenesis.

While enzymes capable of cleaving carbohydrate polymers in the plant cell wall (e.g. cellulases, xylanases, pectate lyases) and esterases that act on the cuticula of plants (cutinases) are generally accepted as important factors for phytopathogenicity of fungi, proteases are mostly disregarded as cell wall degrading enzymes. However, the oxidative crosslinking of hydroxyproline-rich glycoproteins and the accumulation of PR-proteins in reaction to elicitation pathogen attack that have been shown for dicot and grass species alike are examples that show the importance of cell wall proteins in the defense reactions of the plant [BROWN ET AL., 1998; MUTHUKRISHNAN ET AL., 2001; KANG AND BUCHENAUER, 2003]. Particularly during the early infection process – when quick colonization of the host tissue is crucial for successful infection – the pathogen has to cope with rapid cell wall modifications. In synergy with the carbohydrate degrading enzymes proteases are certainly important tools to overcome the plant defense.

Until the recent years research on proteases as factors in pathogenicity has mainly been focused on pathogens of animals and man. Since the extracellular matrix of animal cells – the equivalent to the cell wall of plants - is mainly built of proteins and glycoproteins, it is not surprising that pathogens utlize proteases during the colonization of the host tissue. Proteases have been identified as virulence factors in several pathogenic microorganisms. Examples include filamentous fungi like *Aspergillus fumigatus* which causes diseases of the human respiratory tract [REICHARD ET AL., 2000], yeasts like *Candida albicans* that causes mucosal infections [SCHALLER ET AL., 1999] and bacteria like *Burkholderia pseudomallei* which is responsible for melioidosis in human and animals [LEE AND LIU, 2000].

Proteases associated with pathogenesis have been found in all mechanistic types of proteolytic enzymes. Therefore the following chapter will give a brief overview of the classification of proteases.

## 1.4 General classification of proteases

According to the *Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (NC-IUBMB) proteases (= peptidases) are sorted under the signature 'hydrolases acting on peptide bonds' (EC 3.4). They are generally separated into two groups based on the catalysed cleavage of peptide bonds at the end of a peptide (exopeptidases) or within a peptide (endopeptidases) (see figure 1.2). A special group of peptidases are the omega peptidases that cleave peptide linkages other than those of $\alpha$–carboxyl to $\alpha$-amino groups.

Exopeptidases are further classified as aminopeptidases or carboxypeptidases depending on their action on the amino- or carboxyterminus of the substrate. The number of amino acids which are cleaved from the substrate and the specificity for defined amino acids gives the final classification level.

The large group of the endopeptidases is subdivided based on characteristic amino acids that are essential for the catalytic mechanism into serine endopeptidases, cysteine endopeptidases, aspartic endopeptidases, metalloendopeptidases and threonine endopeptidases. Only relatively few threonine endopeptidases have been described and they are usually included with the serine endopeptidases. Endopeptidases that act on substrates smaller than proteins are referred to as oligopeptidases.

Figure 1.2 gives an overview about the main groups of proteases together with their classification according to the EC nomenclature.

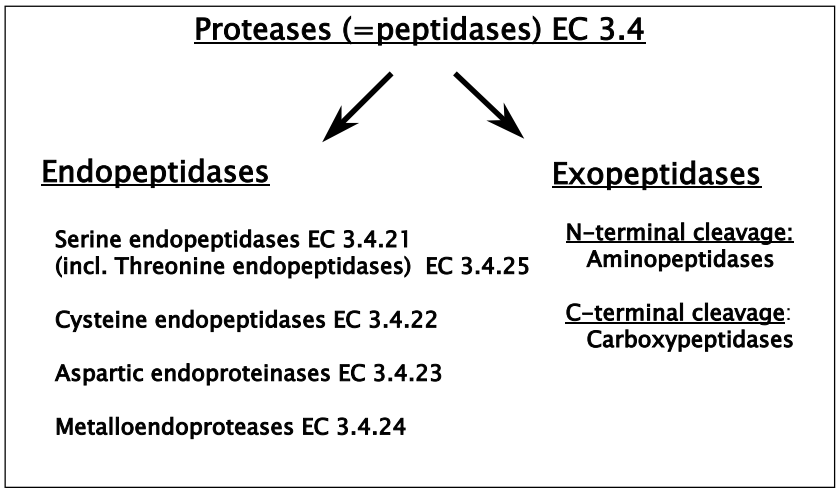


**Figure 1.2: Classification of proteases according to the EC nomenclature**

Only the main groups of peptidases are shown. Further detail is listed in the text.

The five catalytic types of proteases are further subdivided into clans and families based on comparisons of their tertiary structure and the order of the catalytic residues in the amino acid sequence. As an example the serine proteases with the comprised clans and some selected families of proteases are listed in table 1.2.

**Table 1.2: Clans and selected families of the serine proteases.**
According to [BARRETT ET AL., 1998]

| | Characteristic features | Catalytic residues | Examples of comprised families |
|---|---|---|---|
| **Clan SA** | - Contains only endopeptidases<br>- Fold: Double β barrel | His, Asp, Ser | Family S1: Trypsin |
| **Clan SB** | - Contains only family S8<br>- Mostly secreted endopeptidases<br>- Fold: β parallel sheet | Asp, His, Ser | Family S8: Subtilisin |
| **Clan SC** | - Fold: α–β Hydrolase | Ser, Asp, His | Family S10: Carboxypeptidase C |
| **Clan SE** | - Involved in bacterial cell wall biosynthesis and turnover<br>- Fold: Helices and α+β sandwich | Ser, Lys | Family S12: *Streptomyces* R61 D-Ala-D-Ala carboxypeptidase |
| **Clan SF** | - Contains only endopeptidases<br>- Fold: Single β barrel | Ser, Lys, (His) | Family S44: Tricorn protease |
| **Clan SH** | - Contains only family S21<br>- Viral endopeptidases involved in virus prohead assembly<br>- Fold: All β | His, Ser, His | Family S21: Cytomegalovirus assemblin |
| **Clan TA** | - Threonine peptidases<br>- Fold: α,β,β,α sandwich | Thr | Family T1: Proteasome |

## 1.5 *Fusarium graminearum*

The genus *Fusarium* comprises a number of species that are pathogenic to a very wide variety of plants including economically important dicots like potato, bean, tomato and cotton as well as monocots including wheat, barley, rice and maize.

*Fusarium graminearum* (Schwabe) is one of the main causal agents of *Fusarium* head blight (FHB) or scab in cereals. Together with *F. culmorum, F. avenaceae* and several other *Fusarium* species this perthotrophic ascomycete is responsible for severe epidemics in humid and semi-humid regions all over the world [BAI AND SHANER, 1994; PARRY ET AL., 1995; BOTTALICO AND PERRONE, 2002]. *F. graminearum* predominates in North America, Canada and southern Europe while F. culmorum is the prevalent cause of FHB in the cooler regions of northern Europe [MCMULLEN ET AL., 1997; HOFFMANN, 1999].

Apart from wheat *F. graminearum* and its teleomorph *Gibberella zeae* cause scab and root rot of barley, oats and rice as well as ear and stalk rot of maize [Desjardins et al., 1996]. Under laboratory conditions even *Arabitopsis thaliana* could be infected by *F. graminearum* [Urban et al., 2002]. Despite of this broad host range, the major economic impact of *F. graminearum* is seen in FHB of wheat.

Symptoms of the disease range from single bleached spikelets in the head of wheat plants to completely dried spikes (see figure 1.3). Grains of infected heads have significantly reduced weight and the number of kernels per spike is diminished. The starch and protein content of the kernels is reduced in infected plants which impairs the usability for the production of food and beverages [Boyacioğlu and Hettiarachchy, 1995; Nightingale et al., 1999]. Furthermore, the grains are contaminated with mycotoxins that are harmful to animals and humans. Prevalent toxins are the trichothecenes deoxynivalenol (DON) and nivalenol (NIV) as well as the estrogen analog zearalenone (ZEA) [Scheinpflug and Heupel, 1998; Mesterházy et al., 1999]. These toxins are discussed as major virulence factors of *F. graminearum and F. culmorum* since the fungi kill the host tissue at some time during colonization which is accompanied by toxin production [Snijders and Krechting, 1992]. Mutants lacking the ability to produce trichothecenes were shown to be less virulent in field tests with wheat [Desjardins et al., 1996].

**Figure 1.3: Symptoms of *Fusarium* head blight or scab caused by *Fusarium graminearum* in wheat.** The effects range from single affected spikelets (left, indicated by an arrow) to half (middle) and completely dried heads with shrivelled grains.
(Pictures courtesy of M. Nolte, 2001)

The life cycle of *F. graminearum* includes a saprophytic phase and a pathogenic phase (see figure 1.4). In the **saprophytic phase** the fungus grows on post harvesting residues of dead plant material. It overwinters either in the from of ascospores that are formed in perithecia during the sexual cycle or as chlamydospores.

The ascospores get released from the perithecia under favorable wheather conditions which include ambient temperature of 12 to 34°C (preferred 25°C) and a relative humidity of more then 80% for at least 18 hours [SUTTON, 1982; MCMULLEN ET AL., 1997; HOFFMANN, 1999]. The spores are shot into the air and can be dispersed for hundreds of kilometers with wind [BURGESS, 1981]. During the short period between flowering and the beginning development of the kernel (about 10 – 20 days), wheat plants are highly susceptible to *Fusarium* infection [SCHROEDER AND CHRISTENSEN, 1963], especially during the presence of developed anthers [SUTTON, 1982]. If ascospores or rain splash-dispersed macroconidia get in contact with the wheat florets, the fungus enters the **pathogenic phase**.

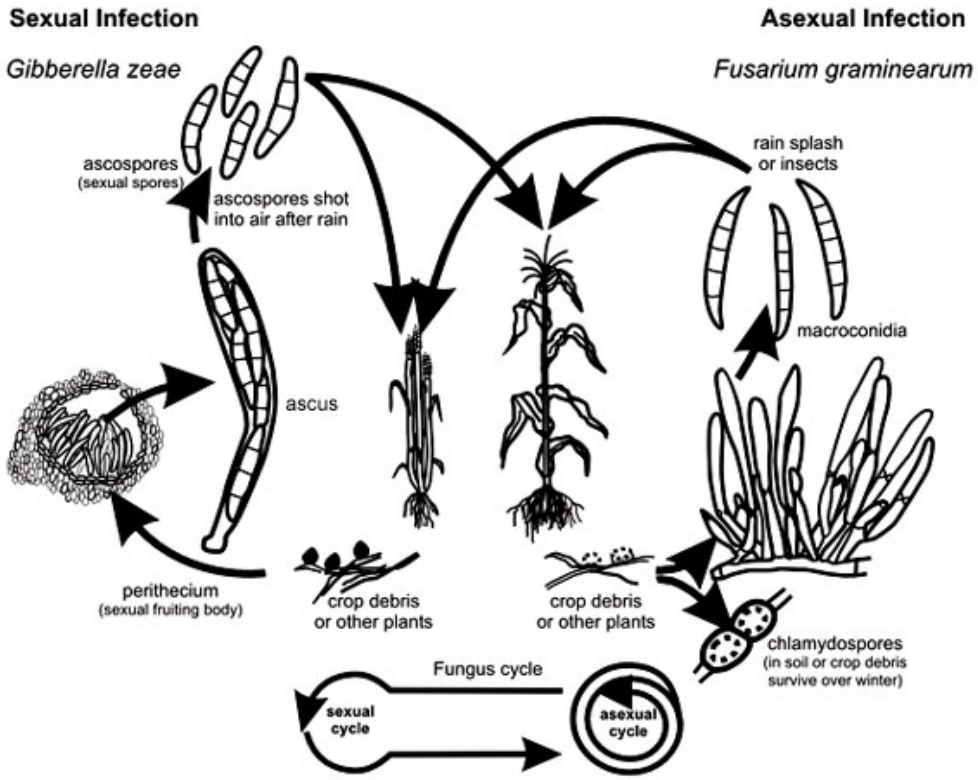


**Figure 1.4: Life cycle of Fusarium graminearum** [SUTTON, 1982]

The infection process of *F. graminearum* is still not elucidated in detail. The classical model of colonization is based on the entrance through dead anthers or stomatal openings followed by penetration of the ovary and succeeding infection of the floral bracts [PRITSCH ET AL., 2000]. The fungus then grows inter- and intracellular in the ovary and the bracts and spreads from one spikelet to the other through the rachis. Direct penetration of the surface of the bracts has also been reported [WANJIRU ET AL., 2002]. After only 2 to 3 days past infection the formation of macroconidia can be observed which can in turn serve as an inoculum for new infections [PRITSCH ET AL., 2000]. The propagation of fungal hyphae during later stages of infection is accompanied by disintegration of host cell organelles, degeneration of host cytoplasm and collapse of some parenchyma cells [WANJIRU ET AL., 2002]. While these drastic effects are accounted to the action of mycotoxins, the degradation of plant cell wall components (cellulose, xylan, pectin) in contact with fungal hyphae were visualized by immuno-gold labeling techniques. Together with the reported insolubilisation of hydroxyproline-rich proteins in wheat cell walls in response to elicitation and infection [EL-GENDY ET AL., 2001; KANG AND BUCHENAUER, 2003] this suggests an important role of cell wall degrading enzymes – including proteases – in the pathogenicity of *F. graminearum.*

## 1.6 Objectives of the present work

Based on the information available, proteases of *F. graminearum* are clearly involved in the degradation of storage proteins in infected wheat kernels. However, a role in the degradation of cell wall structural proteins during the early stages of pathogenesis is also probable, since direct penetration of cell walls and the modification of cell wall carbohydrates by fungal cell wall degrading enzymes have been shown.

In the present work a model system for the induction of extracellular proteases of *F. graminearum* that might be involved in the degradation of structural proteins was to be established. Potential protease induction should be monitored and inducable proteases should be characterized and evaluated with respect to their potential as cell wall degrading enzymes during pathogenesis.

Furthermore, since only two genes encoding proteases were described for members of the genus *Fusarium* at the beginning of this work, a genetic approach to the characterization of fungal proteases should be applied.

# 2 Materials & Methods

## 2.1 Materials

### 2.1.1 Chemicals and materials

The chemicals and reagents that were used for buffers and media were obtained from MERCK KGAA (Darmstadt) and SIGMA-ALDRICH CHEMIE GmbH (Taufkirchen) unless otherwise indicated.

Paper and membrane filters as well as blotting papers were obtained from SCHLEICHER & SCHUELL (Dassel).

### 2.1.2 Organisms – growth conditions and strain maintenance

#### 2.1.2.1 Fungal isolates

For preliminary experiments a strain of *Fusarium graminearum* Schwabe isolated in Wuhan, China was used. It was kindly provided by Dr. Yu-Cai Liao, 'Institut für Biologie II', RWTH Aachen. Since this isolate apparently lost its virulence after some years of subculturing on solid media in our institute according to a petri dish seedling test [NOLTE, 2001], a different strain was used for the experiments shown.

*Fusarium graminearum* Schwabe isolate K59 was kindly provided by the 'Institut für Pflanzenkrankheiten', RFWU Bonn. This strain proved to be highly potent in colonizing wheat spikes and seedlings as well as detached leaves of susceptible cultivars [NOLTE, 2001].

For strain maintenance and short term storage, the fungus was grown on solid medium in petri dishes (CM-agar, see table 2.1). A piece of agar with mycelium measuring about 1 $cm^2$ was transferred top down to a new plate approximately every eight weeks under sterile conditions. The plate was sealed with Parafilm and kept at room temperature on the workbench for several days until it was completely covered by the fungus. It was then stored at 4°C in the dark.

For long term storage, conidia were produced as described in chapter 2.2.1.3. 1 ml of sterile 65% (v/v) glycerol was added per ml of suspension before freezing at -70°C.

Growth conditions for submerged culture will be described in detail in chapter 2.2.1 as they were varied according to the particular experiment.

#### 2.1.2.2 Plant cultivars

Inoculation of detached wheat leaves with *Fusarium graminearum* conidia in a petri dish was performed using the winter wheat cultivar Ritmo (CEBECO) which has a high susceptibility for *Fusarium head blight* (FHB) according to the 'Bundessortenamt', Hannover.

The grains were soaked in tap water for one hour and set out to germinate in a glass dish (∅ 190 mm) on moist filter paper (∅ 150 mm) with the embryo facing upwards. The dish was covered with a glass lid and kept at 25°C in the dark for 24 h. Germinated seeds which appeared to be free of contaminations were then transferred to plastic pots (7x7 cm) with steam sterilized soil (ED 73, BALSTER EINHEITSERDEWERK GMBH, Fröndenberg). The wheat plants were grown in climatic chambers (AR-66L, PERCIVAL SCIENTIFIC, Perry, USA) at 20°C with a day/night cycle of 16/8 h and a light intensity of 15,000 – 20,000 lux. After two to four weeks, detached leaves were used for inoculation as described in chapter 2.2.3.

#### 2.1.2.3 Bacteria

One Shot® TOP10F' chemically competent *Escherichia coli* (INVITROGEN, Karlsruhe) were used for amplification of transformation vectors, for storage and sequencing. They were grown over night in LB medium (DIFCO, Augsburg) at 37°C and 170 rpm in a rotary shaker (Multitron II, INFORS, Einsbach). For long term storage, 1 ml of sterile 65% (v/v) glycerol in LB medium was added to 1 ml of bacterial suspension before freezing at -70°C.

## 2.2 Methods

### 2.2.1 Cultivation of *Fusarium graminearum* in submerged culture

#### 2.2.1.1 CM-medium, minimal medium and protease induction medium

Since the ability of *Fusarium graminearum* to secrete protease(s) under different conditions was a major topic of this work, cultivation of the fungus in submerged culture was the method of choice. This way it was possible to assay for proteolytic activity in the medium at any desired point in time without having to terminate the experiment. Likewise it was feasible to scale the culture method to any desired volume for protein purification experiments.

Early experiments had shown that *Fusarium graminearum* does not secrete significant amounts of proteases when grown in CM-medium (Complete Medium [PONTECORVO, 1953], see below) for two days. To investigate whether protease secretion can be induced, the medium was modified by omitting components that could serve as a source of peptides or amino acids and replacing them with varying amounts of a long-chained protein. As this

represents the sole source of nitrogen in the medium, growth for prolonged periods of time depends on the ability of the fungus to access this source by cleaving the protein with extracellular proteases. Apart from that, the amount of sucrose as the primary carbon source was reduced compared to the CM-medium, increasing the need to utilize the protein substrate.

Gelatin – partially hydrolyzed collagen of animal origin - was selected as the protein of choice as it roughly resembles the hydroxyproline-rich structural proteins that are found in the cell walls of higher plants (see chapter 1.2). It also is easily available and readily soluble while autoclaving the medium.

The different media which were used in protease induction experiments are shown in table 2.1. The pH value normally ranged from 5 to 6 and was not adjusted. All media were autoclaved and stored at 4°C. Before use, they were warmed to room temperature.

#### 2.2.1.2 Preculture

Starting with the fungal stock culture on CM-agar plates, a liquid preculture was inoculated under sterile conditions by adding four pieces (each about 1 $cm^2$) of mycelium-covered agar to 100 ml of CM-medium (see table 2.1) in a 200 ml Erlenmeyer flask. The preculture was incubated for 24 h on a rotary shaker at 150 rpm and 28°C in the dark. At this time small spheres of mycelium (about 1 mm in diameter) were observed in addition to the agar blocks and pieces of split off agar.

This preliminary culture was used to inoculate further flasks by transferring 5 ml per 50 ml of fresh medium under sterile conditions. Care was taken to transfer equal amounts of mycelium by resuspending settled material before each withdrawal and avoiding pieces of agar.

#### 2.2.1.3 Induction of conidia

For long term storage of *Fusarium graminearum* and for inoculation of wheat leaves, conidia were produced by growing the fungus in conidia induction medium [Booth, 1971] (see table 2.1). Several 200 ml Erlenmeyer flasks with 100 ml of medium were inoculated with 10 ml of preculture under sterile conditions and incubated for five days under the same conditions as above. The medium was then filtered using an autoclaved glass funnel stuffed with a piece of cotton wool to remove the mycelium. To pellet the conidia, the medium was transferred into 50 ml Falcon tubes and centrifuged for 10 min at 3000 rpm (~1500 x g) in a swing out rotor (Megafuge 1.OR, Heraeus/Kendro, Hanau). After

discarding the supernatant, the conidia were resuspended in 2 ml of sterile water and pelleted again as above. This step was repeated and the pellet was finally resuspended in 1 ml of water. The concentration of conidia was determined using a Thoma counting cell and adjusted to $10^6$ conidia/ml.

**Table 2.1: Media and stock solutions used for cultivation of *Fusarium graminearum***

| | |
|---|---|
| CM-medium according to [PONTECORVO, 1953]:<br>50 ml Salt stock solution<br>2 ml Vitamin stock solution<br>1 ml Trace element stock solution<br>2 g Peptone (from soybean, MERCK , Darmstadt)<br>1 g Yeast extract (DIFCO, Augsburg)<br>1 g Acid hydrolyzed casein (MERCK, Darmstadt)<br>10 g Sucrose<br>ad 1 l A.dest<br>Autoclave. Store at 4°C or room temperature | Minimal medium:<br>50 ml Salt stock solution<br>2 ml Vitamin stock solution<br>1 ml Trace element stock solution<br>1 g Sucrose<br>ad 1 l A.dest<br>Autoclave. Store at 4°C or room temperature |
| CM-agar<br>CM-medium<br>+ 1.5% (w/v) Agar-Agar (DIFCO, Augsburg)<br>Autoclave. Pour in petri dishes. Store at 4°C | Protease induction medium:<br>Minimal medium<br>+ varying amounts of gelatin<br>(preferred 0,5%(w/v))<br>Autoclave. Store at 4°C or room temperature |
| Salt stock solution:<br>10.4 g/l KCl<br>10.4 g/l $MgSO_4 * 7 H_2O$<br>30.4 g/l $KH_2PO_4$<br>ad 1 l A.dest<br>Store in aliquots of 50 ml at -20°C | Vitamin stock solution:<br>0.5 g/l Biotin<br>16 g/l Para-aminobenzoic acid<br>20 g/l Pyridoxin hydrochloride<br>50 g/l Nicotinic acid<br>Prepare 50 ml of stock solution<br>Store in aliquots of 1 ml at -70°C |
| Trace element stock solution:<br>1 g/l $FeSO_4 * 7H_2O$<br>0.15 g/l $CuSO_4 * 5 H_2O$<br>1.61 g/l $ZnSO_4 * 7 H_2O$<br>0.1 g/l $MnSO_4 * H_2O$<br>0.1 g/l $(NH_4)_6Mo_7O_{24} * 4 H_2O$<br>Prepare 100 ml of stock solution<br>Store in aliquots of 2 ml at -70°C | Conidia induction medium [BOOTH, 1971]:<br>15 g/l Carboxymethyl cellulose<br>1 g/l $NH_4NO_3$<br>1 g/l $KH_2PO_4$<br>1 g/l Yeast extract (DIFCO, Augsburg)<br>0.5 g/l $MgSO_4 * 7 H_2O$<br>ad 1 l A.dest<br>Autoclave. Store at 4°C or room temperature |

#### 2.2.1.4 Induction of protease secretion

100 ml Erlenmeyer flasks containing 50 ml of 'minimal medium' (without protein), 'protease induction media' (with 0.1%, 0.2%, 0.3%, 0.4% or 0.5% (w/v) of gelatin respectively), and CM-medium were inoculated with 5 ml of preculture. The flasks were incubated for two days on a rotary shaker at 150 rpm and 28°C in the dark.

For collecting the growth medium, a coarse separation of mycelium and medium was performed by straining through fluted filters (∅ 125 mm). The medium was then filtered through membrane filters (0,2 µm pore size, ME 24) using a vacuum pump to remove remaining conidia and mycelium. Samples of the collected media were assayed for protease activity as described in chapter 2.2.4.1.

Since protease activity in the medium could be induced using this procedure (see chapter 3.3) and the resulting activity increased with the substrate concentration, medium with 0.5% of gelatin was used as protease induction medium (PI-medium) in the following experiments.

#### 2.2.1.5 Growth rate and kinetics of protease secretion

To determine the growth rate of *Fusarium graminearum* in different media, 100 ml Erlenmeyer flasks with 50 ml of CM-medium or PI-medium respectively were inoculated with 5 ml of preculture and incubated on a rotary shaker at 150 rpm and 28°C in the dark for up to seven days. Samples were taken daily and the content of the respective Erlenmeyer flasks was strained through fluted filters (∅ 125 mm) for coarse separation of mycelium and medium. The fungal mass was then transferred to membrane filters (0,2 µm pore size, ME 24) and the remaining liquid was removed under vacuum assistance before determining the fresh weight of the filter cake.

The medium harvested in the first step was filtered through membrane filters (0,2 µm pore size, ME 24) using a vacuum pump to remove remaining conidia and mycelium. Samples of the collected media were assayed for protease activity as described in chapter 2.2.4.1.

#### 2.2.1.6 Kinetics of initial protease secretion – Mass inoculation

To determine how fast *Fusarium graminearum* reacts to changes in the culture medium, it was necessary to increase the concentration of protease for detection in the initial phase of induction. Since inoculation in the aforementioned experiments was carried out with little starting material, early reactions could not be measured due to dilution effects.

Therefore a *second* preculture step was introduced by inoculating multiple 200 ml Erlenmeyer flasks containing 100 ml of CM-medium with 10 ml of preculture as described above and incubating them for further 24 h under the same conditions as before. The produced fungal mass was separated from the medium under sterile conditions by straining through gauze.

The mycelium was stirred and compressed with a sterile spatula until most of the medium was removed and a compact clot was formed. In early experiments the mycelium was also washed with sterile water to completely remove residual medium, but this was omitted later.

500 ml Erlenmeyer flasks with 200 ml of CM-medium or PI-medium respectively were inoculated with the mycelium harvested from the secondary preculture (about 1 g fresh weight), thus providing enough starting material to detect early stages of protease secretion. The cultures were incubated for three to five days as described above and samples were taken as soon as 1.5 h after transferring the mycelium.

Small samples of medium were withdrawn from the respective Erlenmeyer flasks with sterile pipet tips and transferred to micro tubes. The fungal material was precipitated by centrifugation at 13,000 rpm in a table top centrifuge (Picofuge, EPPENDORF) for 10 minutes. The supernatant was carefully removed and the pellet discarded. It sometimes was necessary to repeat the centrifugation step, since the mycelium did not precipitate readily. It turned out that apart from the nature of the medium, the shape of the micro tubes used also had an effect on the precipitability and 1,5 ml tubes – which had a pointed tip – were more suitable than 2 ml tubes. The clarified medium was quick-frozen in liquid nitrogen and stored at -20°C until further use. The samples were assayed for protease activity as described in chapter 2.2.4.1.

#### 2.2.1.7 Variations in protein derived nitrogen levels

CM-medium contains several sources of amino acids and peptides (casein hydrolysate, peptone, yeast extract). To elucidate whether the supply of available nitrogen in this form is sufficient for the fungus *not* secreting protease in CM-medium, the PI-medium was modified by replacing the long-chained gelatin substrate with predigested gelatin.

A stock solution of digested gelatin was prepared by heating 200 ml of A. dest. with 2.5 g of gelatin to completely dissolve the protein. After cooling, 10 mg of Protease K (8.1 U/mg, SERVA, Heidelberg) were added and the solution was incubated for 20 h at 37°C. The digested gelatin was then sterilized by autoclaving - which also inactivated the protease - brought to a volume of 250 ml with sterile A. dest. and stored at 4°C until used.

This solution of 1% (w/v) predigested gelatin was mixed under sterile conditions with an equal volume of 2x concentrated minimal medium thus resulting in 0.5% predigested PI-medium (pPI-medium). A sample of this medium was inspected using polyacrylamide gel electrophoresis (see chapter 2.2.4.5) to make sure that the gelatin was completely cleaved.

To find out if the mere presence of protein substrate is sufficient for protease induction, CM-medium supplemented with 0.5% (w/v) gelatin (gCM-medium) was also used in one experiment. The fungus was cultivated for three to five days in CM-, gCM-, PI- and pPI-medium under the conditions described above. Transfer of fungal mycelium for inoculation and processing of samples were performed as described in chapter 2.2.1.6.

#### 2.2.1.8 Variations in carbohydrate levels

The effect of different carbohydrate levels on protease secretion was investigated by growing *Fusarium graminearum* in PI-medium and pPI-medium (see above) with different concentrations of sucrose. To simplify the preparation of media, PI-medium and pPI-medium without sucrose were used, which could be complemented by different amounts of a sterile 0,33 g/ml sucrose stock solution. The fungus was cultivated in media containing 0, 1 and 2 g/l sucrose respectively for three to five days under the conditions described above. Transfer of fungal mycelium and processing of samples were performed as described in chapter 2.2.1.6.

#### 2.2.1.9 Isolated wheat cell wall material as substrate

The effect of wheat cell wall material on protease induction during submerged cultivation was investigated by adding 0.5% (w/v) of isolated cell wall material to 200 ml of minimal medium before autoclaving. Briefly, isolation was performed by grinding wheat leaves with 0.5 M potassium phosphate buffer (pH 7.0), centrifuging and extracting the pellet with methanol/chloroform, acetone and ethanol, washing with A. dest. and freeze drying the resulting material according to [MIERAU, 1998]. The cell wall preparation was kindly provided by J. MOLDENHAUER. Transfer of fungal mycelium and processing of samples were performed as described in chapter 2.2.1.6.

### 2.2.2 Cultivation of *Fusarium graminearum* on agar plates

To examine the growth of the fungus on solid substrate containing CM-, minimal- and PI-medium, said media were solidified with 1.5% agar and 1 $cm^2$ pieces of a stock culture on CM-agar were transferred top down to the plates under sterile conditions. The plates were sealed with Parafilm and incubated up to six days at room temperature on the workbench. Pictures were taken daily with a digital camera (OLYMPUS C-4040 Zoom).

### 2.2.3 In planta cultivation – Detached leaf petri dish assay

Under natural circumstances *Fusarium graminearum* infects the heads of wheat plants during the flowering stage (see chapter 1.5). Since the cultivation of adult plants which are required for modeling this system in laboratory experiments requires a lot of resources, a petri dish assay with detached leaves established in our institute by M. NOLTE [NOLTE, 2001] was chosen as a model system for *in planta* experiments.

Wheat plants were grown as described in chapter 2.1.2.2 for three weeks. Leaves were harvested by cutting about 10 cm below the tip and passed through parallel slits in filter paper in sets of four or five as shown in figure 2.1. After fixing them to the paper with thin strips of adhesive tape and trimming protruding parts of the leaves, the filter was placed on tap water-agar plates with the cut ends in contact with the moist substrate. This way, the plant material can be incubated for several days without wilting.

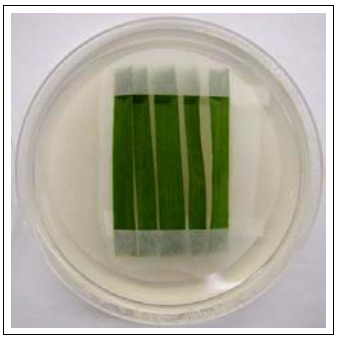

**Figure 2.1: Detached leaf petri dish assay**
Detached leaves were passed through slits in filter paper and placed on moist tap water-agar before spraying them with conidia solution for inoculation.

Inoculation with *Fusarium graminearum* was performed by spraying the leaf surface with a suspension of $10^6$ conidia/ml (see chapter 2.2.1.3) containing 0.04% (v/v) Tween using an airbrush system. One milliliter of suspension was evenly applied per plate and controls were treated with the same volume of 0.04% Tween in water. The petri dishes were sealed with Parafilm and incubated for up to five days in a climatic chamber at 20°C with a day/night cycle of 16/8 h and an illumination of 15,000 – 20,000 lux.

Samples were taken daily after visually inspecting the leaves and taking pictures with a digital camera (OLYMPUS C-4040 Zoom). Three to four leaves per sample were wrapped in aluminum foil, quick-frozen in liquid nitrogen and stored at -70°C until used for RNA extraction. One leaf was cut in segments of about 1 cm, which were quick-stained in a solution of 0.1% (w/v) Fluorescence Brightener 28 (Calcofluor, SIGMA) in 0.1 M Tris/HCl buffer pH 8,5 without destaining them first. After briefly washing with water, the development of the fungus and colonization of the plant tissue was investigated microscopically (OLYMPUS BX 40) under UV illumination (OLYMPUS UV illuminator).

Destaining was omitted since it was shown by N. PEDE [PEDE, 2003] that conidia appear to be washed away while destaining at least during the first days of colonization. Furthermore, the red chlorophyll autofluorescence of the leaf cells enhances the contrast when examining the growth of the brightly blue stained mycelium and conidia on top of the leaves. This advantage lessens though, when the leaves start to develop brownish spots during later stages of infection as the affected tissue shows an orange to yellowish autofluorescence which decreases the contrast.

### 2.2.4 Protein biochemical methods

#### 2.2.4.1 Photometric assay for protease activity

Assays were performed in triplicates with denaturated samples as comparative samples using an azo-dye labeled substrate. Six aliquots of 25 to 100 µl growth medium per sample were brought to 100 µl with A. dest. in 1.5 ml micro tubes. Three tubes were heated to 80°C for 10 minutes to inactivate enzyme activity. After cooling, 50 µl of azocasein solution and 50 µl of incubation buffer (see table 2.2) were added to each tube with a Multipette (EPPENDORF).

The samples were incubated at 40°C under gentle agitation for 30 to 60 minutes allowing the substrate to be digested. 500 µl of 5% aqueous trichloroacetic acid were then added with a Multipette to precipitate non-digested protein and the mixture was incubated for 10 minutes at room temperature. The precipitate was pelleted by centrifuging for 5 minutes at 13,000 rpm in a table top centrifuge (Picofuge, EPPENDORF) and 400 µl of the supernatant which contained the dye labeled fragments of the digested substrate were transferred to 1.5 ml plastic microcuvettes (RATIOLAB, Dreieich). 500 µl of test buffer were added and the optical density at 400 nm was determined using a double ray photometer (Uvikon 933, KONTRON / BIOTEK, Neufahrn) with a water filled reference cuvette. Protease activity was calculated using the following formula with one unit of activity defined as a change in absorbance of 1 per hour.

$$Protease\,activity\left[\frac{U}{ml}\right] = \bar{E} - \bar{E}_0 \cdot \frac{1000}{v} \cdot \frac{60}{t}$$

$\bar{E}$ = mean of the absorbance of non-denaturated samples
$\bar{E}_0$ = mean of the absorbance of denaturated samples
$v$ = sample volume in µl
$t$ = incubation time in min

**Table 2.2: Solutions used for the standard photometric protease assay**

| | |
|---|---|
| Azocasein solution:<br>20 mg/ml Azocasein (Sulfanilamide-azocasein, SIGMA) | Incubation buffer:<br>200 mM Tris, 20 mM $CaCl_2$ pH 7.8 |
| Stop solution:<br>5% (w/v) Trichloroacetic acid | Test buffer:<br>500 mM Tris, pH 8.8 |

### 2.2.4.2 pH-profile and temperature optimum

For determining the pH-optimum of protease activity, four different incubation buffers were used (see table 2.3). The temperature optimum was measured by incubating the samples at different temperatures after adding the substrate solution and the standard incubation buffer. Apart from these changes, the assays were performed as described above.

**Table 2.3: Incubation buffers used for determining the pH-profile of protease activity**

| | |
|---|---|
| pH 4 – 6<br>200 mM Na-citrate, 20 mM $CaCl_2$ | pH 6 – 8<br>200 mM Bis-Tris, 20 mM $CaCl_2$ |
| pH 8 – 9.5<br>200 mM Tris, 20 mM $CaCl_2$ | pH 9.5 – 11.5<br>200 mM CAPS, 20 mM $CaCl_2$ |

### 2.2.4.3 Inhibitor studies

To investigate the type of protease which is secreted when cultivating *Fusarium graminearum* in PI-medium, aliquots of the growth medium were preincubated with different protease inhibitors before determining the activity. Medium was harvested two days after inoculation with preculture (see chapter 2.2.1.5), and distributed to micro tubes in aliquots of 375 µl. Stock solutions of the inhibitors listed in table 2.4 were added and the mixtures were brought to a final volume of 400 µl with A.dest. Since some of the inhibitors were dissolved in methanol, one sample was supplemented with 10 µl of this solvent as a control. After incubating for 15 minutes at room temperature, protease activity was determined as described in chapter 2.2.4.1 and set in relation to the activity of a sample that was only supplemented with water.

**Table 2.4: Protease inhibitors used for inhibitor studies**

| Name | Inhibition specificity | Solvent | Stock solution | Final concentration |
|---|---|---|---|---|
| Bestatin | Metalloproteases | MeOH | 3 mM | 150 µM |
| Phosphoramidon | Metalloproteases | $H_2O$ | 340 µM | 10 µM |
| EDTA | Metalloproteases | $H_2O$ | 2.7 mM (1 mg/ml) | 10 µM |
| Soybean trypsin inhibitor | Serine proteases (Trypsin-like) | $H_2O$ | 1 mg/ml | 50 µg/ml |
| Apronitin | Serine proteases (Trypsin, Chymotrypsin) | $H_2O$ | 150 µM (1 mg/ml) | 10 µM |
| PMSF | Serine proteases | MeOH | 100 mM | 2 mM |
| Chymostatin | Serine- / Cysteine proteases | $H_2O$ | 1.6 mM (1 mg/ml) | 100 µM |
| Leupeptin | Serine- / Cysteine proteases | $H_2O$ | 2.1 mM (1 mg/ml) | 100 µM |
| E-64 | Cysteine proteases | $H_2O$ | 2.8 mM (1 mg/ml) | 10 µM |
| Pepstatin | Aspartic proteases | MeOH | 1.46 mM (1 mg/ml) | 10 µM |

#### 2.2.4.4 Cation exchange perfusion chromatography

For partial purification of the protease secreted during growth of the fungus in PI-medium, perfusion chromatography was used. Medium which was harvested two days after mass inoculation (see chapter 2.2.1.6) was submitted to cation exchange chromatography using a BioCAD Sprint system with a SCOUT column selector and AFC2000 combined fraction collector / Sample injector (APPLIED BIOSYSTEMS, Foster City, USA).

POROS® 20 HS column material - a strong cation exchanger which allows high flow rates - was used for separation. Chromatography was performed with a flow rate of 6 to 8 ml/min in a MES / HEPES / sodium acetate buffer system (33 mM each, diluted to 11 mM during the run) adjusted to pH 4.5. Proteins that bound to the column were eluted with a NaCl gradient ranging from 0 to 2 M over 12 column volumes. Protein content was observed at 280 nm. Fractions of 2 to 2.5 ml were collected and assayed for protease activity as described in chapter 2.2.4.1 using 50 to 100 µl of the fraction. Before applying the samples to the column, they were centrifuged for 5 minutes at 13,000 rpm in a table top centrifuge to remove particles that precipitated during freezing.

For separation on acrylamide gels, selected fractions were desalted using PD-10 columns (AMERSHAM PARMACIA, Freiburg) which were equilibrated with A. dest. The eluate was freeze-dried and resuspended in 1/50 to 1 volumes A. dest. compared to the total volume of the injected sample thus resulting in a concentration factor of 1 to 50. The samples were then submitted to SDS-PAGE and zymography (see chapters 2.2.4.5 – 2.2.4.9).

Since the protease activity was found in the binding fractions while a major part of the protein contained in the PI-medium did *not* bind to the column (see chapter 3.7.1), multiple injections of the sample were performed before starting the gradient. This way the bound protein could be concentrated and eluted from the column in larger amounts. To elucidate the maximum capacity of the column, up to 20 injections of 495 µl each were made and the flowthru of selected injections was collected. Protease activity in these fractions was assayed to determine the maximum load to be injected.

**Table 2.5: Program used for cation exchange perfusion chromatograpy (multiple injections)**

Column material: POROS® HS 20 cation exchange
Column volume (CV): 1.662 ml
UV detector wavelength: 280 nm
Flow rate: 6 - 8 ml /min

Solutions:
**A**: $H_2O$ **B**: MES/HEPES/NaAc (33 mM each) **C**: 3 M NaCl

| | | | |
|---|---|---|---|
| Equilibration: | 66% **A**, 34% **B** | 4 CV | |
| Loading: | Injection of 495 µl sample (with AFC 2000) | | ┐ repeat |
| Washing: | 66% **A**, 34% **B** | 3 CV | ┘ 1 to 20 times |
| Extra washing: | 66% **A**, 34% | 1 CV | |
| Elution: | from 66% **A**, 34% **B** | | |
| | to 66% **C**, 34% **B** | 12 CV | |
| Cleaning: | 66% **C**, 34% **B** | 2.5 CV | |
| | 66% **A**, 34% **B** | 6 CV | |

### 2.2.4.5 SDS polyacrylamide gel elecrophoresis (SDS-PAGE)

Gel electrophoresis of media samples and FPLC fractions was performed in discontinuous SDS polyacrylamide gels according to Laemmli [LAEMMLI, 1970] as well as gradient gels. Gels measuring about 8.5 x 7 cm were cast using the BIORAD, Mini protean II system with 1.5 mm spacers.

For discontinuous SDS-PAGE a 12% separating gel and a 5% stacking gel were used (see table 2.6). Gradient gels covered a range of 5 – 15% acrylamide/bisacrylamide and were complemented after polymerization with a small 5% gel prepared with separating gel buffer. The protein samples to be separated were mixed with ¼ volume of non-reducing 4x loading buffer, incubated for at least 15 minutes at room temperature and transferred into the wells of the gel. One lane was loaded with prestained broad-range size markers (BIORAD, München) ranging from about 7 kDa to 210 kDa. Discontinuous gels were run in 1x electrode buffer at 20 mA per gel until the proteins reached the border of the stacking

gel and 30 mA per gel afterwards. Gradient gels were uniformly run at 30 mA per gel. To preserve enzyme activity for subsequent zymography, electrophoresis was performed at 4°C. After the separation was completed, the gels were submitted to Coomassie or silver staining or used for zymography (see below).

**Table 2.6 Solutions used for SDS-PAGE**

| | |
|---|---|
| Separating gel buffer (4x):<br>1.5 M Tris<br>0.4% (w/v) SDS<br>pH 8.8 | Stacking gel buffer (4x):<br>0.5 M Tris<br>0.4% (w/v) SDS<br>pH 6.8 |
| Loading buffer (4x):<br>0.32 M Tris<br>8% (w/v) SDS<br>20% (v/v) Glycerol<br>0.04% (w/v) Bromophenol blue | Electrode buffer (10x)<br>250 mM Tris<br>1.9 M Glycine<br>2% (w/v) SDS<br>pH 8.3 – 8.4 |

**Table 2.7 Composition of acrylamide gels used for protein separation (amounts for one gel measuring about 85 x 70 x 1.5 mm)**

| | |
|---|---|
| 12% separating gel<br>2.4 ml $H_2O$<br>1.75 ml Separating gel buffer (4x)<br>2.8 ml Acrylamide / bisacrylamide (30% / 0.8% solution, ROTH Karlsruhe)<br>20 µl TEMED (100%)<br>15 µl APS (25% in $H_2O$)<br>~7 ml | 5% stacking gel<br>1.75 ml $H_2O$<br>0.75 ml Stacking gel buffer (4x)<br>0.5 ml Acrylamide / bisacrylamide (30% / 0.8% solution, ROTH Karlsruhe)<br>10 µl TEMED (100%)<br>7 µl APS (25% in $H_2O$)<br>~3 ml |
| 5% Gradient gel solution (reservoir of mixer):<br>2.9 ml $H_2O$<br>1.25 ml Separating gel buffer (4x)<br>0.833 ml Acrylamide / bisacrylamide (30% / 0.8% solution, ROTH Karlsruhe)<br>15 µl TEMED (100%)<br>10 µl APS (25% in $H_2O$)<br>~5 ml | 15% Gradient gel solution (mixing chamber of mixer):<br>1.1 ml $H_2O$<br>1.25 ml Separating gel buffer (4x)<br>2.5 ml Acrylamide / bisacrylamide (30% / 0.8% solution, ROTH Karlsruhe)<br>125 µl Glycerol<br>15 µl TEMED (100%)<br>10 µl APS (25% in $H_2O$)<br>~5 ml |

### 2.2.4.6 'Slow' Coomassie staining of acrylamide gels

Coomassie staining was carried out using a 'one step' method which takes longer than other staining protocols but results in lower background thus removing the need for a destaining step. The gel was incubated in 12% aqueous trichloroacetic acid to denaturate the proteins, rinsed with water and then submerged in the staining solution which was prepared by mixing four parts of the Coomassie staining stock solution with one part methanol. After incubating for one hour to one day, the gel was very briefly rinsed with methanol to remove adhering particles and then washed with water.

**Table 2.8: Solutions used for Coomassie staining of acrylamide gels**

| Coomassie staining stock solution: | Comassie suspension: |
|---|---|
| 20 ml 80% $H_3PO_4$<br>100 g $(NH_4)_2SO_4$<br>ad 980 ml $H_2O$<br>Mix with Coomassie suspension (will not solve completely). Resuspend settled particles before use. | 5 g Coomassie® brilliant blue suspended in 20 ml $H_2O$ |

### 2.2.4.7 Silver staining of acrylamide gels

Silver staining was performed according to H. VORUM [VORUM, 1999]. The gel was incubated for two hours in fixing solution and washed three times for 20 minutes in washing solution. It was then submerged for two minutes in sensitizing solution and washed three times for 5 minutes in water. The gel was stained with silver nitrate solution for 20 minutes followed by washing twice for 1 minute with water. The bands were visualized with developing solution until the desired contrast was reached and the reaction was terminated by incubating in stop solution for five minutes. All steps were carried out with gentle agitation under a fume hood.

**Table 2.9: Solutions used for silver staining of acrylamide gels**

| Fixing solution: | Washing solution: |
|---|---|
| 50% (v/v) Methanol<br>12% (v/v) Acetate<br>0.05% (v/v) Formaldehyde | 35% (v/v) Ethanol |
| | Sensitizing solution:<br>0.02% (w/v) $Na_2S_2O_3$ |
| Silver nitrate solution:<br>0.2% (w/v) $AgNO_3$<br>0.076% (v/v) Formaldehyde<br>Prepare fresh. | Developing solution:<br>6% (w/v) $Na_2CO_3$<br>0.05% (v/v) Formalydehyde<br>0.0004% (w/v) $Na_2S_2O_3$ |

### 2.2.4.8 Standard zymography

A method for determining the apparent molecular weight of enzymes which degrade a polymeric substrate is zymography. It uses standard electrophoresis procedures with acrylamide gels which have the substrate copolymerized with the gel matrix during preparation of the gel. It is important to use non-reducing loading buffer when preparing the samples since reducing agents may permanently destroy the tertiary structure of the enzyme. The denaturation caused by SDS in the buffers is usually reversible by incubating three times for 15 minutes in 2.5% Triton X®-100 solution and subsequent rinsing with water. The gel is then washed in a renaturation buffer designed for the specific enzyme to be investigated for 15 minutes and incubated in renaturation buffer at a suitable temperature for several hours to several days. After incubation, the substrate is stained with an appropriate reagent, resulting in transparent bands against a dark background where active enzymes have digested the substrate.

The substrate used was gelatin at a final concentration of 0.1% (w/v). A stock solution of 2% gelatin in $H_2O$ was autoclaved and stored at room temperature. 50 µl/ml of this stock solution was added to the gel solution when preparing acrylamide gels while adjusting the amount of water respectively.

Preliminary experiments had shown, that the protease activity which can be observed in the growth medium when cultivating *Fusarium graminearum* in PI-medium is insensitive to SDS in concentrations usually employed in SDS-PAGE. The washing step with Triton X®-100 was therefore omitted and the gel was only washed with renaturation buffer (50 mM Tris, 5 mM $CaCl_2$, pH 8.5). It was incubated under gentle agitation in renaturation buffer at 40 °C over night. Staining of the protein substrate was performed by coomassie staining as described in chapter 2.2.4.6.

### 2.2.4.9 Blotting zymography

Since the insensitivity of the protease to SDS resulted in digestion of the substrate even during the electrophoresis, it was almost impossible to correlate enzyme activity to sharp bands. Instead, a transparent lane was formed as the substrate was cleaved by the migrating enzyme. Since performing the separation at 4°C did not resolve this problem and a reversible inhibitor of the protease which could be added to the sample was not available, a different method for zymography was investigated.

Washing a standard SDS acrylamide gel used for separation with renaturing buffer and placing a gelatin containing acrylamide gel on top of it (overlay-gel method) before incubating this 'sandwich' in a moist chamber at 40°C over night did not give a satisfying result. Therefore the proteins separated during SDS-PAGE were transferred into the substrate-containing gel by electroblotting using a 'semi-dry blotting apparatus' (AMERSHAM-PHARMACIA, Freiburg). The gel was not washed with renaturing buffer before this procedure since SDS is required for the transfer.

After wetting the electrodes of the apparatus with water, six pieces of gel blotting paper cut to the size of the gel were soaked with anode buffer $A_1$ and placed on the anode. Three pieces of paper soaked with anode buffer $A_2$ were added and the substrate containing gel was placed on top. It was covered with the gel used for separation followed by nine pieces of paper soaked in cathode buffer. Care was taken to avoid the formation of air bubbles during the assembly and the stack was compacted by gently rolling with a glass rod.

Transfer of the proteins was carried out by applying 0.8 mA/cm$^2$ while limiting the apparatus to a maximum of 100 V and 9 W. After one hour the stack was disassembled and the substrate containing gel was incubated and stained as described above.

**Table 2.10: Buffers used for electroblotting**

| | |
|---|---|
| Anode buffer $A_1$:<br>0.3 M Tris<br>20% (v/v) Methanol<br>pH 10.4 | Anode buffer $A_2$:<br>25 mM Tris<br>20% (v/v) Methanol<br>pH 10.4 |
| Cathode buffer:<br>40 mM 6-Aminocaproic acid<br>20% (v/v) Methanol<br>0.01% (w/v) SDS<br>pH 7.6 | |

## 2.2.5 Molecular biology

### 2.2.5.1 Isolation of fungal DNA

Mycelium for DNA isolation was harvested from cultures grown for varying times in CM-medium as described above by straining the content of the Erlenmeyer flask through fluted filters and removing residual medium using membrane filters (0.2 µm pore size, ME 24) under vacuum assistance. Aliquots of about 100 to 150 mg fresh weight were cut from the filter cake with a sterile spatula, wrapped in aluminum foil and quick-frozen in liquid nitrogen. The samples were stored at -70°C until used.

DNA was isolated using a phenol/chloroform based protocol (T. CONZE, personal communication). Care was taken to make sure that all reagents and buffers as well as laboratory material were free of DNase activity. Solutions were prepared using the highest available quality of chemicals with autoclaved ultrapure water. Mortars, pistils, pipet tips and micro tubes were autoclaved prior to use.

The frozen mycelium was ground to a fine powder in a precooled mortar with liquid nitrogen. It was then quickly transferred to a sterile Falcon tube containing a mixture of 2.5 ml 2x isolation buffer and 2.5 ml 2x urea solution (see below) using a cold sterile spatula. The sample was submerged in the mixture by gently inverting the tube several times before 2.5 ml of phenol (pH 8) were added. After carefully mixing again, the suspension was allowed to settle for a few moments. 5 ml of phenol / chloroform (1:1) were then added and the tube was inverted several times before centrifuging for 10 minutes at 3000 rpm (~1500 x g) in a swing-out rotor at 4°C (Megafuge 1.OR, HERAEUS/KENDRO, Hanau).

The aqueous upper phase containing the DNA was carefully transferred to a new Falcon tube avoiding the white interphase pellet. If the pellet was very prominent, the phenol / chloroform extraction was repeated and the aqueous phase was again transferred to a new tube. The DNA was precipitated by adding 0.7 volumes of ice-cold 2-propanol and centrifuging for 120 minutes at 2500 rpm (~1000 x g) in a swing-out rotor at 4°C.

The supernatant was discarded and the pellet was washed with 1 ml of ice-cold 70% Ethanol before centrifuging for 15 minutes as before. After discarding the supernatant, the tubes were centrifuged briefly to collect remaining ethanol, which was then carefully removed. The pellet was air-dried and resuspended in 100 µl of autoclaved water. DNA concentration was measured photometrically (EPPENDORF Biophotometer) at 260 nm and DNA purity was estimated using the 260 / 280 nm ratio. DNA quality was also visually verified using agarose electrophoresis (see chapter 2.2.5.6). The samples were stored at 4°C until used.

**Table 2.11 Solutions used for DNA isolation**

| 2 x isolation buffer: | 2 x urea solution: | Phenol (pH 8, SIGMA) |
|---|---|---|
| 0,6 M NaCl<br>0,1 M Tris<br>40 mM EDTA<br>4% (w/v) Sarcosyl<br>1% (w/v) SDS<br>(also used for RNA) | 10 M Urea<br>warm slightly before use to avoid crystallization<br>(also used for RNA) | Phenol / Chloroform (1:1) |
| | | Ice-cold 2-propanol |
| | | Ice-cold 70% Ethanol<br>(also used for RNA) |

### 2.2.5.2 Standard PCR

PCR was performed using RedTaq polymerase (SIGMA). The primers used in this work are listed in appendices VIII - IX together with a graphic representation of their position in the target sequence and the annealing temperatures for selected primer combinations. Standard reaction mixtures contained in the following order:

| | |
|---|---|
| 17.5 µl | $H_2O$ |
| 2 µl | DNA solution (~100 ng/µl) |
| 1 µl | of each primer (25 µM) |
| 1 µl | dNTP-mix (2.5 µM each) |
| 2.5 µl | 10x buffer (as supplied) |
| 1 µl | RedTaq DNA polymerase (1 U) |
| 25 µl | |

The components were gently mixed in a 200 µl micro tube and centrifuged briefly. PCR was carried out in an Eppendorf PCR thermoblock using the following standard program:

| | | | | |
|---|---|---|---|---|
| | Premelting: | 2 min | 92°C | |
| ┌ | Melting: | 30 s | 92°C | |
| 25-30x | Annealing: | 30 s | 55 - 65°C | (varying temperatures depending on the primers used) |
| └ | Elongation: | 1 min | 72°C | (or 1 min per 1000 bp of expected amplificate) |
| | Final elongation: | 5 min | 72°C | |
| | Hold: | ∞ | 4°C | |

In cases where the PCR was only used to check for the presence of an amplificate, the reaction volume was reduced to 12.5 µl using only half of the listed volumes. For preparative amounts of PCR product, i.e. for purification and cloning of the fragment, the quantities were doubled to reach a total volume of 50 µl. Products were examined by separation on agarose gels as described in chapter 2.2.5.6.

#### 2.2.5.3 Touch-down PCR

With primers that were deduced from non-homologous sequences, a variation of the standard PCR was used. A high annealing temperature was chosen for the first cycle which was then decreased with each repeat. This way the specificity of primer binding was slowly reduced to make sure that the product with the highest possible homology to the primers was produced first - albeit in only small amounts - which could then serve as template for amplification at lower temperatures. Thus the desired product could be enriched while suppressing amplificates derived from unspecific binding.

Primers were deduced from an alignment of ***Fusarium oxysporum* f. sp. Lycopersici** and ***Fusarium* spec. strain S-19-5** protease genes (see chapter 3.8). These primers named **Fus S-19-5 P Upper** and **Fus S-19-5 P Lower** (see appendix IX) were used in touch-down PCR with genomic DNA from *Fusarium graminearum* as template, decreasing the alignment temperature from 65°C to 60°C in 25 cycles with an elongation time of 1 minute. The resulting bands were cut from the gel and the DNA was purified and sequenced as described below (chapters 2.2.5.7 – 2.2.5.9).

#### 2.2.5.4 Inverse PCR

A method that can give information about genomic sequences flanking a known sequence is inverse PCR [OCHMAN ET AL., 1990]. It uses primers that are complementary to those employed in standard PCR, i.e. extension proceeds outward from the core region. The template for this technique consists of DNA that was cut with a restriction endonuclease producing sticky ends. The restricted DNA is religated with a high degree of dilution, which favors the formation of circular DNA molecules by joining both ends of a single fragment.

If the circular fragment is of a suitable length - which depends on the distribution of the particular restriction site in the genome - the regions adjacent to the known sequence can be amplified with standard PCR procedures. The result is a fragment that comprises the flanking regions fused together. After sequencing the amplificate, the primer sequences and the restriction site of the endonuclease can be used to identify the separate areas and assemble them with the starting sequence.

After several unsuccessful attempts at extending the sequence obtained from PCR and touch-down PCR testing different primer combinations and endonucleases, **Sal I** was chosen as the restriction endonuclease to be used. It cuts within the known sequence which usually is undesirable as this limits the prospective extension of the sequence. If one primer is designed to be located right 'inside' of to the restriction site pointing 'outwards', and the other one is at the other side of the known sequence (see figure 2.2) the amplificate can only provide information about *one* adjacent region. On the other hand,

chances to produce *any* amplificate at all are increased. Since one end of the restricted fragment is known to be close to one primer, the second restriction site can be farther from the other primer and still result in a range that is not too big to be amplified by PCR after religation.

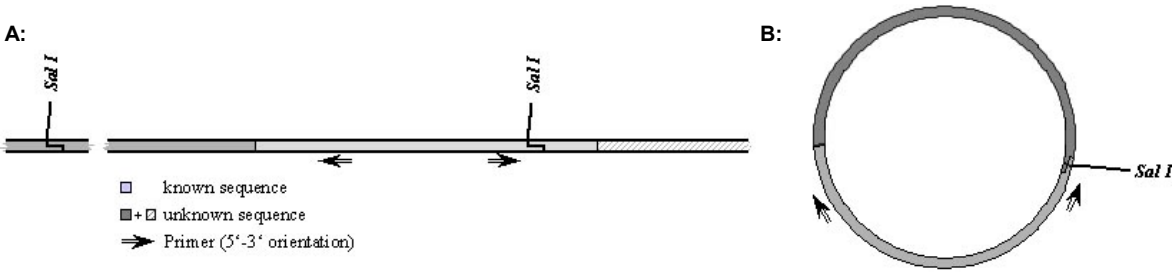


**Figure 2.2: Schematic representation of the applied variation of inverse PCR**

The restriction enzyme cuts within the known sequence (A) thus limiting the extension of the sequence information to only *one* flanking region that can be amplified after religation (B).

For digestion of DNA, the following components were added to a 1.5 ml micro tube in the given order and incubated at 37°C for 4 h.

| | |
|---|---|
| 33 - 41 µl | $H_2O$ |
| 2 - 10 µl | genomic DNA (~10 µg total) |
| 5 µl | 10x 'One-phor-all buffer+' (AMERSHAM-PHARMACIA, Freiburg) |
| 2 µl | Sal I (10 U/µl, MBI FERMENTAS) |
| 50 µl | |

The restriction enzyme was inactivated by heating to 80°C for 15 min and an aliquot of 5 µl was run on a 1,5% agarose gel to visually verify the digestion (see chapter 2.2.5.6).

Ligation of the obtained fragments was performed with a high degree of dilution by adding the following to the remaining 45 µl of digested DNA and incubating at 16°C over night:

| | |
|---|---|
| 177.5 µl | $H_2O$ |
| 25 µl | 10x ligation buffer (as supplied) |
| 2.5 µl | T4 ligase (2 U/µl, PROMEGA) |
| 250 µl | |

The ligated DNA was precipitated by adding 10.5 µl of 5M NaCl (final concentration 200 mM) and 175 µl of ice-cold 2-propanol (0.7 volumes) followed by centrifugation for 15 minutes at 13,000 rpm and 4°C in a table top centrifuge (Heraeus Biofuge fresco, KENDRO, Hanau). The supernatant was discarded and the pellet was washed with 0.5 ml of ice-cold 70% Ethanol. After centrifugation for 10 minutes as above the ethanol was removed completely. The pellet was air-dried and resuspended in 30 µl of autoclaved $H_2O$.

PCR was performed as described in chapter 2.2.5.2 using the primers **FS-19-5 IPCR Up3** and **FS-19-5 IPCR Dw3** (see appendix IX) running 30 cycles with an annealing temperature of 60°C and an elongation time of 2 minutes. The product was separated on a 1.8% agarose gel as described in chapter 2.2.5.6 and the prospective candidate for sequencing was cut from the gel, eluted and sequenced as described below (chapters 2.2.5.7 – 2.2.5.9).

### 2.2.5.5 TAIL PCR

Another method for extending a known sequence is TAIL PCR [LIU ET AL., 1995]. It uses a combination of three nested specific primers and a short arbitrary degenerate primer in subsequent amplifications. Specific product is favored over amplificates resulting from the degenerate primer alone by running complex PCR programs with alternating cycles of high and low stringency. In the primary PCR step the desired product is usually not detectable since it is only amplified *linearly* during the high stringency cycles. Secondary and tertiary PCR each employ highly diluted product of the preceding amplification as template thus enriching the desired product.

The primers used in TAIL PCR 1, 2 and 3 were **FS-19-5 Up3**, **FS-19-5 IPCR Dw** and **FS-19-5 IPCR Dw3** respectively. As arbitrary degenerate primers **DP1**, **DP2** and **DP3** were tested in separate experiments. For primer sequences see appendix IX. Reaction mixtures contained in the following order:

| | |
|---|---|
| 18.5 µl | $H_2O$ |
| 1 µl | DNA solution (~150 ng/µl) *for primary PCR*<br>or<br>1:40 dilution of preceding PCR product<br>*for secondary and tertiary PCR* |
| 1 µl | specific primer (5 mM => 0.2 mM final conc.) |
| 1 µl | arbitrary degenerate primer (50 mM => 2 mM final conc.) |
| 1 µl | dNTP-mix (2.5 µM each) |
| 2.5 µl | 10x buffer (as supplied) |
| 1 µl | RedTaq DNA polymerase (1 U) |
| 25 µl | |

The PCR programs used are shown in table 2.12. After separation of the final product on a 1.8% agarose gel (see chapter 2.2.5.6), the prospective candidate for sequencing was cut from the gel and the DNA was eluted and sequenced as described below (chapters 2.2.5.7 – 2.2.5.9). A size shift of bands between secondary and tertiary PCR corresponding to the difference in binding positions of the specific primers is a good indication of the desired product.

**Table 2.12 Temperature programs used in TAIL PCR**

| TAIL PCR 1: | | | TAIL PCR 2: | | TAIL PCR 3: | |
|---|---|---|---|---|---|---|
| 2 min | 92°C | 5 s 94°C ┐ | 2 min | 94°C | 2 min | 94°C |
| 1 min | 95°C | 1 min 59°C │ | 5 s | 94°C ┐ | 10 s | 94°C ┐ |
| 15 s | 94°C ┐ | 2 min 72°C │ | 1 min | 59°C │ | 1 min | 44°C 20 x |
| 1 min | 59°C 5 x | 5 s 94°C │ | 2 min | 72°C │ | 2 min | 72°C ┘ |
| 2 min | 72°C ┘ | 1 min 59°C 12 x | 5 s | 94°C │ | 5 min | 72°C |
| 15 s | 94°C | 2 min 72°C │ | 1 min | 59°C 10 x | ∞ | 4°C |
| 3 min | 30°C | 5 s 94°C │ | 2 min | 72°C │ | | |
| 2 min | 72°C | 1 min 44°C │ | 5 s | 94°C │ | | |
| 5 s | 94°C ┐ | 2 min 72°C ┘ | 1 min | 44°C │ | | |
| 1 min | 44°C 10 x | 5 min 72°C | 2 min | 72°C ┘ | | |
| 2 min | 72°C ┘ | ∞ 4°C | 5 min | 72°C | | |
| | continued ⇨ | | ∞ | 4°C | | |

### 2.2.5.6 Standard agarose gel electrophoresis

Agarose gel electrophoresis was used to examine the quality of genomic DNA and to separate PCR products. 1 – 2% (w/v) agarose were dissolved in 50 ml of TAE buffer (see below) by boiling in a micro wave oven. After cooling for several minutes, the solution was cast in a plastic tray and allowed to solidify for about 30 minutes. The tray was then placed in an electrophoresis apparatus containing TAE buffer.

1 - 10 µl of the samples were brought to 10 µl with sterile $H_2O$, mixed with 2 µl 6x electrophoresis buffer (MBI FERMENTAS) and transferred to the wells of the gel. One lane was loaded with 1 µl marker DNA (100 bp ladder plus, MBI FERMENTAS) mixed with 9 µl $H_2O$ and 2 µl 6x buffer. The samples were separated by applying a voltage of 5 V/cm for 1 to 2 hours. The gel was then stained for 10 to 20 minutes in an ethidium bromide solution (0.5 µg/ml) and documented under UV illumination using either a Polaroid® camera or an imaging system with a thermo printer (Image Master, AMERSHAM-PHARMACIA, Freiburg).

**Table 2.13: TAE buffer used for gel electrophoresis**

| TAE buffer (50x stock solution) | EDTA stock solution: |
|---|---|
| 0,2 M Tris base | 0,5 M EDTA pH 8,0 |
| 5,5% (v/v) Glacial acetic acid | |
| 100 ml/l EDTA stock solution | |

#### 2.2.5.7 Purification of PCR fragments

PCR fragments for cloning and sequencing were produced in a total reaction volume of 50 µl. Five adjacent lanes of an agarose gel were each loaded with 10 µl sample mixed with 2 µl 6x buffer and separation was carried out as described above. The band of interest was cut with a sterile scalpel under UV illumination and placed in a pre-weighted sterile micro tube. Elution of the DNA from the agarose and purification was performed with a commercially available kit (EasyPure®, BIOZYM, Oldendorf) according to the manufacturers instructions. The DNA was recovered in 15 µl of sterile $H_2O$.

#### 2.2.5.8 Cloning of PCR fragments and colony PCR

Purified PCR fragments were cloned into the pCR®2.1-TOPO vector (INVITROGEN, Karlsruhe see appendix XI). This vector has a cloning site with single 3'-thymidine overhangs and covalently bound topoisomerase I allowing direct cloning of PCR products derived from *Taq* polymerase.

Cloning was performed mainly as described by the manufacturer ('TOPO TA cloning kit', INVITROGEN, Karlsruhe) but dividing the given volumes for the ligation reaction in half. The following components were added to a 200 µl micro tube in the given order, mixed gently by pipetting up and down and incubated for 15 minutes at room temperature:

| | |
|---|---|
| 1.25 µl | $H_2O$ |
| 1 µl | purified PCR product |
| 0.5 µl | salt solution (as supplied) |
| 0.25 µl | TOPO vector |
| 3 µl | |

The tubes were placed on ice and 1 µl of the solution was added to 25 µl of One Shot® TOP10F' chemically competent *E. coli* (½ of the supplied aliquots) which were slowly thawed in an ice bath. After gently tapping the tube several times to mix the contents, the bacteria were incubated on ice for 15 minutes, heat-shocked for 30 seconds in a 42°C water bath without shaking and transferred back to the ice.

250 µl of SOC-medium (as supplied) or LB-medium (DIFCO, Augsburg) were added and the bacteria were incubated for 1 h at 37°C and 170 rpm in a rotary shaker (Multitron II, INFORS, Einsbach) to allow expression of ampicillin resistance. Two different volumes of the suspension (25 and 75 µl) were then plated on LB-agar plates containing ampicillin, IPTG and X-Gal ('selective plates' for blue-white-screening, see below) and incubated at 37°C over night.

Several well-separated white colonies were picked from the plates using sterile pipet tips, transferred to a fresh selective plate and incubated at 37°C for another day. Bacteria that developed a bluish shade were not used for further steps. Some of the remaining white colonies were selected and submitted to colony PCR to ensure successful insertion of the desired fragment into the vector. PCR was set up as described in chapter 2.2.5.2 using the same primers and annealing temperature as in the production of the insert, but omitting the template DNA. Instead, bacteria were added to the reaction tube by piercing a colony with a sterile pipet tip and gently pipetting the contents of the tube up and down several times. The PCR product was separated on an agarose gel as described and some of the clones that showed the desired fragment were chosen for sequencing.

**Table 2.14: Selective plates used in blue-white-screening**

| | |
|---|---|
| LB$^{amp50}$ IPTG X-Gal plates:<br><br>LB-agar (DIFCO, Augsburg)<br>Autoclave. cool to about 60°C.<br>Add 1 µl ampicillin stock solution per ml medium.<br>Pour into petri dishes. After cooling, spread:<br>40 µl of X-Gal stock solution and<br>40 µl of IPTG stock solution per plate. | Ampicillin stock solution:<br><br>50 mg/ml ampicillin in $H_2O$ |
| | X-Gal stock solution<br><br>40 mg/ml X-Gal in dimethylformamide |
| | IPTG stock solution:<br><br>100 mM IPTG in $H_2O$ |

### 2.2.5.9 Plasmid purification and sequencing of cloned PCR fragments

Overnight cultures were prepared from bacteria carrying the vector with the insert to be sequenced. The respective colonies were picked with sterile pipet tips which were then dropped into 15 ml Falcon tubes containing about 7 ml of LB-medium with 50 µg/ml ampicillin. The tubes were incubated overnight at 37°C and 170 rpm in a rotary shaker with the caps only closed lightly to allow minimum gas exchange. 2 ml of the suspension were transferred to a sterile 2 ml micro tube and centrifuged for 5 minutes at 13,000 rpm in a table top centrifuge (Picofuge, EPPENDORF). The supernatant was discarded and 2 ml of suspension were added on top of the pellet and centrifuged as above for another 5 minutes. After removing the supernatant, plasmids were purified from the bacteria using a commercially available kit ('QIAprep spin miniprep kit', QUIAGEN, Hilden) according to the manufacturers instructions. The DNA was eluted from the supplied columns with 50 µl of sterile $H_2O$ in the last step. The concentration was measured photometrically (EPPENDORF Biophotometer) at 260 nm and DNA purity was estimated using the 260 / 280 nm ratio.

Sequencing was kindly performed by D. AHLERT at our institute using the didesoxy method [SANGER et al., 1977]. The sequencing reaction was carried out in a PCR thermoblock (GeneAmp® PCR system 2400, PERKIN ELMER, Rodgau-Jügesheim) with **M13** primers (see appendix IX) that were 5'-labeled with the fluorescence dye IRD 800. Gel separation and evaluation of the fluorescence pattern employed the LI-COR 4000 sequenator (MWG-BIOTECH, Ebersberg) at the 'Institut für Botanik', Westfälische Wilhelms-Universität Münster.

Sequencing of the cDNA fragment generated with RT-PCR (see chapter 2.2.5.11) was carried out by MWG-BIOTECH, Ebersberg.

### 2.2.5.10 Isolation of fungal RNA

For RNA isolation, aliquots containing a sufficient amount of mycelium were withdrawn from the respective cultures at different points in time using sterile 5 ml pipet tips and passed through membrane filters (0.2 µm pore size, ME 24) under vacuum assistance. Aliquots of 100 to 150 mg were cut from the filter cake with a sterile spatula, wrapped in aluminum foil and quick-frozen in liquid nitrogen. The samples were stored at -70°C until used.

Preliminary attempts to isolate RNA from *Fusarium graminearum* samples using a commercially available extraction solution (peqGOLD Trifast, PEQLAB, Erlangen) suggested that this method is not suitable for filamentous fungi since the quality of RNA was poor. It was however successfully used to extract RNA from wheat leaves inoculated with the basidiomycete *Puccinia graminis* (M. KOHLHOFF, personal communication).

RNA from *Fusarium* was therefore isolated using a urea and phenol / chloroform based protocol similar to that used for DNA isolation (T. CONZE, personal communication). Care was taken to make sure that all reagents and buffers as well as laboratory material were free of RNase-activity. Solutions were prepared using the highest available quality of chemicals with autoclaved ultrapure water. Mortars, pistils, pipet tips and micro tubes were autoclaved and heated to 120°C over night.

The frozen mycelium was ground to a fine powder in a precooled mortar with liquid nitrogen. It was then quickly transferred to a sterile Falcon tube containing a mixture of 2.5 ml isolation buffer and 2.5 ml urea solution using a cold sterile spatula. The sample was submerged in the mixture by gently inverting the tube several times before 2.5 ml of phenol (pH 4.3, SIGMA) were added. After carefully mixing again, the suspension was allowed to sit for a few moments. 5 ml of phenol / chloroform (1:1) were then added and the tube was inverted several times before centrifuging for 10 minutes at 3000 rpm (~1500 x g) in a swing-out rotor at 4°C (Heraeus Megafuge 1.OR, KENDRO, Hanau ).

The aqueous upper phase containing the RNA was carefully transferred to a new Falcon tube avoiding the white interphase pellet. If the pellet was very prominent, the phenol / chloroform extraction was repeated and the aqueous phase was again transferred to a new tube. An equal volume of a 4 M LiCl solution was added, giving a final concentration of 2 M LiCl. The RNA was precipitated at 4°C overnight.

After centrifuging for 120 minutes at 2500 rpm (~1000 x g) in a swing-out rotor at 4°C the supernatant was discarded, the pellet was resuspended in 0.57 ml of RNAse-free water and transferred to a sterile 2 ml micro tube. 1.425 ml of 100% EtOH (= 2.5 volumes) and 57 µl of sodium acetate solution (= 1/10 volume) were added and the tube was inverted several times. The RNA was reprecipitated by centrifuging for 120 minutes at 4000 rpm (~1500 x g) in a table top centrifuge at 4°C (Heraeus Biofuge fresco, KENDRO, Hanau).

The supernatant was discarded and the pellet was washed with 1 ml of ice-cold 70% EtOH before centrifuging for 15 minutes at 3300 rpm (~1000 x g) in a table top centrifuge at 4°C. After discarding the supernatant, the tubes were centrifuged briefly to collect remaining Ethanol at the bottom, which was then carefully removed. The pellet was air-dried and resuspended in 50 to 100 µl of RNAse-free water. RNA concentration was measured photometrically (EPPENDORF Biophotometer) at 260 nm and RNA purity was estimated using the 260 / 280 nm ratio. The samples were stored at -20°C until used.

For some experiments, remaining DNA residues were digested with DNase (RQ1 RNase-free DNase, PROMEGA) by incubating 1 to 10 µg of RNA with the supplied 10x buffer and 1 to 10 U of DNase brought to a total volume of 10 µl with $H_2O$. After 10 to 15 minutes at 37°C the reaction was terminated by adding 1 µl of the supplied Stop-solution and the DNase was denaturated at 65°C for 10 minutes.

**Table 2.15: Solutions used for RNA isolation**

<table>
<tr><td rowspan="4">2 x isolation buffer:<br>0,6 M NaCl<br>0,1 M Tris<br>40 mM EDTA<br>4% (w/v) Sarcosyl<br>1% (w/v) SDS<br>(also used for DNA)</td><td rowspan="2">2 x urea solution:<br>10 M Urea<br>warm slightly before use to avoid crystallization<br>(also used for DNA)</td><td>LiCl solution:<br>4 M Lithium chloride</td></tr>
<tr><td>100% Ethanol (room temperature)</td></tr>
<tr><td rowspan="2">Ice-cold 70 % Ethanol<br>(also used for DNA)</td></tr>
<tr><td>Phenol (pH 4,3 SIGMA)</td><td rowspan="2">Sodium acetate solution:<br>3 M Sodium acetate</td></tr>
<tr><td>Phenol / Chloroform (1:1)</td></tr>
</table>

### 2.2.5.11 cDNA production and RT-PCR

cDNA was prepared from total RNA that was isolated as described in chapter 2.2.5.10 from mycelium grown in PI-medium for 14 hours after mass inoculation. Poly-A-RNA was transcribed to DNA with an RNA-dependent DNA polymerase lacking RNase H activity (M-MLV reverse transcriptase, RNase H minus, point mutant, PROMEGA) using anchored oligo $(dT)_{23}$-primers (from 'enhanced Avian RT-PCR kit', SIGMA). In a first step the following components were added to a 200 µl micro tube in the given order:

| | |
|---|---|
| 10 µl | $H_2O$ |
| 2 µl | RNA solution (~1 µg/µl) |
| 2 µl | $dX(dT)_{23}$-primer (70 µM, X=A,T,G,C) |
| 14 µl | |

The mixture was centrifuged briefly and heated for 10 minutes at 70°C to melt RNA secondary structures and snap-cooled on ice for 5 minutes to allow annealing of primers. The reaction mixture was completed by adding in the following order:

| | |
|---|---|
| 5 µl | 5x buffer (as supplied) |
| 5 µl | dNTP-mix (25 mM each, SIGMA) |
| 1 µl | reverse transcriptase (200 U) |
| 25 µl | |

After mixing, the tubes were centrifuged briefly and incubated for 60 minutes at 40°C followed by 15 minutes at 70°C to inactivate the enzyme.

The produced cDNA was used as a template for PCR with primer combinations that enclose a putative intron sequence. This way, sequencing of the obtained product could verify the existence of the expected intron and PCR fragments derived from cDNA could be distinguished from amplificated genomic DNA (gDNA) by their size.

The primers used were **FgP Up** and **FS-19-5 Dw3** (see appendix IX) which give an amplificate of 849 bp with gDNA and a predicted amplificate of about 800 bp with cDNA. PCR conditions were as described in chapter 2.2.5.2 using 2 µl of cDNA as template instead of gDNA. The PCR product was separated on a 2% agarose gel and visually examined with UV irradiation after staining with ethidium bromide.

A band with the expected size of 800 bp was cut from the gel and the DNA was purified and sequenced as described above (chapters 2.2.5.7 – 2.2.5.9).

### 2.2.5.12 Quantitative expression analysis

Since the identity of the putative cDNA fragment was confirmed by comparison with the genomic sequence, RT-PCR could be used for qualitative analysis of the gene expression during fungal growth on the plant.

Total RNA was isolated from leaves inoculated with *Fusarium graminearum* conidia (see chapter 2.2.3) which were harvested daily from 1 to 5 days past inoculation (dpi). RNA from water treated leaves was used as a control. As described in chapter 2.2.5.11 cDNA was prepared from these samples and PCR was performed with the given primer pair. Since these primers result in a cDNA-derived PCR product which can easily be distinguished from the fragment amplified with genomic DNA as the template, DNase digestion was omitted during the RNA preparation.

PCR products were separated on a 2% agarose gel and visually examined under UV irradiation after staining with ethidium bromide.

## 2.3 Computer aided methods

### 2.3.1 Documentation of polyacrylamide gels, agarose gels, macroscopic and microscopic images

SDS-PAGE gels and Polaroid® images of agarose gels were scanned using a HEWLETT-PACKARD DeskScan 4c/T equipped with a transilluminating unit. Microscopic images which were photographed using slide films were scanned with a NIKON LS-2000 slide scanner.

Agarose gels were also documented using an imaging system (Image Master, AMERSHAM-PHARMACIA, Freiburg) as thermo prints and digital images.

Macroscopic images were taken using a digital camera (OLYMPUS C-4040 Zoom) and transferred to a computer.

The obtained images were processed with imaging software to enhance the quality for printing. The employed methods – like excising the relevant parts and enhancing the contrast – did not change the validity of the images for documentation of the results.

### 2.3.2 Analysis of DNA and amino acid sequences

Data obtained from DNA sequencing was analyzed with the 'DNAstar' software (DNASTAR INC., Madison, Wisconsin, USA). The module 'SeqMan' was used to check and manually edit the trace files resulting from the sequencing and to assemble overlapping fragments of DNA sequences. The module 'EditSeq' was used for simple processing of the sequences and for translation into amino acid codes. The software 'pDRAW32' (http://www.geocities.com/acaclone) was used to draw a map of assembled sequencing fragments and of primer positions.

### 2.3.3 Databases

Database research was performed with the databases and tools available through the online resources of the European Bioinformatics Institute (EBI) which can be reached at 'http://www.ebi.ac.uk'.

For comparing the sequences obtained during this work with the databases, the programs 'BLAST' ('Basic Local Alignment Search Tool') [Altschul et al., 1997; Altschul et al., 1990] and 'FASTA' [Pearson, 1990; Pearson and Lipman, 1988] were used.

### 2.3.4 Sequence alignment

Sequence alignments of DNA or amino acid sequences were performed with the program 'ClustalW' [Thompson et al., 1994] either online (http://www.ebi.ac.uk/clustalw) or with the locally installed interface 'ClustalX' [Thompson et al., 1997] which was used to export colored alignments and phylogenetic tree diagrams. ClustalW was also used as part of the sequence analysis software 'BioEdit' [Hall, 1999].

### 2.3.5 Prediction of protein characteristics based on amino acid sequences

The prediction of putative sorting signal sequences in a given amino acid sequence and the most probable target compartment was performed using the programs 'SignalP 2.0' (http://www.cbs.dtu.dk/services/SignalP-2.0) [Nielsen et al., 1997] and 'PSORT II' (http://psort.ims.u-tokyo.ac.jp) [Nakai and Horton, 1999; Nakai and Kanehisa, 1992].

The theoretical isoelectric point and molecular weight were calculated based on amino acid sequences using the program 'ProtParam' [Bjellqvist et al., 1994; Bjellqvist et al., 1994] available at http://ca.expasy.org. Investigation of sequence motifs was performed using the program 'InterPro' which integrates a wide range of separate services (http://www.ebi.ac.uk/interpro) [Mulder et al., 2003] that interpret conserved motifs to classify protein domains.

# 3 Results

The major goal of this work was to elucidate the inducability of secreted proteases of *Fusarium graminearum* and to characterize such proteases on biochemical and molecular levels. Cultivation of the fungus had to be established at our institute and growth under different conditions with respect to nutrient availability was investigated. Emphasis was placed on nitrogen depletion and the ability of the fungus to degrade protein substrate. The results will be presented in chapters 3.1 to 3.6 including data about basic biochemical properties of proteolytic activity induced in a specific medium. Chapter 3.7 presents the partial purification of a protease from the medium using cation exchange chromatography. Cloning and sequencing of a protease gene from *F. graminearum* as well as molecular characterization of this gene will be presented in chapter 3.8 and 3.10 In chapters 3.11 and 3.12, the cloning of a fragment of the respective cDNA and qualitative expression studies during fungal growth in submerged culture and in a model system for *in planta* growth are presented.

## 3.1 General characterization of fungal growth

### 3.1.1 Growth on CM agar

Stock cultures of *F. graminearum* were cultivated on CM-agar (see figure 3.1). About five days after inoculation, the plate was completely covered by the fungus with the mycelium changing color over time. Aerial hyphae were white at early stages and developed a pink hue after longer cultivation. The dense mycelium which grew close to the substrate was slightly orange during the first days and then turned darker while the agar became red to brownish. The cultures often showed ring-like structures with less prominent aerial hyphae and stronger pigmentation of the agar. While storing the plates at 4°C in the dark for several weeks, the mycelium flattened and conidia formation was observed.

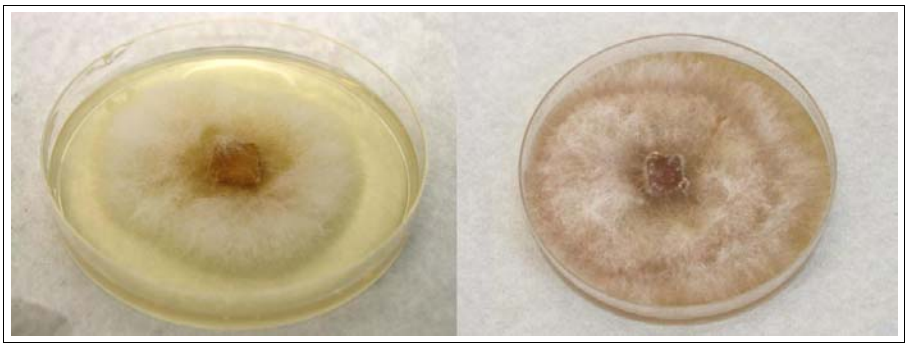

**Figure 3.1:** ***Fusarium graminearum* grown at room temperature on CM-agar for four (left) and six days.** The color of the mycelium ranged from white to slightly orange and pink. The agar also developed a red to brownish appearance after several days of cultivation. The strong formation of aerial hyphae is clearly visible.

### 3.1.2 Submerged culture in CM-medium

The fungus grew readily when cultivated in liquid CM-medium. One day after inoculation with pieces of agar from the stock culture, a fine layer of mycelium was observed growing on the surface of the blocks, and small spheres of about 1 mm in diameter – most probably derived from fragments of hyphae or from conidia – were suspended in the medium. For further experiments, multiple flasks of fresh medium were inoculated with these small colonies. This way it was possible to eliminate the carry-over of agar pieces from the stock culture and an even distribution of mycelium for subsequent experiments could be ensured. Moreover, this preculture step served to make sure that all parallel experiments were performed with mycelium that was adapted to liquid medium and in the same state of growth.

The increase of fungal biomass – as measured by the fresh weight of mycelium - was observed within five days past inoculation (dpi) of CM-medium with aliquots of the preculture. The fresh weight at the time of inoculation was not determined and set to zero since only very little mycelium was transferred. As shown in figure 3.2 exponential growth lasted until two dpi after which a static growth phase was entered. At five dpi a decrease in fresh weight was observed indicating the beginning of the decline phase. The fresh weight of the mycelium reached a maximum of about 2.4 g per flask at four dpi.

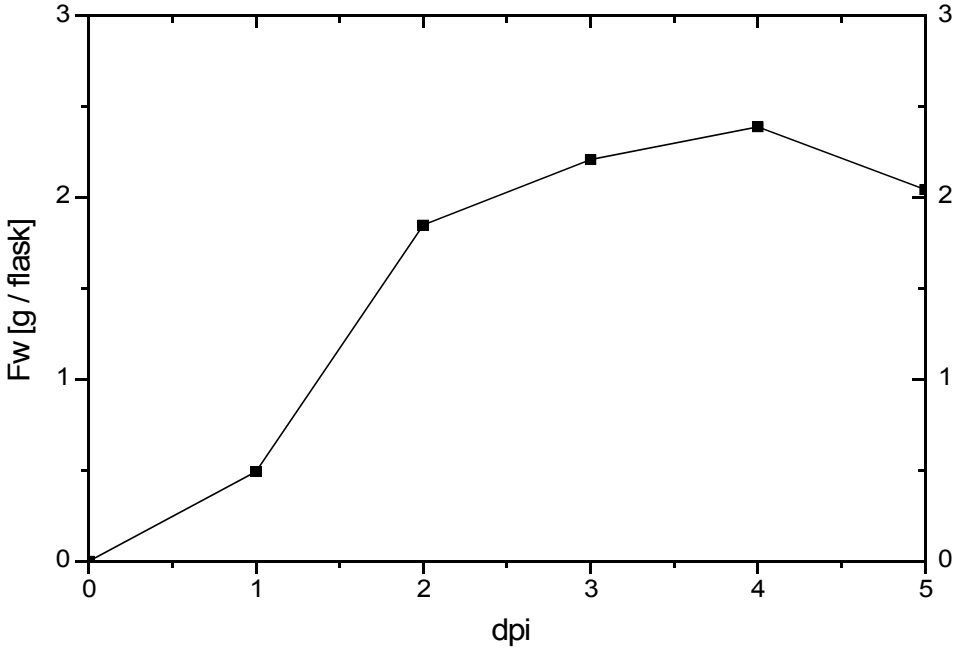


**Figure 3.2: Growth of *Fusarium graminearum* in submerged culture with CM-medium.**
Flasks containing 50 ml of CM-medium were inoculated with preculture and incubated for up to five days. Mycelium was harvested daily to determine the fresh weight of the produced fungal biomass. Shown is the mean value of two measurements from one experiment. dpi = days past inoculation, Fw = fresh weight

The morphology of the mycelium mainly stayed spherical throughout the experiment with the colonies having a loose appearance and growing to a size of up to 0.5 – 1 cm in diameter. At later stages, mycelium which had a filamentous appearance was also observed floating in the medium, giving it a turbid look. The color of the mycelium was white to pale yellow in the beginning of the experiment. This could be due to the yellow to orange color of the CM-medium itself. Over time, the mycelium darkened and became brownish towards the end of cultivation.

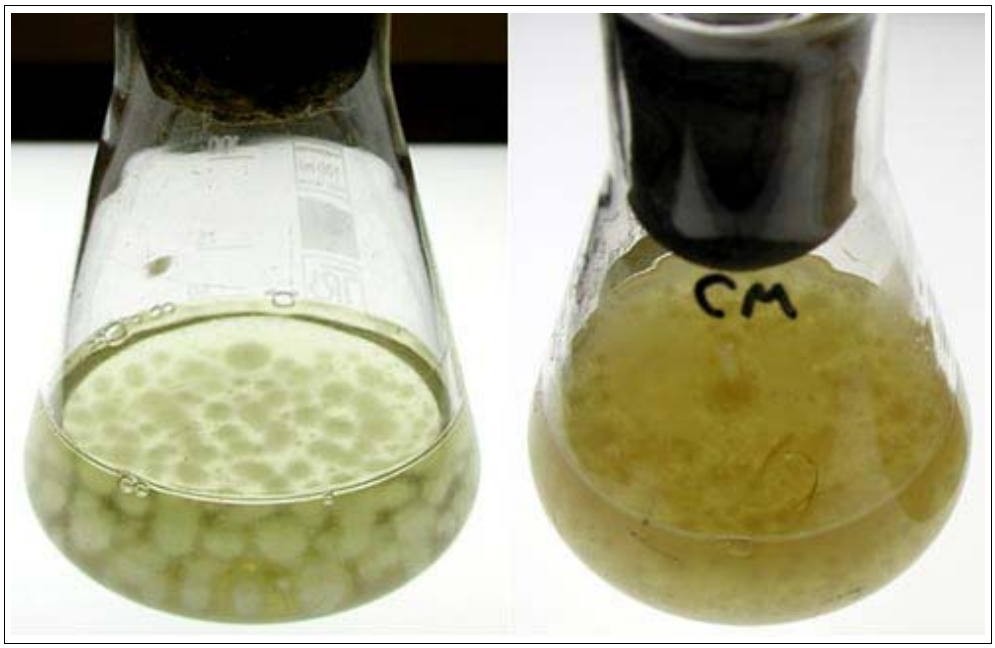


**Figure 3.3:** ***Fusarium graminearum*** **grown in submerged culture with CM-medium.**
Pictures were taken one day (left) and four days after inoculation of the medium with preculture. The change in color of the mycelium and the increase in turbidity of the medium at day four is visible.

### 3.1.3 Formation of conidia

*F. graminearum* did not sporulate in CM-medium within five days of cultivation. However, when grown in conidia induction medium (see chapter 3.2.1.3), massive formation of conidia was observed after about five days. When they were pelleted during harvesting, the conidia appeared slightly red to brownish. Under the microscope, conidiophores were visible on the mycelium and the characteristic multiseptate 'banana-shaped' macroconidia were seen. The mycelium had the usual highly branched appearance with distinct septae which was observed in all media used in this work.

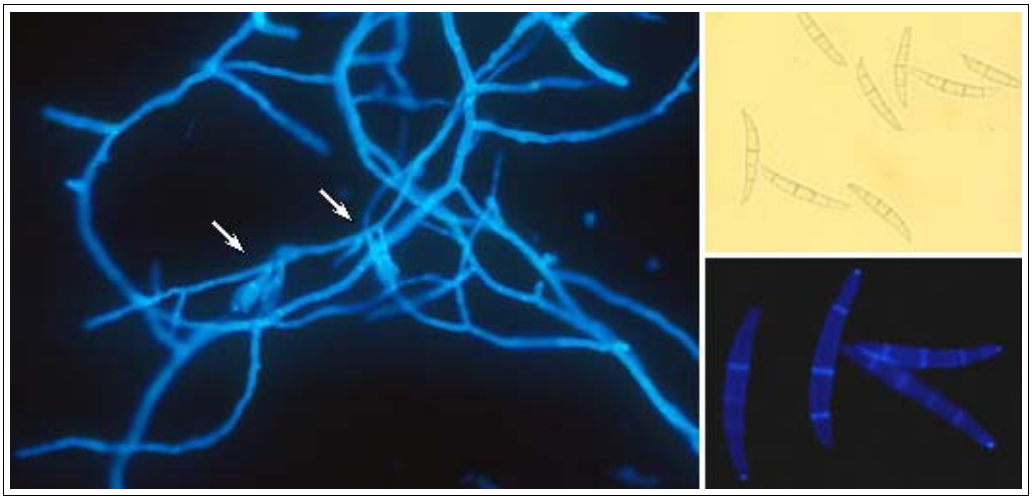

**Figure 3.4 Conidia formation in conidia induction medium.**

The fungus was cultivated for five days in conidia induction medium. An aliquot was stained with Calcofluor (see chapter 2.2.3) and examined under the microscope in bright-field (top right) and with UV irradiation. The arrows indicate conidiophores where conidia are produced. The small inlets show several conidia with the typical shape and septation. (magnification 200x and 400x)

## 3.2 Optimization of the photometric protease assay

The assay used for determining proteolytic activity in the growth medium during submerged culture is based on the digestion of a dye-labeled protein substrate. After incubation with the sample, non-digested substrate is precipitated and the optical density of the supernatant containing the cleaved fragments is determined photometrically as a measure of protease activity.

To examine the range of activity for which this assay is suitable, different concentrations of Proteinase K were incubated with the substrate for incubation times between 30 and 60 minutes as described in 2.2.4.1. The difference in absorption at 400 nm between the heat-denaturated comparative samples and the active samples ($\Delta E_{400}$) was determined. In addition, different volumes of a protease-containing sample of fungal growth medium were assayed the same way.

It turned out, that the relation between the absorption at 400 nm and the protease activity in the sample was linear for up to one hour for standard activities obtained in induction media. If $\Delta E_{400}$ was higher than 0.25, the respective assay was repeated with less sample or with a shorter incubation time.

The three parallel measurements that were routinely performed ususally showed very little deviation from the arithmetic mean of the calculated values, making this a reliable and reproducible method.

## 3.3 Development of protease induction medium

The growth rate of *F. graminearum* in CM-medium was highest between one and two days after inoculation with preculture, therefore medium for protease induction assays was harvested at two dpi. It turned out, that by assaying 100 µl of growth medium from CM-cultures with an incubation time of 60 minutes as described in chapter 2.2.4.1, no significant protease activity could be measured.

However, when the nitrogen and carbon sources in the medium were limited and gelatin as a long-chained protein substrate was offered, protease activity was clearly detectable in samples of growth medium at two dpi. The induced activity increased with the concentration of the substrate as shown in figure 3.5.

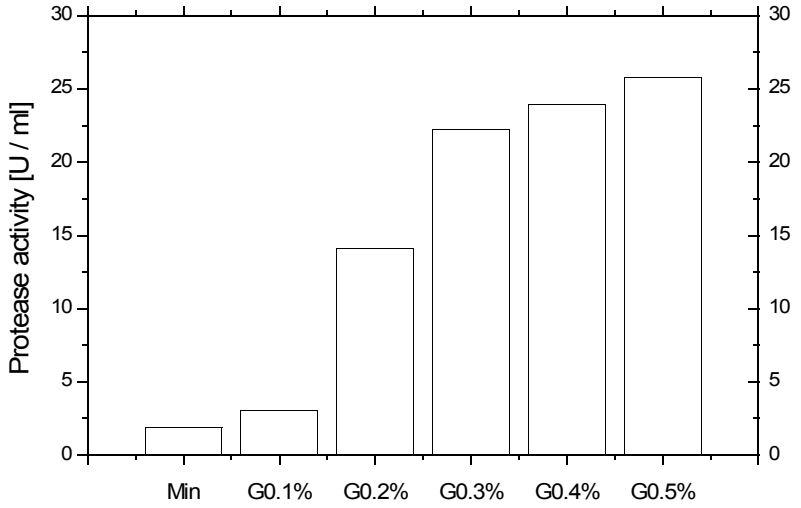


**Figure 3.5: Induction of protease activity in the growth medium with gelatin**

50 ml each of minimal medium and media containing different concentrations of protein (gelatin) as the single source of nitrogen and the major source of carbon were inoculated with preculture and cultivated for two days before assaying for protease activity in the medium. Shown is the mean of three parallel measurements from one experiment. Abbreviations: Min = minimal medium, G0.1% – G0.5% = minimal medium containing 0.1 to 0.5% (w/v) of gelatin respectively.

Furthermore, the growth rate of the fungus – measured as fresh weight of the produced fungal biomass at two dpi – increased with the gelatin concentration as well (see figure 3.6). In minimal medium without protein, the fungus showed a very limited increase in fungal biomass. When the protease activity per flask is set in relation to the fresh weight of mycelium per flask, the strong secretion of protease induced in induction media containing 0.2% gelatin and over gets obvious (see figure 3.7).

As the total induced protease activity in the medium was highest in minimal medium containing 0.5% gelatin, this was used as protease induction medium (PI-medium) for all further experiments even though the protease activity per fresh weight was highest for 0.3% gelatin.

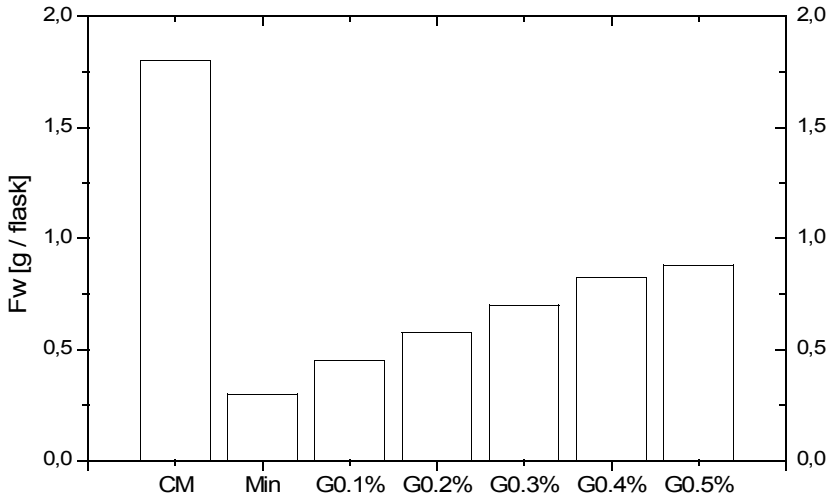


**Figure 3.6: Fresh weight of mycelium grown for two days in different media.**

50 ml each of CM-medium and media containing different concentrations of protein (gelatin) as the single source of nitrogen and the major source of carbon were inoculated with preculture and cultivated for two days before determining the fresh weight of the mycelium. Shown is the mean of two parallel measurements from one experiment. Abbreviations: CM = CM- medium, Min = minimal medium without gelatin, G0.1% – G0.5% = minimal medium containing 0.1 to 0.5% (w/v) of gelatin respectively.

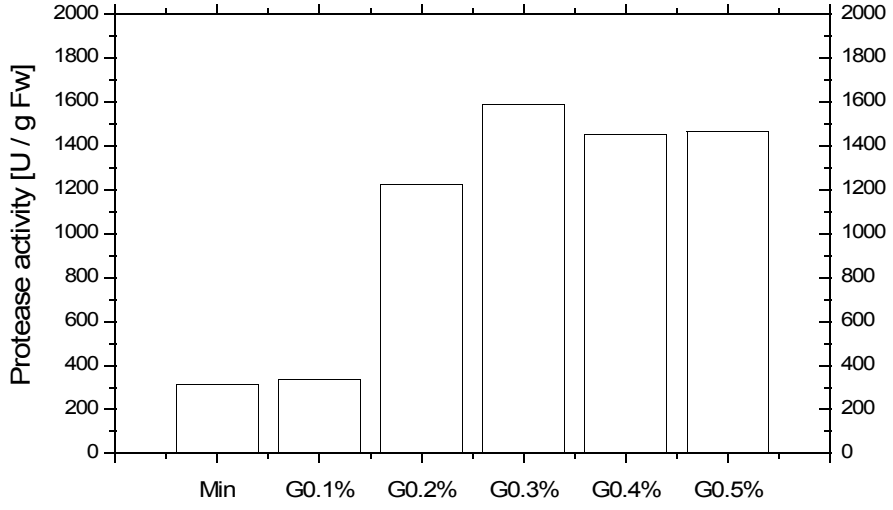


**Fig 3.7: Protease activity per fresh weight.**

The protease activity per fresh weight is calculated from the values depicted in the previous figures. Abbreviations: Min = minimal medium without gelatin, G0.1%–G0.5% = minimal medium containing 0.1 to 0.5% (w/v) of gelatin respectively.

## 3.4 Characterization of growth in protease induction medium

### 3.4.1 Growth on PI-agar compared to CM- and minimal-agar

Solidified CM-, PI- and minimal medium as well as CM-medium supplemented with 0.5% gelatin (gCM-medium) were inoculated with a stock culture of *F. graminearum* and incubated at room temperature on the work bench. Apart from the minimal-agar, all plates could serve as a substrate for fungal cultivation (see figure 3.8, top row). The very limited growth observed on minimal-agar only occurred close to the inoculum and did not extent further than a few millimeters.

Growth on CM-agar was abundant and quick and showed aerial hyphae and dense mycelium as described in chapter 3.2.1. The fungal morphology on PI-medium differed markedly from that on CM-agar and growth was much slower. Aerial hyphae were rare and the mycelium showed a very dense, almost yeast-like appearance. While the fungus took on a red to brownish hue after prolonged cultivation on CM-agar, this already was the case for PI-agar after about four days. The CM-agar itself turned yellow to orange and red over time. PI-agar developed a deep red color.

These differences can also be seen in the bottom row of figure 3.8, which shows a plate that was cast using the four different substrates and inoculated with a piece of stock culture. After four days, the fungus had overgrown about one half of the quarters containing CM- and gCM-agar with strong formation of aerial mycelium on the edges. On the other two quarters growth proceeded much slower and aerial hyphae were only spreading in from the adjacent fields.

At six days past inoculation the CM- and gCM-segments were completely covered and mycelium from these had also entered the PI- and minimal-agar-containing quarters making a quantification difficult. The change in color of the agar can clearly be seen at the bottom of the plate (figure 3.8, bottom right). Minimal- and CM-agar turned yellow to orange while PI-agar turned dark red. Interestingly, gCM-agar also developed a dark red color, indicating that this is an effect related to the gelatin content.

Under the binocular both PI- and minimal agar showed very dense and highly branched mycelium that appeared very thin and had only short branches (see figure 3.9). On CM- and gCM-agar, the aerial mycelium with its longer branches and thicker hyphae was seen.

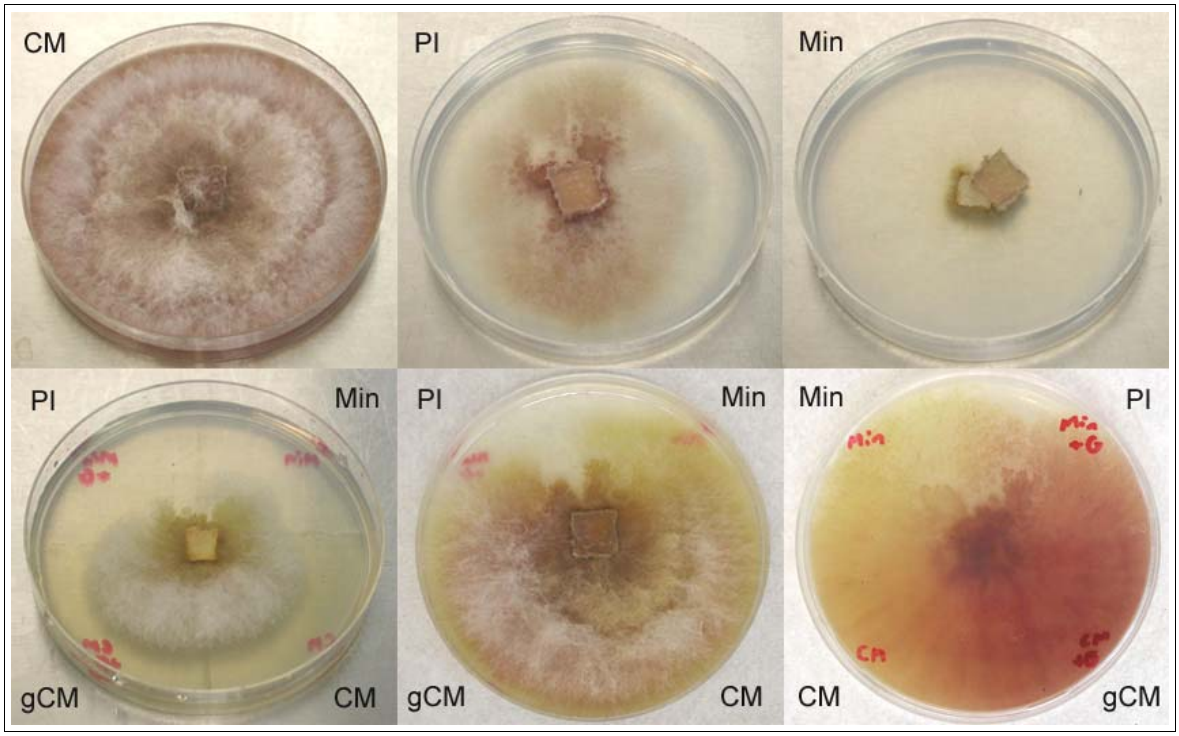


**Figure 3.8:** ***Fusarium graminearum*** **grown on different substrates for four and six days.**

CM-, CM- with 0.5% gelatin (gCM-), PI- and minimal medium were solidified with 1.5% agar, inoculated with a piece of stock culture on CM-agar and incubated at room temperature on the workbench. The top row shows mycelium after six days of growth. The bottom row shows a plate consisting of four separately cast media after four days (left) and six days of growth (middle: top view of the mycelium, right: bottom view of the agar). Clearly visible is the almost complete lack of aerial hyphae on PI-agar compared to CM-agar as well as the very limited growth on minimal agar. On PI- and minimal-agar a dense, almost yeast-like growth was observed. The different colors of the agar after six days are obvious.

**Figure 3.9:** **Mycelium grown on PI- (left) and CM-agar.**

Mycelium that was grown for four days as described above was inspected under the binocular and photographed with a digital camera that was held to the eyepiece. The left part of the picture shows dense and highly branched mycelium on PI-agar. On the right, aerial hyphae on CM-agar are shown. Magnification: 50x

### 3.4.2 Submerged culture in PI-medium compared to CM- and minimal medium

Growth in submerged culture with PI-medium was significantly reduced in comparison with CM-medium (see figure 3.10). With about 1.25 g/flask at days three to five, the maximum fresh weight was only about one half of that reached in CM-medium and the growth curve showed a lag phase of about one day before the exponential growth began. The static growth phase was reached at three dpi and lasted until the end of the experiment.

On the other hand, the fungus clearly showed a stronger growth in PI-medium than in minimal medium. With about 0.3 g/flask, only a very limited increase in biomass was observed during the first two days of growth in this medium followed by a steady decrease.

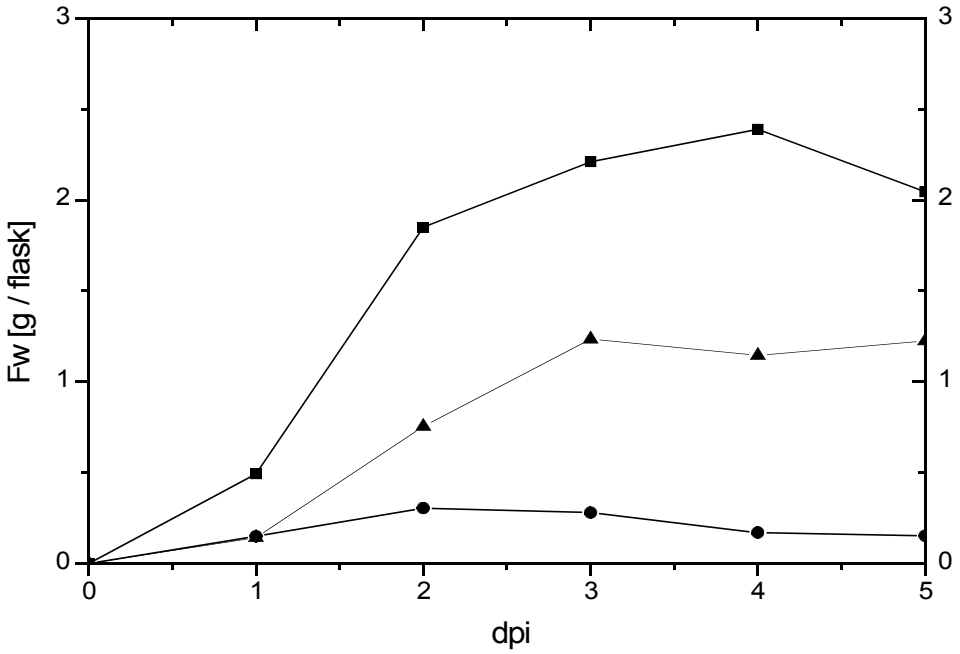


**Figure 3.10: Comparison of growth in CM-, Minimal-, and PI-medium.**

50 ml of CM- (■), minimal (●) and PI-medium (▲) respectively were inoculated with preculture and cultivated for up to five days. Mycelium was harvested daily to determine the fresh weight of the produced fungal biomass. Shown is the mean value of two measurements from one experiment. dpi = days past inoculation, Fw = fresh weight

The morphology of mycelium grown in PI-medium differed markedly from that grown in CM-medium (see figure 3.11). A compact spherical nature of the colonies was maintained throughout the experiment, and little to no filamentous material was observed. The spheres did not grow to the same size as those in CM-medium. Furthermore, the mycelium and medium developed a light to dark red color from about two days past inoculation on, which intensified over time. This is shown in more detail in chapter 3.5.5 below.

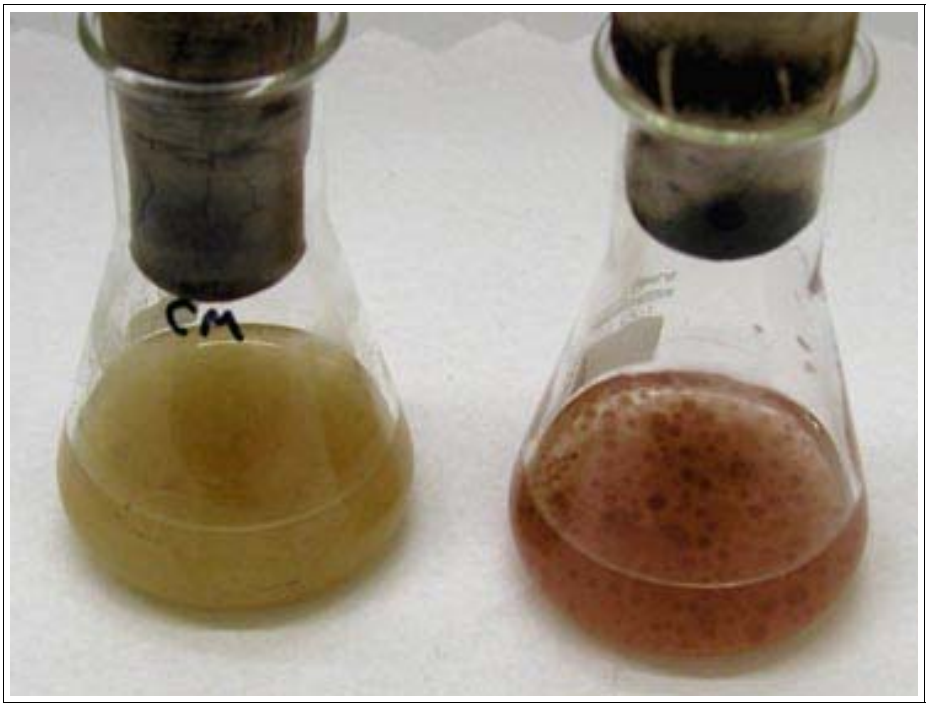


**Figure 3.11:** ***Fusarium graminearum*** **grown for four days in CM- (left) and PI-medium.**
50 ml of CM- and PI-medium respectively were inoculated with preculture and incubated for four days. The difference in the macroscopic appearance of the mycelium is obvious.

Under the microscope, hyphae that were grown in PI-medium appeared thinner than those from CM-medium. During the last days, conidia formation was observed, but the amount was less than in conidia induction medium and differed between separate experiments.

## 3.5 Kinetics of protease secretion

### 3.5.1 Overview of protease secretion – daily samples

As shown in figure 3.12, protease activity was strongly induced in PI-medium after a lag phase of one day. Between one and three dpi the activity quickly rose from almost zero U/ml to about 10 U/ml. In the following two days a steady decline in protease activity was observed, reaching 4 U/ml at five dpi. In CM-medium only a slight variation in protease activity was observed with the overall level being very low. However, it has to be stated, that in a few experiments a transient increase was also measured in CM-medium which showed a similar time course as in PI-medium, but lagged about one day behind and reached a significantly lower maximum. Usually though, protease activity was not induced in CM-medium.

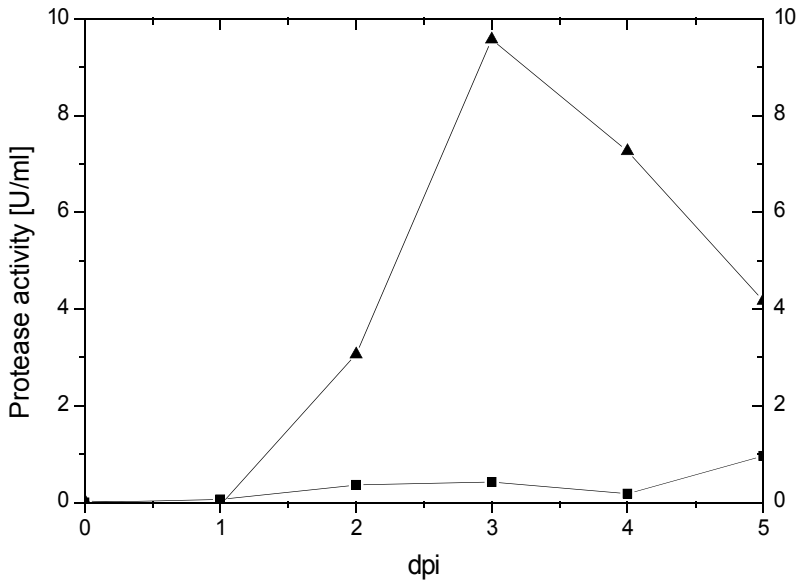


**Figure 3.12: Kinetics of protease induction with CM- and PI-medium.**

50 ml of CM- (■) and PI-medium (▲) respectively were inoculated with preculture and cultivated for up to five days. Medium was harvested daily and assayed for protease activity. Shown is the mean value of three measurements each for two flasks per day (PI-medium) or one flask per day (CM-medium) from one experiment. dpi = days past inoculation

### 3.5.2 Basic characterization of the protease activity induced in PI-medium

#### 3.5.2.1 pH-optimum

The pH-optimum of the protease activity present in PI-medium at two days past inoculation with preculture was determined using four different buffers as shown in figure 3.13. A very broad maximum ranging from about 6.5 to 10.5 was found with a steep decrease in activity below 6.5. Within the examined range, no distinct peaks were observed that would indicate the presence of multiple proteases with different pH-optima.

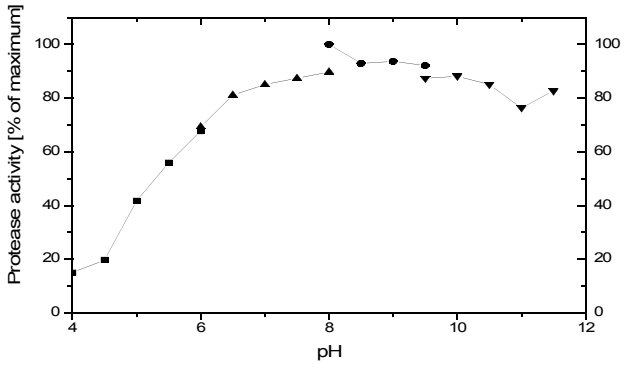


**Figure 3.13: pH-dependency of the protease activity induced in PI-medium.** The protease activity was determined in a sample taken after two days past inoculation with preculture. The buffers used were Citrate (■), Bis-tris (▲), Tris (●) and CAPS (▼). Shown is the mean of two measurements from one experiment normalized as percent of the maximum determined value.

#### 3.5.2.2 Temperature optimum

The temperature optimum of the protease activity present in PI-medium at two dpi was determinined by assaying at different temperatures between 10 and 80°C. Figure 3.14 shows an increase in protease activity up to 60 °C with a rapid decrease at higher temperatures.

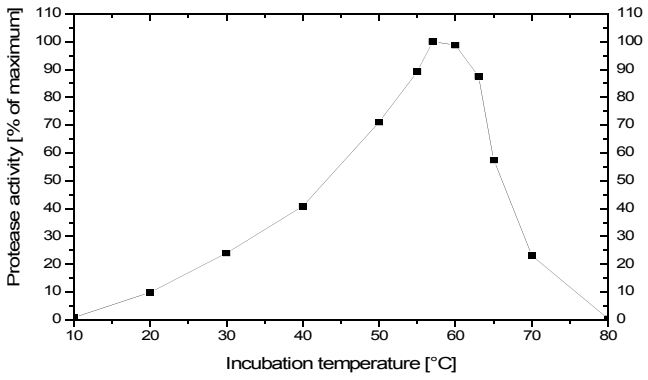


**Figure 3.14: Temperature-dependency of the protease activity induced in PI-medium.** The protease activity was determined at different incubation temperatures in a sample taken after two days past inoculation with preculture. Shown is the mean of two measurements from one experiment normalized as percent of the maximum determined value.

### 3.5.2.3 Inhibitor studies

Samples of PI-medium harvested at two days past inoculation with preculture were preincubated with a selection of protease inhibitors before assaying for protease activity to investigate the type of protease that is secreted. The used inhibitors are selective for different classes or families of proteases. The general range of inhibitory action is given in figure 3.15 which shows the effect of the employed substances on the protease activity compared to the untreated control.

Bestatin and phosphoramidone as inhibitors of metalloproteases clearly showed no effect, while EDTA apparently increased protease activity in the sample. This could be due to an enhanced solubility of the azocasein substrate with EDTA, interfering with the assay. E-64 and pepstatin, which exclusively inhibit cysteine and aspartic proteases, did not significantly affect protease activity in the sample. Inhibitory effects are obvious though in the groups of serine and serine / cysteine protease inhibitors. Soybean trypsin inhibitor did not show a significant inhibition, but preincubation with apronitin and leupeptin lead to a decrease in protease activity of about 20 and 35% respectively compared to the untreated control. Chymostatin and PMSF caused a decrease of about 85 and 90% respectively. The observed inhibition profile indicates, that the activity measured in PI-medium relates to a serine protease.

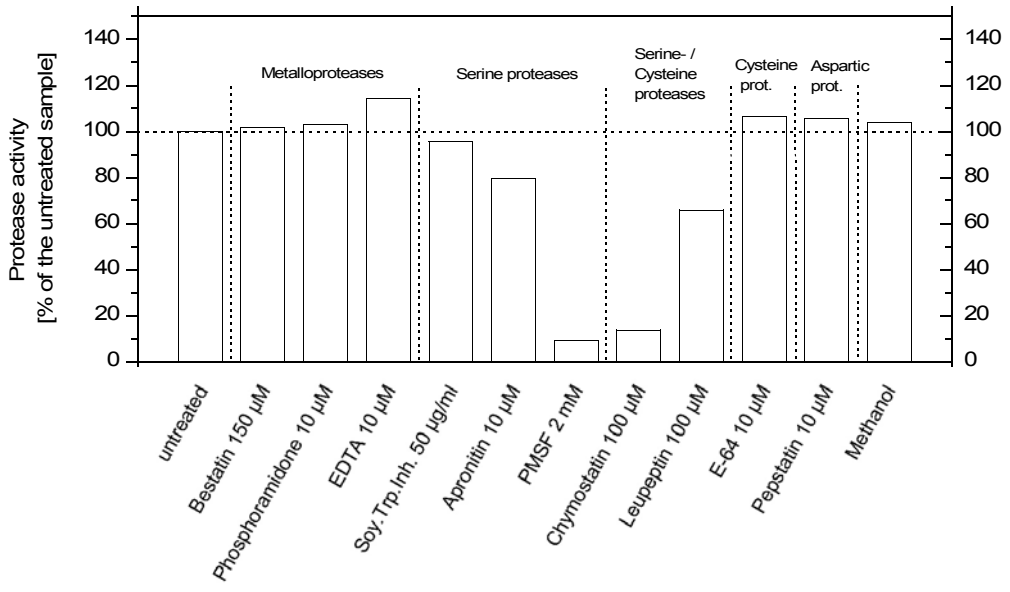


**Figure 3.15: Inhibition of protease activity in PI-medium with specific inhibitors.**

A sample of PI-medium harvested after two days past inoculation with preculture was preincubated for 15 minutes with the listed inhibitors before assaying for protease activity. Methanol was included as a control since it was used as a solvent for some inhibitors. The measured activity was normalized as percent of the untreated control. Shown is the mean of three measurements from one experiment. EDTA: ethylene-diaminetetraacetic acid, Soy.Trp.Inh: Soybean trypsin inhibitor, PMSF: Phenylmethylsulfonylfluoride, E-64: trans-epoxysuccinyl-L- leucylamido(4-guanidino)-butane

### 3.5.3 Kinetics of initial protease secretion – Mass inoculation

Even though it was known from the previous experiments, that protease secretion could be induced by growing *F. graminearum* in PI-medium, it was unclear whether the observed lag phase during the first day was due to slow adaptation of the mycelium to the change in medium or due to dilution effects because of the small amount of transferred mycelium. To elucidate this question, about 1 g of mycelium was grown in a secondary preculture with CM-medium and transferred to CM- and PI-medium respectively. This way, even small amounts of protease activity could be visualized during the initial phase of induction.

It turned out, that the fungus does react very fast to the change in medium (see figure 3.16). At four hours past inoculation (hpi) an increase in protease activity in the medium was observed. It quickly reached about 1 U/ml at six hpi and then stayed on a plateau of about 1.2 U/ml for twelve hours. A rapid increase from 1.2 to almost 5 U/ml was measured within in the following three hours. In CM-medium, protease was not secreted in significant amounts as described for the previous experiments.

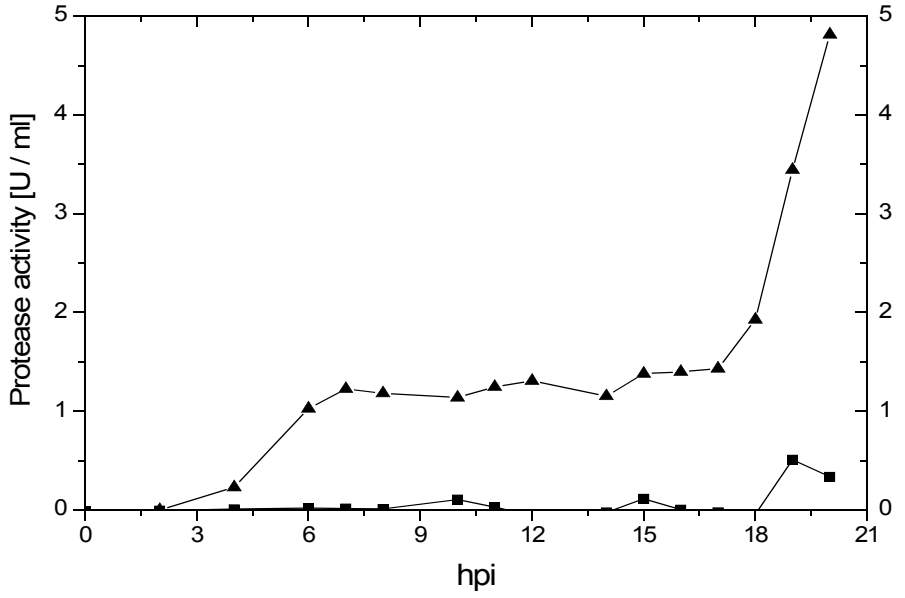


**Figure 3.16: Initial protease secretion after mass inoculation of CM- and PI-medium.**
200 ml of CM- (■) and PI-medium (▲) respectively were inoculated with the complete mycelium of a secondary preculture and cultivated for up to 20 hours. Samples of medium and mycelium were withdrawn with sterile pipet tips and assayed for protease activity. Shown is the mean of three parallel measurements from one experiment. hpi = hours past inoculation

The extended coverage of protease activity in CM- and PI-medium during four days past inoculation in short intervals (see figure 3.17) showed an similar time course with a first increase in protease activity at about 6 hpi in PI-medium, a plateau at 0.8 U/ml lasting until about 30 hpi followed by a strong increase to almost 20 U/ml at 50 hpi and a shallow decrease thereafter, reaching about 10 U/ml at 96 hpi. In CM-medium, no significant protease activity was observed.

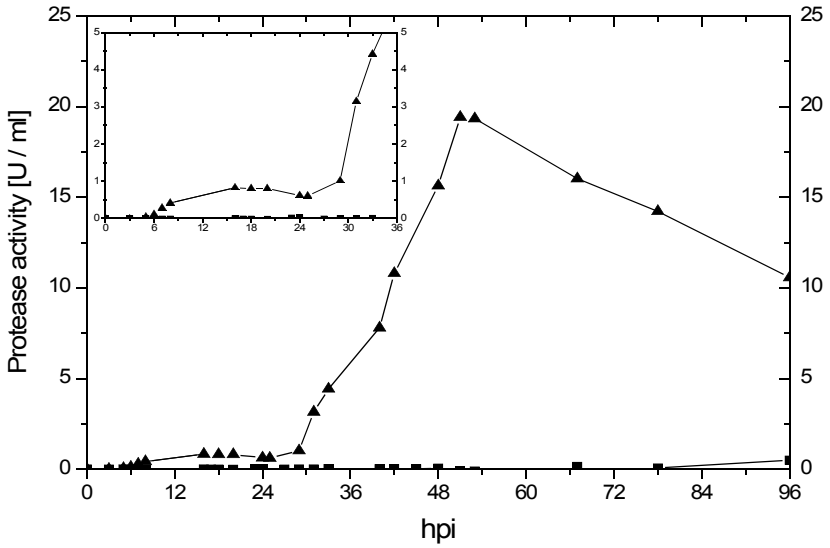


**Figure 3.17: Protease secretion after mass inoculation of CM- and PI-medium.**

200 ml of CM- (■) and PI-medium (▲) respectively were inoculated with the complete mycelium of a secondary preculture and cultivated for up to 96 hours. Samples of medium were withdrawn with sterile pipet tips and assayed for protease activity. Shown is the mean of three parallel measurements from one experiment. The inlet is an enlarged diagram of the data obtained in the first 36 hours of the experiment. hpi = hours past inoculation

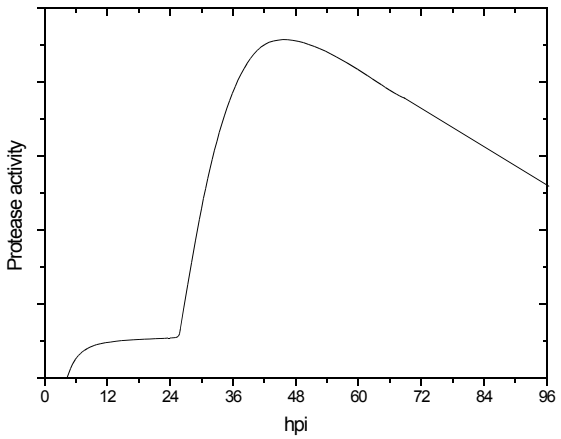


**Figure 3.18: Schematic representation of the phases observed for protease activity during cultivation in PI-medium.**

Figure 3.18 shows a generalized model for the phases of protease secretion during the growth of *F. graminearum* in PI-medium verified in several experiments.

Protease activity was detectable first after 3 to 6 hours of growth, leading to a plateau that lasted for 12 to 24 hours (named 'plateau phase' hereinafter) and a second transient increase which reached its maximum at about 36 to 50 hpi followed by a shallow decrease in protease activity (named 'peak phase' hereinafter).

### 3.5.4 Variations in nitrogen and carbohydrate levels – Mass inoculation

The distinct phases of protease secretion during the cultivation in PI-medium raised the question, whether these are the result of a regulation that reacts to the availability of different components of the medium. The major components that are limited in PI-medium compared with CM-medium are amino acids and peptides as sources of nitrogen and carbon. Also sucrose as a source of carbon and energy is reduced. Since the protein substrate is the major factor which defines the induction medium, an experiment for varying the availability of nitrogen in the medium was set up.

'pPI-medium' – a medium similar to PI-medium, with the same amount of gelatin (0.5%) – was prepared, but in this case the protein was predigested with Protease K (see chapter 2.2.1.7). 200 ml of the media were each inoculated with the mycelium from a secondary preculture and cultivated for up to three days.

The data shown in figure 3.19 is from the series of mesurements, that is depicted in figure 3.17 above, this time comparing PI-medium and pPI-medium. The plateau phase starting at about six hpi in PI-medium was lacking in pPI medium. Interestingly though, the peak phase with its maximum at about 50 hpi was observed in both media.

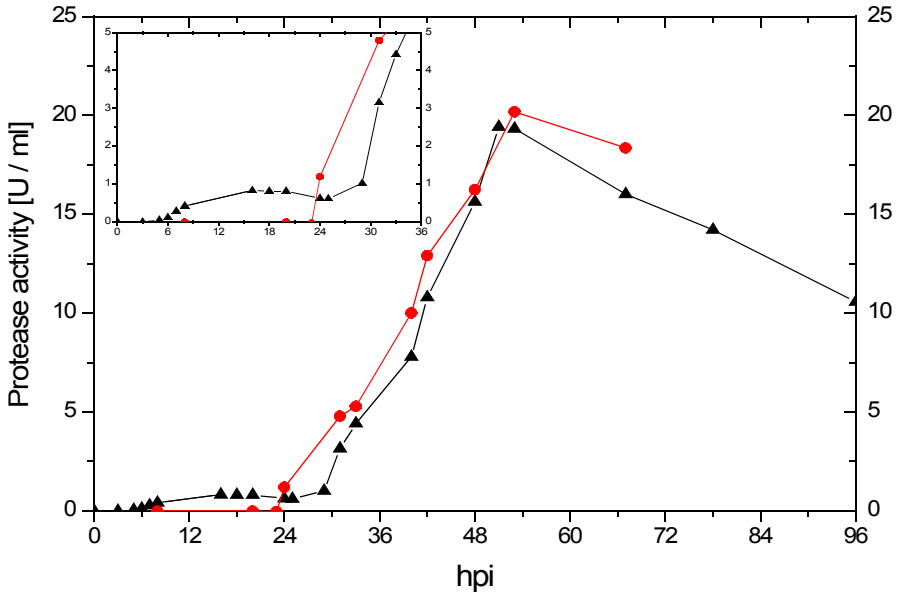


**Figure 3.19: Protease secretion after mass inoculation of PI- and pPI-medium.**

200 ml of PI- (▲) and pPI-medium (●) respectively were inoculated with the complete mycelium of a secondary preculture and cultivated for up to 96 hours. Samples of medium were withdrawn with sterile pipet tips and assayed for protease activity. Shown is the mean of three parallel measurements from one experiment. The inlet is an enlarged diagram of the data obtained in the first 36 hours of the experiment.
hpi = hours past inoculation

A different experiment which varied both, available nitrogen (as predigested protein) and carbon (as sucrose) was set up by complementing sucrose-free PI- and pPI-medium with a sucrose stock solution to give final concentrations of 0, 1 and 2 g/l. Figures 3.20 and 3.21 show the time course of protease activity obtained during the cultivation of the fungus in these six media after mass inoculation.

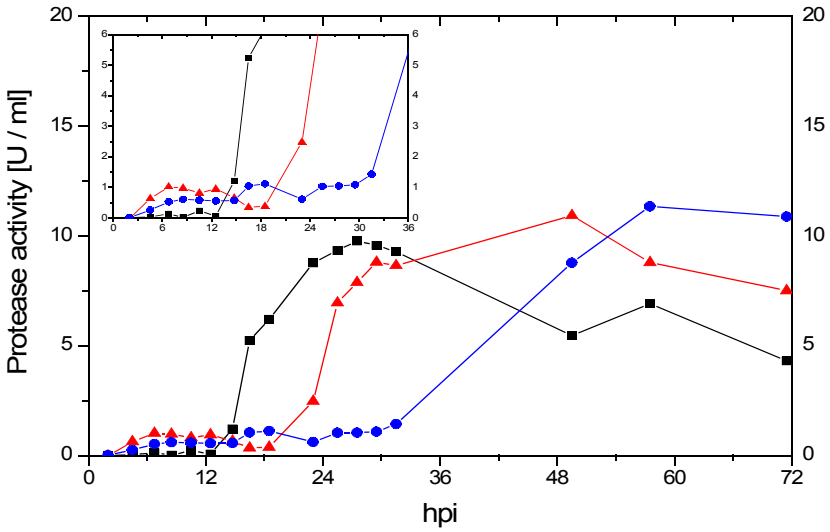


**Figure 3.20: Protease secretion after mass inoculation of PI-medium containing different concentrations of sucrose.** 200 ml of PI-medium without sucrose (■) and with 1 g/l (▲) or 2 g/l (●) of sucrose respectively were inoculated with the complete mycelium of a secondary preculture and cultivated for up to 72 hours. Samples of medium were withdrawn with sterile pipet tips and assayed for protease activity. Shown is the mean of three parallel measurements from one experiment. The inlet is an enlarged diagram of the data obtained in the first 36 hours of the experiment. hpi = hours past inoculation

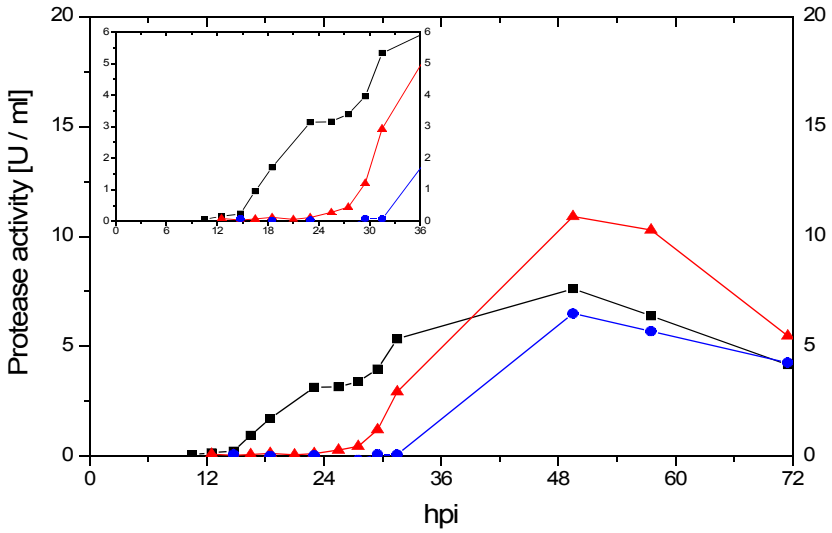


**Figure 3.21: Protease secretion after mass inoculation of pPI-medium containing different concentrations of sucrose.** 200 ml of pPI-medium without sucrose (■) and with 1 g/l (▲) or 2 g/l (●) of sucrose respectively were inoculated, cultivated and assayed for protease activity as above. Shown is the mean of three parallel measurements from one experiment. The inlet is an enlarged diagram of the data obtained in the first 36 hours of the experiment. hpi = hours past inoculation

This experiment was only conducted once, therefore the available data is limited to the time courses shown and no generalizations can be made. Nevertheless, the results are consistent when compared with each other and provide some information on the regulation of protease secretion in relation to nitrogen and carbon availability.

Starting with the medium which imposes the strongest limitation with regard to substrate availability (PI-medium without sucrose, ■ in figure 3.20), lack of the plateau phase during the first hours of growth and a very early start of the peak phase at about 15 hpi was remarkable. The maximum of the peak was determined as about 10 U/ml at 28 hpi.

A concentration of 1 g/l sucrose (standard PI-medium, ▲ in figure 3.20) resulted in protease secretion which followed a time course similar to that described above for other experiments: A plateau phase of 1 U/ml starting at about 4 hpi and lasting for about 15 hours and a peak phase which started at about 21 hpi. Unfortunately, the maximum of the peak phase is not comprised in the data collected for this experiment, but it is assumed to be around 40 hpi. Unusual is the slight decrease in protease activity towards the end of the plateau phase, which was not observed during the cultivation in PI-medium in other experiments.

A further increase in sucrose to 2 g/l (● in figure 3.20) resulted in a prolonged plateau phase with about 0.6 U/ml starting at 4 hpi and lasting for at least 28 hours. The exact start of the peak phase is not comprised in the data, but assumed to be around 32 hpi. The maximum was determined as about 11 U/ml at 58 hpi.

With the readily available amino acids and peptides in pPI-medium, the plateau phase was lacking regardless of the sucrose concentration. The peak phase however was always present. As described above for PI-medium, the increase in activity which marks the beginning of the peak phase started at different times during the growth in the different media.

In pPI-medium without sucrose (■ in figure 3.21) activity started to increase at about 15 hpi which matches the results obtained for the corresponding PI-medium. With 1 g/l sucrose (Standard pPI-medium, ▲ in figure 3.21), the start of the peak phase was at about 30 hpi in medium, which is clearly later than in the corresponding PI-medium in this experiment, but still within the usual range depicted in the generalized model (see figure 3.18 and annotations). In medium complemented with 2 g/l sucrose (● in figure 3.21) the peak phase started between 32 and 50 hpi, but the exact time was not determined.

Summarizing the results of this experiment, it can be stated that an increased amount of sucrose available in the medium delays the start of the peak phase. The exact timing cannot be elucidated with the data available, but a rough estimate is that an increase of 1 g/l leads to a delay of about 6 to 12 hours under the conditions employed. Furthermore, availability of predigested protein in the medium results in absence of the plateau phase. The observed lack of the plateau phase in PI-medium without sucrose – which contains *non-digested* protein substrate – is an exception to this rule.

### 3.5.5 Growth in CM- and PI-medium – Mass inoculation

Investigation of the growth rate of *F. graminearum* during cultivation in CM- and PI-medium was repeated for mass inoculation experiments. Therefore several flasks containing 200 ml of medium were mass inoculated as described above and cultivated for up to 42 hours past inoculation. The increase of fungal biomass – as measured by the fresh weight of mycelium – was determined by harvesting the content of one flask per medium for each sample. The fresh weight at the time of inoculation was not determined and set to 0.8 g which is the approximate amount of mycelium transferred. Figure 3.22 shows a linear increase of biomass from 0.8 to about 6 g/flask within 14 hpi for CM-medium followed by a static growth phase until 42 hpi. In PI-medium the fresh weight increased linearly from 0.8 to almost 4 g/flask within 22 hpi and then remained on this value until 42 hpi.

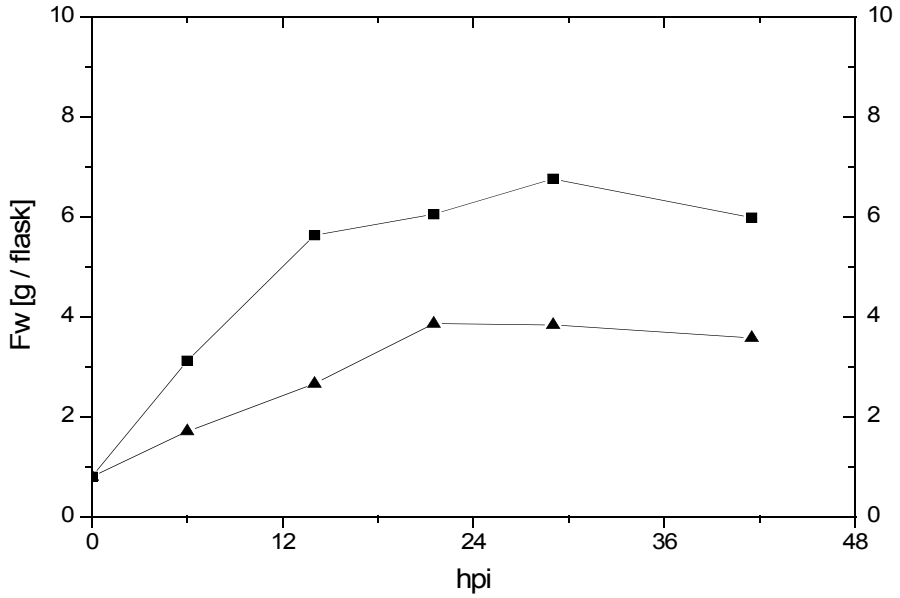


**Figure 3.22: Comparison of growth in CM-, and PI-medium after mass inoculation.**

200 ml of CM- (■) and PI-medium (▲) respectively were inoculated with secondary preculture and cultivated for up to 42 hours. Mycelium was harvested to determine the fresh weight of the produced fungal biomass. Shown is the fresh weight obtained from one flask per sample. hpi = hours past inoculation, Fw = fresh weight

The macroscopic appearance of mycelium cultivated for up to 48 hpi in CM- and PI-medium after mass inoculation is shown in figure 3.23. As described above for cultures inoculated with preculture, mycelium and medium turned dark red in PI-medium while the fungus gradually turned brownish in CM-medium and the culture became turbid during the late stages of incubation. The red hue of the mycelium in PI-medium became obvious at about 24 hpi which correlates with the start of the peak phase of protease activity. Furthermore the mycelium turned yellow at about 6 hpi at which time the plateau phase usually started. In CM-medium the mycelium appeared yellowish from the start of the experiment, which was due to the color of the medium itself.

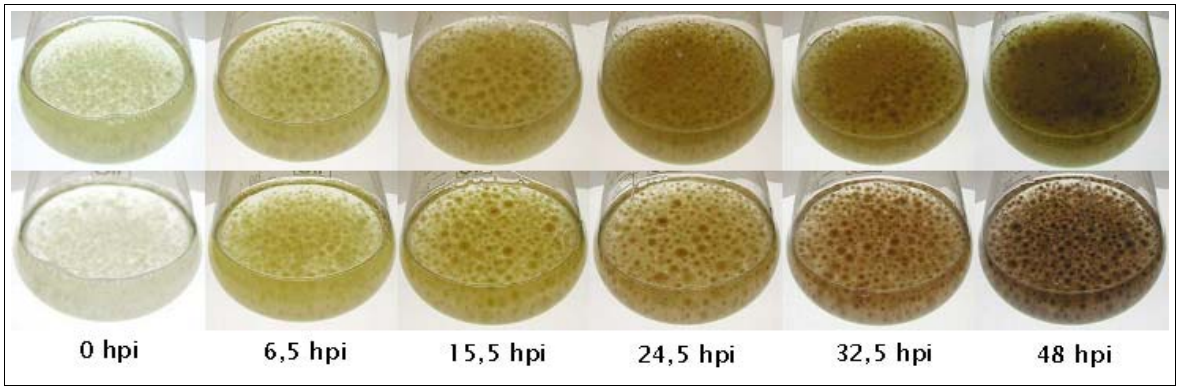


**Figure 3.23:** ***Fusarium graminearum*** **grown in CM- and PI-medium after mass inoculation.**
200 ml of CM- (top row) and PI-medium (bottom row) respectively were inoculated with the complete mycelium of a secondary preculture and cultivated for up to 48 hours. Pictures of the cultures placed on a transilluminator were taken at using a digital camera.

### 3.5.6 pH of the medium during cultivation in PI- and pPI-medium with variations in carbon levels – Mass inoculation

Since the change in color of the medium and mycelium in PI-medium was a recurring observation, and the timing correlated with the significant increase in protease activity, the role of variations in pH during cultivation was taken into account. When adding a few drops of concentrated NaOH-solution to a sample of mycelium-containing PI-medium which was harvested early during the experiment shown in chapter 3.5.4, the color instantly changed to red. The observed variations during the growth in PI-medium apparently resulted from a substance that acts as a pH-indicator.

Therefore the pH of the remaining samples which were taken for the determination of protease activity was measured. The results are shown in figure 3.24 in comparison with the obtained data from the protease assays.

In all tested media (PI- and pPI-medium with 0, 1 and 2 g/l sucrose respectively) the pH was about 5 at the beginning of the experiment. pPI medium without sucrose (■ in figure 3.24, right) showed a slightly higher value at 2 hpi, but regarding the steep ascent of the pH during the next hours, it can be assumed to have originated close to 5.

In the three variations of PI-medium, a sigmoidal increase in pH was observed, which started at about 4, 16 and 23 hpi and reached pH 6 at about 15, 23 and 31 hpi in medium with 0, 1 and 2 g/l sucrose respectively. It then slowly aproximated pH 8.2, reaching this value at about 72 hpi. The points in time for which pH 6 was measured, correlate with the start of the peak phases of protease activity in the three media.

In the variations of pPI-medium, the start of the peak phases correlate with a pH of 7, after which pH 8.2 was approximated as above. The development of the pH during the early stages of cultivation in the pPI-media differs markedly from that in PI-medium. It shows a fast increase from the beginning of the experiment on, with only a slight delay of about 2 hours each with increasing sucrose concentration in the media. At about 12 hpi the increase became constantly slower towards the end of the experiment.

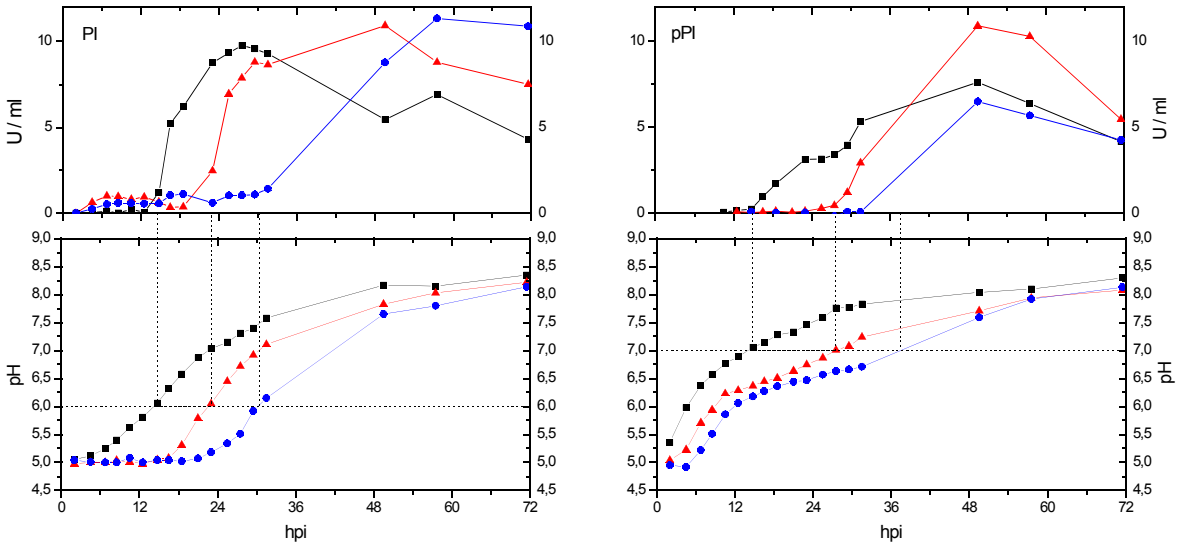


**Figure 3.24: Comparison of the changes in pH and protease activity in PI- and pPI-medium after mass inoculation.**

200 ml of PI-medium (left row) and pPI-medium (right row) without sucrose (■) and with 1 g/l (▲) or 2 g/l (●) of sucrose respectively were inoculated with the complete mycelium of a secondary preculture and cultivated for up to 72 hours. Samples of medium were withdrawn with sterile pipet tips and assayed for protease activity (top diagrams). Shown is the mean of three parallel measurements from one experiment. Furthermore, the pH in the samples of medium was determined (bottom row). The dotted lines serve as an aid for the comparison of the diagrams. hpi = hours past inoculation

### 3.5.7 Protease activity and pH of the medium during growth in minimal medium containing wheat cell wall material – Mass inoculation

In one experiment, mycelium from a secondary preculture was transferred to 500 ml Erlenmeyer flasks containing 200 ml of minimal medium with a suspension of 0.5% (w/v) wheat leaf cell wall material (see chapter 2.2.1.9) and cultivated for up to 72 hours. The fresh weight of the fungal biomass was not determined, but visual estimation showed growth of the fungus at low levels. Significant protease activity was not observed during the cultivation period, therefore the respective diagram was omitted here.

The change in pH of the medium during the growth of *F. graminearum* in the presence of cell wall material is shown in figure 3.25. The shape of the graph is comparable with that obtained when growing the fungus in pPI-medium (▲ in figure 3.24, right row) with a fast increase at about 6 hpi that became slower after about 12 hpi. The pH approximated a maximum value of about 6.5 which was significantly lower than in the previous experiments. Furthermore, the starting pH of 4.5 at the beginning of the experiment was lower than with pI- and pPI-medium. The pH did not reach the threshold of 7.0 at which protease activity increased in pPI-medium (see above) and only slightly exceeded 6.0 – the pH at which protease activity increased in PI-medium.

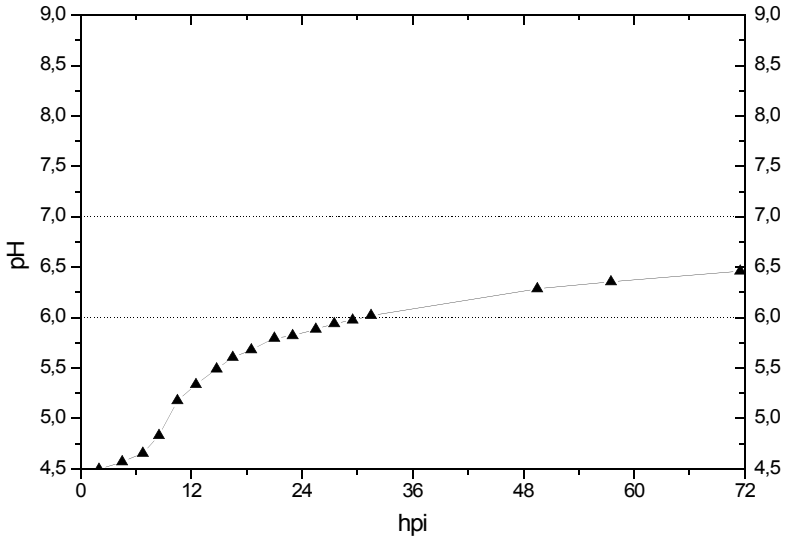


**Figure 3.25: pH of the medium during cultiviation of *Fusarium graminearum* in the presence of wheat cell wall material.** 200 ml of minimal medium containing a suspension of 5% (w/v) wheat cell wall preparation (see chapter 2.2.1.9) were each inoculated with the complete mycelium of a secondary preculture. Samples of medium were withdrawn with sterile pipet tips for determination of the pH. Shown is the mean of two measurements from two experiments. The dotted lines serve as an aid for comparison with the results in figure 3.24.

## 3.6 SDS-PAGE of crude media samples

To visualize the digestion of the gelatin substrate during cultivation and to investigate the protein pattern in the medium, samples taken at different points in time were separated in a 5 – 15% gradient polyacrylamide gel and the gel was silver stained after the separation (see chapters 2.2.4.5 and 2.2.4.7).

As shown in figure 3.26 protein digestion started as soon as six hours past inoculation which corresponds to the start of the plateau phase of protease activity. The almost completely stained first lane (0 hpi) contains protein of large and small molecular weights alike, most probably gelatin and gelatin fragments. Some distinct bands can be seen below the continuous smear. At six and ten hpi, a shift from very large sized fragments to smaller fragments is observed, indicating proceeding digestion of the substrate in the medium. During the fast increase in protease activity at 21.5 hpi only fragments of medium to small sizes are seen in the sample while a very prominent band at about 71 kDa is obvious. This band is also clearly visible at 36 hpi, but it is less intense in this sample. Furthermore, the shift towards smaller fragments continues compared to 21.5 hpi. With enhanced contrast, two bands appearing at six and ten hpi attract attention. The bands measuring about 30 and 74 kDa are only visible in these two samples.

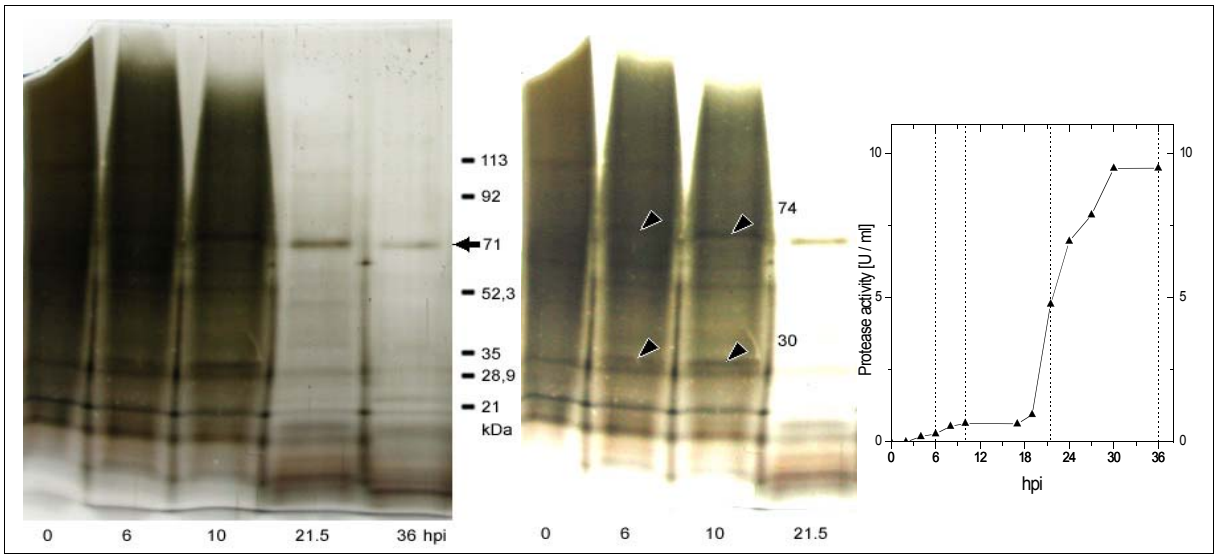


**Figure 3.26: SDS-PAGE of crude media samples taken during cultivation of *Fusarium graminearum* in PI-medium.**

Samples were harvested at the given points in time and separated in a 5-15% acrylamide/bisacrylamide gel using non-reducing sample buffer. 37.5 µl of medium were loaded per lane. The arrow in the left picture indicates a very prominent band of about 71 kDa. The diagram in the middle shows the same gel as on the left, but with revised contrast and brightness. The 36 hpi lane was omitted in this picture since it yielded no further information. Arrowheads mark bands of about 74 and 30 kDa respectively. Depicted on the right is the time course of protease activity in the medium during cultivation. The dotted lines indicate the times at which samples were taken.

## 3.7 Partial purification of a protease from PI-medium

### 3.7.1 Cation exchange chromatography

FPLC with cation exchange column material was used to partially purify a protease from PI-medium harvested 46 hpi after mass inoculation. At about this time, the maximum protease activity is observed as shown in experiments described above. Figure 3.27 shows the obtained chromatogram and the protease activity determined in the collected fractions.

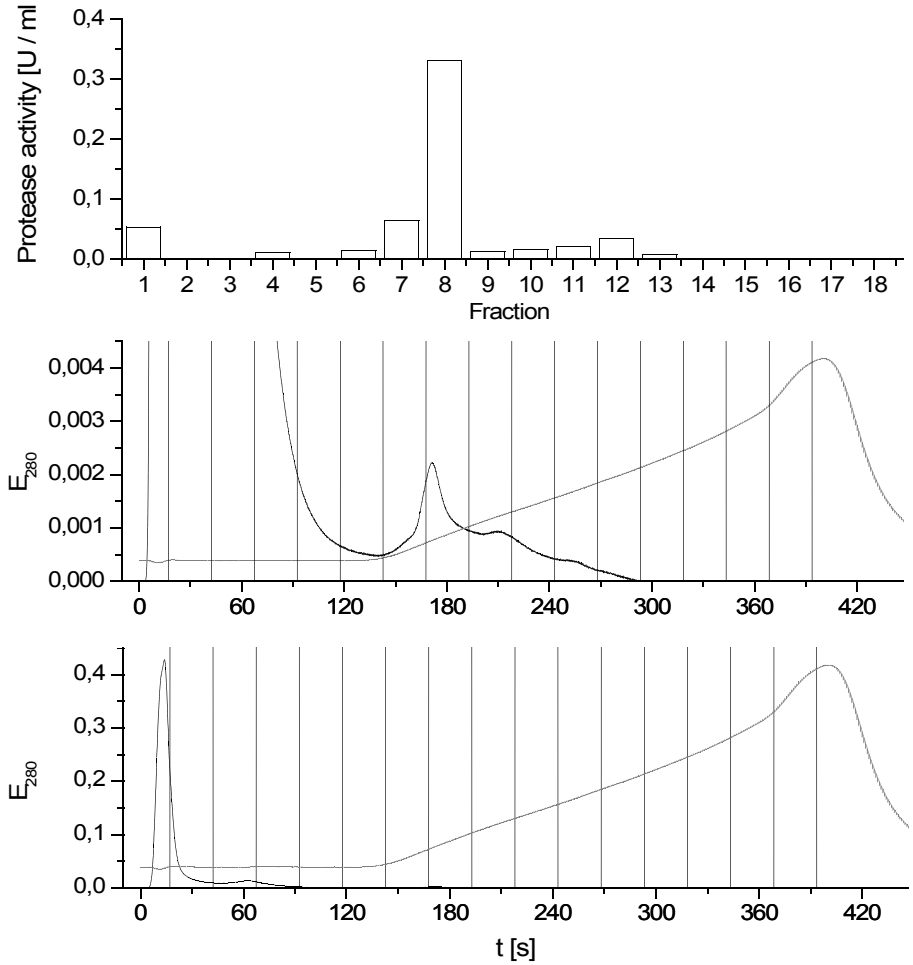


**Figure 3.27: Cation exchange chromatography: Chromatogram and protease activity of collected fractions.**

495 µl of crude, centrifuged PI-medium harvested 48 hours after mass inoculation were separated in MES / HEPES / sodium acetate buffer (pH 4.5) with 0 to 2 M NaCl as the eluent (dotted line). Elution was performed over a range of twelve column volumes. The diagram in the middle is an enlargement of the lower graph (100x), showing the relation of the protein content in the flowthrough and the binding fractions as measured by the absorption at 280 nm. The upper diagram shows the protease activity assayed in the collected 2.5 ml fractions.

The chromatogram in Figure 3.27 clearly shows, that the major part of protein in the sample does not bind to the column and is found in the flowthrough. It takes a massive change in scale (factor 100x) from the lower to the middle diagram to make the eluted peaks visible. However, most of the protease activity is concentrated in a single fraction (fraction 8 in the diagram). Only very little activity was measured in the flowthrough.

An exact determination of the protein concentration in the crude medium and the fractions was not carried out. Instead, a rough estimate of the protein content was performed using the peak area of the absorption at 280 nm. The protein concentration in the fractions was too low to be determined using the standard Bradford assay (BRADFORD, 1976), therefore more time-consuming methods comprising the precipitation and concentration of protein contained in the fractions would have had to be used. Since the chromatography could be used to remove the bulk of protein originating from the gelatin substrate and only a partial purification was aspired, this estimation was considered sufficient.

Calculation of the peak area with the analytical software of the BioCAD SPRINT system used for FPLC resulted in a ratio of about 0.55% bound protein to 99.45% protein in the flowthrough. The area calculated for the most active fraction reflected about 27% of the bound protein or 0.15% of the total protein in the sample.

Since most of the protein did not bind to the column while the binding fraction showed protease activity, multiple injections of sample were performed to concentrate the protease from the medium. To investigate the maximum number of injections before exceeding the columns binding capacity, up to 20 injections of 495 µl of sample each were carried out with automatic sample injection and fractions containing the flowthrough of selected injections were assayed for protease activity.

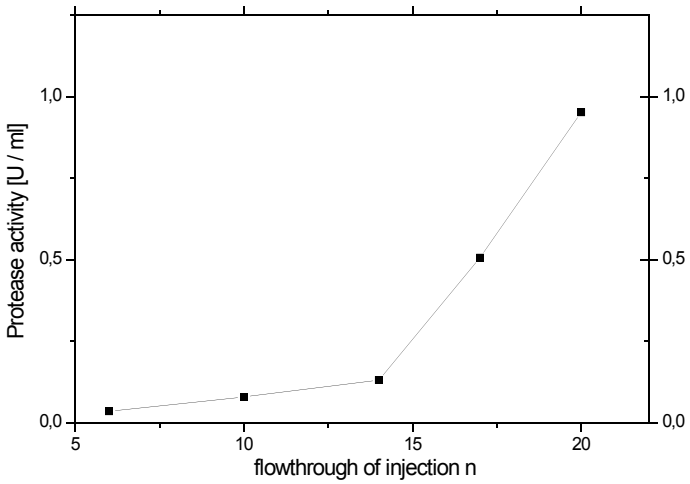


**Figure 3.28: Protease activity in the flowthrough of multiple sample injections.** 2 ml fractions containing the flowthrough of selected applications of 495 µl PI-Medium harvested after 24 hours past mass inoculation on a cation exchange column were assayed for protease activity.

As shown in figure 3.28, the column capacity was exceeded with 17 injections resulting in increased protease activity in the flowthrough, while the flowthrough of injection 14 still showed only little activity.

Figure 3.29 shows the obtained chromatogram and the protease activity determined in the collected fractions after multiple injections of PI-medium harvested at 48 hours past mass inoculation. A total of 20 injections of 495 µl each were applied in this experiment, thus exceeding the maximum capacity of the column material. The chromatogram shows only the flowthrough of the final injection omitting the previous peaks which all had similar shape and area.

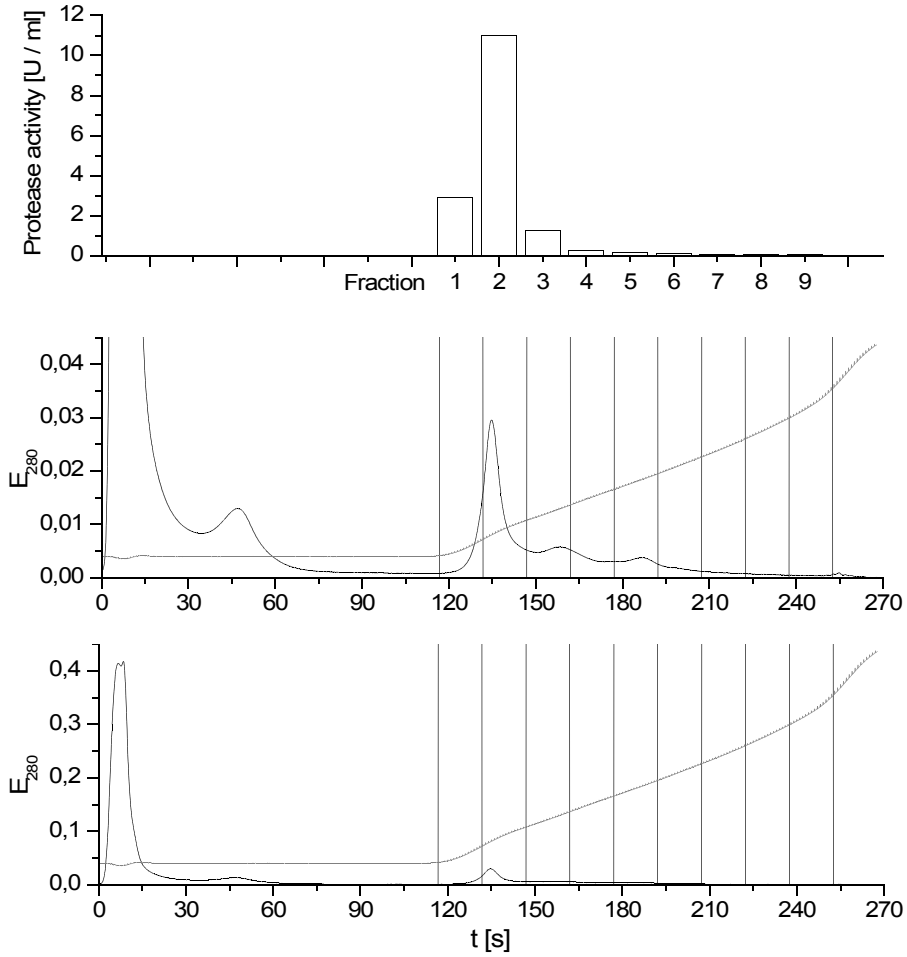


**Figure 3.29: Cation exchange chromatography: Chromatogram and protease activity of collected fractions of multiple sample injections.** 20 injections (495 µl each) of crude, centrifuged PI-medium harvested 48 hours after mass inoculation were separated in MES / HEPES / sodium acetate buffer (pH 4.5) with 0 to 2 M NaCl as the eluent (dotted line). Elution was performed over a range of twelve column volumes. Only the flowthrough of the final injection is shown in the chromatogram. The diagram in the middle is an enlargement of the lower graph (10x), showing the relation of the protein content in the flowthrough and the binding fractions as measured by the absorption at 280 nm. The upper diagram shows the protease activity assayed in the collected 2.5 ml fractions.

The peak of the flowthrough looks similar to that obtained with a single injection (see figure 3.28 above), but is less wide, since a higher flow rate was employed in this experiment. Furthermore, the peaks eluted from the column are comparable with those depicted in figure 3.28, but are more pointed due to the higher amount of protein concentrated from the applied samples.

The ordinate of the chromatogram was only scaled down by a factor of ten between the lower and the middle graph to visualize the eluted peaks. This demonstrates the concentrating effect of multiple injections compared to a single injection (see figure 3.28 with a scaling factor of 100x). The area of the bound protein peaks compared to the total area of the 20 flowthrough peaks is 0.67% to 99.33% as computed by the analytical software of the BioCAD SPRINT system, with the most active fraction accounting for about 50% of the bound protein and 0.33% of the total protein.

The major protease activity is focused to a peak at the start of the gradient which was split into two fractions of 2 ml each. The activity that was determined in these fractions adds up to almost 14 U/ml or 56 U total in the pooled fractions. Concerning the fact, that 9.9 ml of medium with an activity of about 17 U/ml were applied to the column which is a total of about 168 U, this is a recovery rate of 33%. Since the column capacity was exceeded in this experiment, the potential recovery rate would be even higher with optimal column loading.

### 3.7.2 SDS-PAGE and zymography of FPLC fractions

The active fraction of a cation exchange separation of PI-medium (48 hpi) with a total of 20 injections of 495 µl each was desalted, freeze dried and resuspended in A.dest at 1/50 of the original volume of applied crude medium. The concentrated sample was submitted to gel electrophoresis in a 12% polyacrylamide gel containing 0.1% gelatin as well as standard gradient gels ranging from 5 to 15% polyacrylamide.

After separation, the gelatin-containing gel was incubated for 3 hours at 40°C and stained afterwards. As shown in figure 3.30 (left), a clear band against a blue background can be seen at 174 kDa together with a faint band at 129 kDa. Protease activity was not present in samples which were preincubated with 2 mM PMSF (not shown) indicating that it resulted from serine proteases (see chapter 3.5.2.3).

A transparent lane extending up to the edge of the gel is visible above the bands. This phenomenon has been observed in all zymographies with protein copolymerized into the separating gel. As the protease obviously is active regardless of the SDS contained in the gel and the sample buffer, it digests the substrate while migrating in the gel. This results in the clear lane, and unfortunately makes determining the apparent molecular weight of the protease impossible since in most cases, a distinct band is not visible. Even *if* a band is formed, the position in the gel does not always reflect the actual size, because of intractions between the enzyme and its substrate, which is a known phenomenon in zymography [MICHAUD, 1998]

Since this method of zymography was not applicable, a separation in standard SDS polyacrylamide gels with subsequent electroblotting into a substrate-containing gel was performed. Gradient gels ranging from 5 to 15% polyacrylamide were used, since this way the transfer during the blotting process is more uniform for proteins of different size than with continuous 12% gels.

Figure 3.30 (middle) shows the receiving gel after incubation for five hours at 40°C followed by coomassie staining. A clear band can be observed at about 72 kDa which corresponds to a very prominent band in the silver stained gradient gel (right). This is most probably identical to the band observed in crude media samples during the peak phase (see figure 3.26) which was determined as 71 kDa. However, the smear and low molecular weight bands in the crude medium are not present in the FPLC fraction. Only a less intense band of about 52 kDa and a faint band at about 47 kDa are visible. Thus a partial purification of the protease has been achieved. Furthermore a faint transparent band is visible in the zymogram at about 27 kDa, which can not be correlated to a band in the silver stained gel.

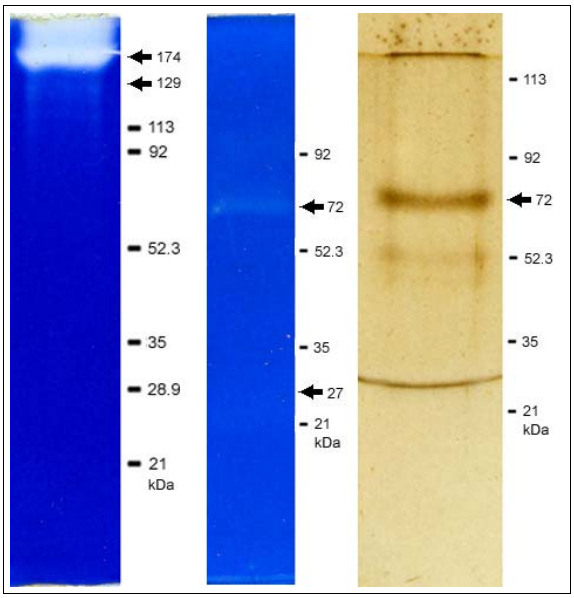


**Figure 3.30: SDS-PAGE and zymography of the active FPLC fraction**

The most active fraction of a cation exchange chromatography with 20 injections (495µl each) of PI-medium harvested at 48 hours past mass inoculation was desalted using a PD-10 column, freeze dried and resuspended in 200 µl of A. dest, thus resulting in a concentration factor of about 50. 15µl of sample were separated in a 12% polyacrylamide gel containing 0.1% gelatin and incubated for 3 h at 40°C before staining with coomassie (left). 30 µl of sample were separated in 5-15% gradient polyacrylamide gels and silver stained (right) or blotted into a 12% polyacrylamid gel containing 0.1% gelatin and incubated for 5 h at 40°C before staining with coomassie (middle). Non-reducing sample buffer was used in each case. The silver stained gel shows a dark stripe at the bottom, the origin of which is not clear. However, it is not related to protein contained in the sample, since it extends over the width of the lane.

## 3.8 Cloning and sequencing of a protease gene

At the beginning of this work, only two genomic sequences encoding proteases of *Fusarium* species were known in the nucleotide databases accessible through the online resources of the European Molecular Biology Laboratory (EMBL). Namely, the extracellular serine protease *prt1* of the tomato pathogen *Fusarium oxysporum* f. sp. lycopersici (EMBL accession number AF074391) and an alkaline protease of a not specified *Fusarium* species – strain S-19-5 – which was published in a Japanese patent (EMBL accession numbers E05653 and S71812).

An alignment of the aforementioned sequences was used to deduce primers for a first attempt to amplify a fragment of a protease gene using *F. graminearum* genomic DNA (gDNA). These primers named **Fus S-19-5 Upper** and **Fus S-19-5 Lower** (see figure 3.3.1 and appendix IX) were employed in touch down PCR with decreasing the annealing temperature from 65 to 60°C.

```
F. oxysporum      5'-GGTCGAGTACATTGAGCAGGAT-3'     F. oxysporum      5'-CCGTCAACCAGGCTGCC-3'
F. sp. S-19-5     5'-GGTCGAGTCTATTGAGCAGGAT-3'     F. sp. S-19-5     5'-CCGTGAACCAGGCTGCC-3'
consensus            ********  ************      consensus            **** ************
Forward primer    5'-GGTCGAGTIIATTGAGCAGGAT-3'     Reverse Primer    3'-GGCAITTGGTCCGACGG-5'

Fus S-19-5 P Upper (Tm = 58.8°C)                   Fus S-19-5 P Lower (Tm = 57.6°C)

                                                                              I = Inosine
```

**Figure 3.31: Deduction of primers for touch down PCR.**

Genomic DNA sequences of two protease genes from *Fusarium oxysporum* f. sp. lycopersici and *Fusarium sp.* strain S-19-5 respectively were aligned for primer deduction.

The PCR yielded two fragments of about 550 and 340 base pairs (bp) respectively. Cloning and sequencing resulted in no significant homologies for the 550 bp fragment when performing a BLAST query against nucleotide databases. The 340 bp fragment however showed a high homology to the *Fusarium oxysporum* sequence and to several other fungal proteases including Proteinase K of *Tritirachium album*, the alkaline protease of *Fusarium* sp. S-19-5, the subtilisin-like protease PR1G of *Metarhizium anisopliae* var. anisopliae, a serine protease from *Paecilomyces lilacinus,* a serine protease of *Verticillium lecanii* and others. All of these organisms are fungi that belong to the phylum ascomycota.

When aligning the genomic sequences of *F. oxysporum* and *F. sp. S-19-5* with their respective cDNA sequences, two and three introns respectively became obvious. The lengths of these regions were 60 and 53 bp for F. oxysporum and 56, 61 and 59 bp for *F. Sp.* S-19-5. Interestingly, the 340 bp fragment spanned the second and third exon of these sequences, but was completely lacking the intermediary intron.

Translation of the 340 bp sequence into amino acids and querying against protein databases revealed very high homologies to serine proteases of the subtilisin family. The fragment comprised a highly conserved cysteine residue as well as an aspartate and a histidine which are part of the catalytic triad of the subtilases.

The attempt to extend the 340 bp fragment using inverse PCR with the primer pair **FS-19-5 IPCR Up / FS-19-5 IPCR Dw** (see appendix IX and X) and several restriction enzymes was not successful, therefore new primers for standard PCR were deduced from an alignment of the cDNA sequences of six subtilisin-like proteases showing homologies on protein level (see figure 3.3.2).

These primers, named **Fus S-19-5 Up1** / **Up2** and **Fus S-19-5 Low1** / **Low2**, were used in all combinations for PCR with an annealing temperature of 44°C and an elongation time of 2 min. The resulting fragments were cloned and sequenced. The primer pair **Up1 / Low2** yielded a fragment of 940 bp that was homologous to the sequences used for primer deduction and comprised the 340 bp fragment.

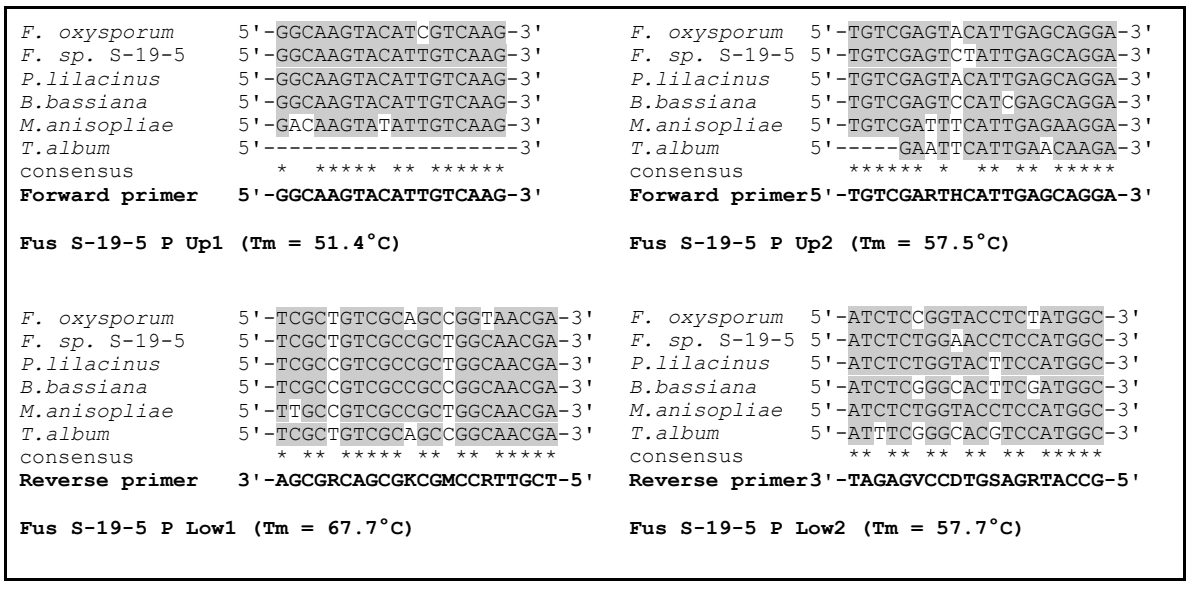


**Figure 3.32: Deduction of primers for standard PCR.**

cDNA sequences of subtilisin-like proteases of *Fusarium oxysporum*, *Fusarium sp*. S-19-5, *Paecilomyces lilacinus, Beauveria bassiana, Metarhizium anisopliae and Tritirachium album* were aligned for primer deduction. D = A/G/T, K= G/T, M= A/C, R=A/G, S=G/C , V= A/G/C.

Since another attempt to extend the sequence obtained from *F. graminearum* DNA by inverse PCR (with the primer pair **FS-19-5 IPCR Up2 / Dw2**, see appendix IX and X) was not successful, the strategy outlined in chapter 2.2.5.4 was employed. The restriction enzyme *Sal I,* which cuts within the 940 bp fragment – about 180 bp from the 3'-end – was

selected to digest the DNA prior to the ligation step. The primer **FS-19-5 IPCR Up3** was used for inverse PCR together with the primer **FS-19-5 IPCR Dw3**, which is located close to the restriction site as depicted in figure 2.2. This way the extension of the DNA sequence was limited to the 5'-region, but the chances to get any result at all were increased.

PCR with an annealing temperature of 60°C and an elongation time of 2 min yielded a fragment of about 600 bp which overlapped with the known 940 bp fragment. The combined sequence spanned 1338 bp and included the start of the coding region based on alignments with gDNA and mRNA of the *F. oxysporum* protease. However, since the 3'-end of the gene was still not available, TAIL PCR as another method to extend a known core sequence was chosen. Table 2.1 shows the three nested specific primers that were used together with the unspecific primers **DP1**, **DP2** and **DP3** in three parallel experiments. **FS-19-5 Up3** is the reverse complement of **FS-19-5 IPCR Up3** used for inverse PCR.

**Table 3.1: Primers used for TAIL PCR.**

For the three specific primers, the position in the known sequence of the 1338 bp fragment – relative to the 5'-end – is listed. The distance between these primers is given, since it can be used to select a prospective canditate for sequencing from the fragments obtained in the TAIL PCR steps. The primers are numbered 1 - 3 according to their application in TAIL PCR steps one to three. N = A/G/C/T, S = C/G, W = A/T

| Primer | Position in 1338 bp fragment | Distance to preceeding primer |
|---|---|---|
| 1) FS-19-5 Up3 (Tm = 57.3°C)<br>5'-GAT CCC AAT CGT TCA GGG TA-3' | 577 | - |
| 2) FS-19-5 IPCR Dw (Tm = 56.7°C)<br>5'-TAC GGT GTC GCC AAG AAG A-3' | 905 | 328 |
| 3) FS-19-5 IPCR Dw2 (Tm = 58.8°C)<br>5'-AAC GAA GCC GTC GAC GCT T-3' | 1142 | 237 |
| DP1 (Tm = 47°C)<br>5'-WAG TGN AGW ANC ANA GA -3' | - | - |
| DP2 (Tm = 47°C)<br>5'-NGT CGA SWG ANA WGA A-'3' | - | - |
| DP3 (Tm = 49°C)<br>5'-NCA GCT WSC TNT SCT T-3' | - | - |

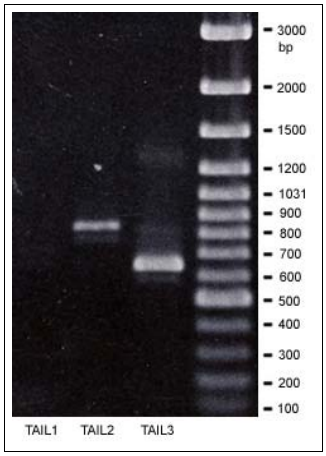


**Figure 3.33: Agarose gel electrophoresis of TAIL-PCR amplificates.** TAIL1-3 represent the succeeding PCR steps.

In combination with the unspecific primer **DP2** the nested PCR steps resulted in one distinct band of about 880 bp in step two, and one band measuring about 650 bp in step three (see figure 3.33). The difference in size between these fragments corellated with the predicted shift due to the distance between the primers.

Sequencing of this fragment showed, that it overlapped with the fragments obtained before. The combined sequence of all fragments measured 1752 bp and contained the 3'-end of the coding region based on alignments with gDNA and mRNA of the *F. oxysporum* protease. The complete sequence is listed in appendix I.

The primers **Fg-P Up** and **Fg-P Dw** which enclose the putative coding region and the primers **Fg-P Up2** and **Fg-P Dw2** and **Dw3** which are located at the ends of the combined sequence were designed to amplify the complete sequence for resequencing (see table 3.2 and appendix IX). Figure 3.34 shows the fragments which were assembled and the position of the used primers.

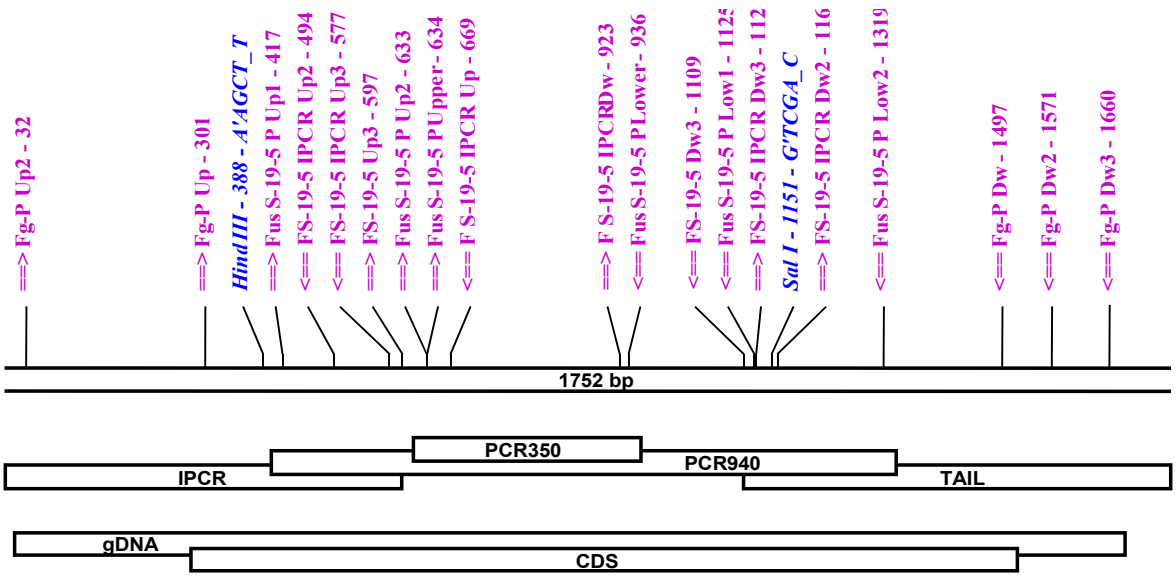


**Figure 3.34: Map of the assembled fragments and the position of used primers.**

Abbreviations: PCR350: Fragment obtained with touch down PCR, PCR940: Fragment obtained with standard PCR, IPCR: Fragment obtained with inverse PCR, TAIL: Fragment obtained with TAIL PCR, gDNA: resequenced range of genomic DNA comprising the putative coding sequence (CDS). The location of the restriction sites of Hind III and Sal I within the sequence are also given. The positions listed for the primers are the respective 3'-ends.

**Table 3.2: List of primers that were used for resequencing of the assembled genomic sequence.**

| | | |
|---|---|---|
| Fg-P Up | 5'-ATG CGT TCC GCT ACT CTT CTC G-3' | (Tm = 62.°C) |
| Fg-P_Up2 | 5'-TGG CGA TCA TCG GAG CAG-3' | (Tm = 62.2°C) |
| Fg-P Dw | 5'-CTA AAC AAG CAG CTC TTG GCT GTC-3' | (Tm = 62.7°C) |
| Fg-P_Dw2 | 5'-GCG AAT CAA AGG TCA AGC GAA TA-3' | (Tm = 61°C) |
| Fg-P_Dw3 | 5'-ACC CGA CTG AGT CCA ACA AAC A-3' | (Tm = 62.7°C) |

## 3.9 Molecular characterization of the obtained gene

Alignment of the *F. graminearum* sequence with gDNA and cDNA of the *prt1* gene from *F. oxysporum* showed high homology with more than 80% identity over extended regions of the genes and a total of 68% identity in the range ot the 1752 bp genomic fragment (see appendix IV). Furthermore, the comparison showed that the gene of *F. graminearum* comprises only one intron, while *prt1* contains two (see figure 3.35 and appendix V for the complete alignment).

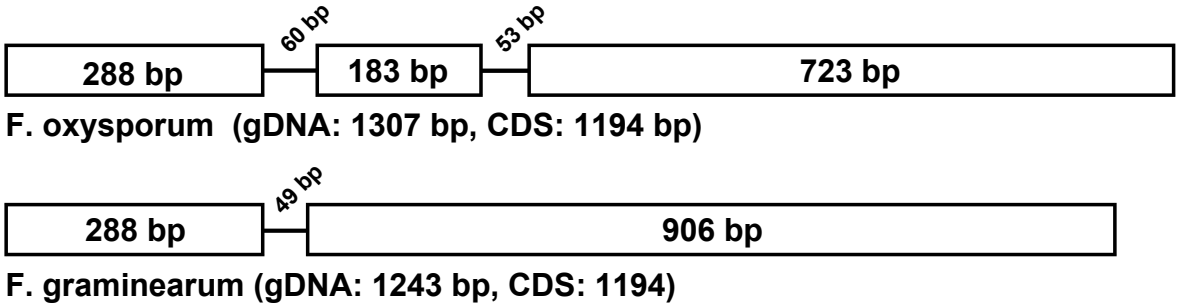


**Figure 3.35: Schematic representation of the exon / intron structure of *prt1* from *Fusarium oxysporum* f. sp. lycopersici and the sequenced gene of *Fusarium graminearum*.** Open boxes indicate exons while introns are represented by lines. The annotations give the length of the respective regions in basepairs. gDNA: length of the genomic DNA from start to stop codon including the introns. CDS: coding sequence excluding the introns.

The putative coding sequence that was assembled based on this data comprises 1194 base pairs. These encode for 398 amino acids in the case of the *F. oxysporum* gene. The *F. graminearum* gene contains a stop codon at excactly the same position within the last exon, but another one eight triplets before that. Since this region of the DNA was sequenced eight times with clones from four different experiments, a sequencing error seems unlikely. Nevertheless, the protruding seven amino acids were enclosed in the sequence due to the conformity of the aformentioned stop codons. The complete protein sequence comprising a total of 389 amino acids (397 including the contentious triplet) is listed in appendix III.

The deduced amino acid sequences of the two genes showed 80% identities and 91% similarities. Due to the obvious relation between these sequences, the protease gene which was sequenced in this work will hereinafter also be named ***prt1***.

When querying the deduced protein sequence against the program 'InterPro' (see chapter 2.3.5), it was clearly classified as a serine protease belonging to the subtilase family. The characteristic conserved regions around the three amino acids that make up the catalytic triad of subtilases and the peptidase S8 domain were located (see figure 3.36 and appendix III).

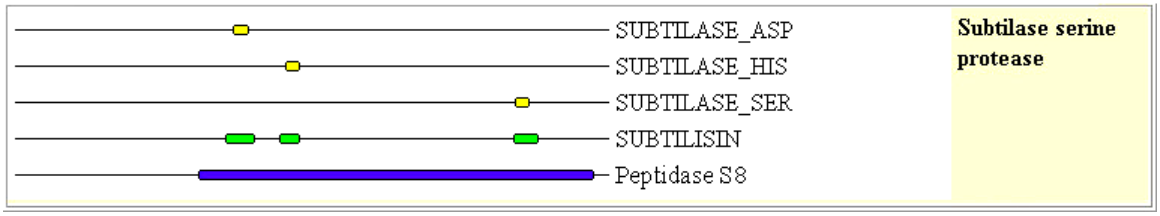


**Figure 3.36: Schematic overview of the identified conserved regions in the deduced amino acid sequence of *prt1* from *Fusarium graminearum.*** The diagram was adapted from the results of an InterProScan query.

Alignment of the deduced protein sequence with other members of the subtilase family of serine proteases revealed close relationships between *F. graminearum* and *F. oxysporum prt1* and subtilisin-like proteases from *Cephalosporum acremonium* (CAH), *Metarhizium anisopliae* (PR1K fragment) and *Tritirachium album* (Proteinase T fragment) as shown in figure 3.37. These proteins showed higher homologies to each other than to other subtilases. Interestingly, the protease of *Fusarium sp.* S-19-5 grouped together with Proteinase R and Proteinase K from *Tritirachium album* and three proteases of *Beauveria bassiana* (= *Tritirachium shiotae*) instead of the subtilases of the other *Fusarium* species.

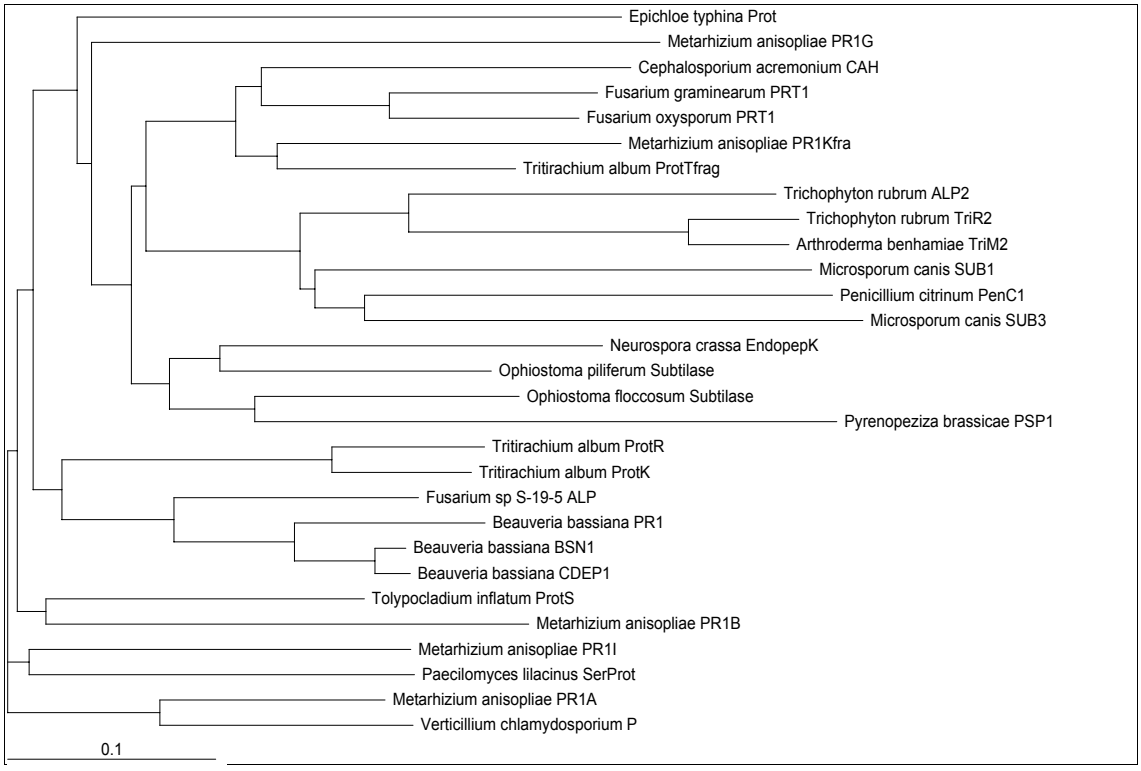


**Figure 3.37: Phylogram of selected fungal subtilisin-like proteases.**

Sequences of proteases that showed homologies with *F. graminearum prt1* in a BLAST query against protein databases were aligned using ClustalW and the pylogram was built with ClustalX. See chapter 2.3.4 for details on the programs. The distances between the tips and branching points of the phylogram are a measure for the similarity of the sequences.

Using the program 'SignalP' (see chapter 2.3.5), a putative signal peptide in the sequence of the *F. graminearium prt1* protein was identified. It consists of 15 amino acids at the N-terminus and most probably tags the protease for secretion as predicted by the program 'Psort II' (see chapter 2.3.5) based on known sorting signals of fungi and animals. A similar signal peptide is also annotated in published protein sequences of other subtilases, some of which are shown in figure 3.38 and appendix VI.

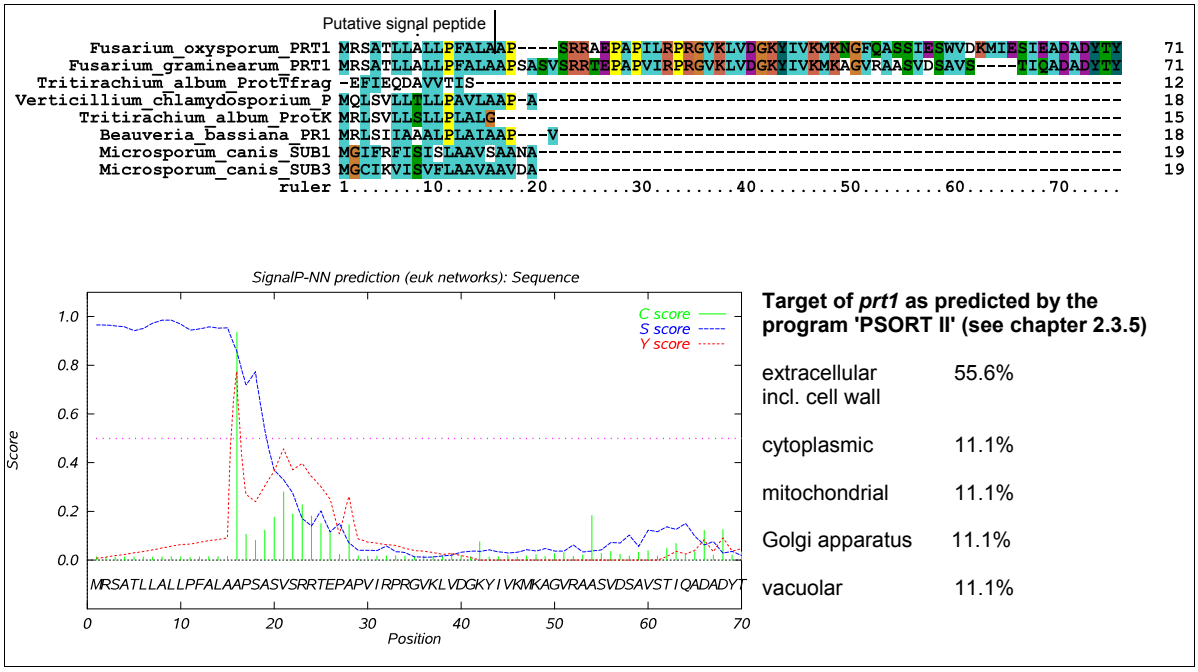


**Figure 3.38: Top: Sequence alignment of the deduced amino acid sequence of *Fusarium oxysporum* and *Fusarium graminearum prt1* genes with published sequences of signal peptides of subtilisin-like proteases.**

The annotation marks the putative cleavage site of the signal peptide in the prt1 genes.

**Bottom: Signal peptide prediction using 'SignalP' (see chapter 2.3.5).**

Green: 'C-Score' gives the probability of a cleavage site at this position. Blue: 'S-Score' gives the probability of a signal peptide at this position. Red: 'Y-Score' = combined combination of the two scores. The peak between the positions 15 and 16 marks the putative cleavage site of the signal peptide.

Available data on subtilases often includes a propeptide that is cleaved to form the functional protease. Alignment of the *F. graminarum prt1* protein sequence with several published sequences of mature proteins without the propeptide indicates a putative cleavage site following position 108 (in relation to the complete amino acid sequence including the signal peptide) that was also discussed for the *F. oxysporum* protein [Di Pietro et al., 2001]. Removal of the 93 amino acid prosequence leaves a mature protease of 278 amino acids (286 including the contentious triplet mentioned above). Appendix VII shows the comparison of an N-terminal part of the *prt1* proteins with some available sequences as well as the putative cleavage sites of the signal peptide and the propeptide.

Computation of the expected biochemical characteristics of the mature protein with the program 'ProtParam' (see chapter 2.3.5) resulted in a predicted molecular weight of 28.3 kDa and a theoretical pI of 4.59. If the propeptide was included, the results were 38.3 kDa and 4.86 respectively. If the seven amino acids after the contentious stop codon were considered, a weight of 39.2 kDa and a pI of 4.77 including the propeptide or 29.1 kDa and a pI of 4.51 excluding the propeptide were obtained.

## 3.10 Molecular characterization of the 5'-flanking region of *prt1*

Investigation of the 279 bp sequence upstream of the start codon revealed two putative targets for transcription factors of the GATA-family that recognize the consensus sequence 5'-(A/T/C)GATA(A/G)-3'. They are located at positions -273 and -154 in relation to the start of the coding sequence and are also found in the upstream region of *F. oxysporum prt1* at almost the same positions. A degenerative third one with the sequence 5'-CCATAG-3' was found at position -226 by alignment with the *F. oxysporum prt1* upstream region. Several other putative binding sites for transcription factors mediating nitrogen, carbon and ambient pH regulation were identified in the F. oxysporum sequence. They are listed in appendix VIII and will be described further in chapter 4.6.

## 3.11 Cloning and sequencing of a cDNA fragment of *prt1*

Total RNA from *F. graminearum* was prepared from mycelium grown in PI-medium for 14 hours after mass inoculation as described in chapter 2.2.1.6. At this time, the increase in protease activity in the medium was maximal as shown in chapter 3.5.3. The primers used for the amplification of cDNA after the reverse transcription were **Fg-P Up**, which is located at the start codon of the putative coding region, and **FS-19-5 Dw3** (see appendix IX and X). These primers comprise the intron predicted from alignments with *prt1* from *F. oxysporum*, thus resulting in a fragment of 849 bp for gDNA and 801 bp for cDNA without the intron.

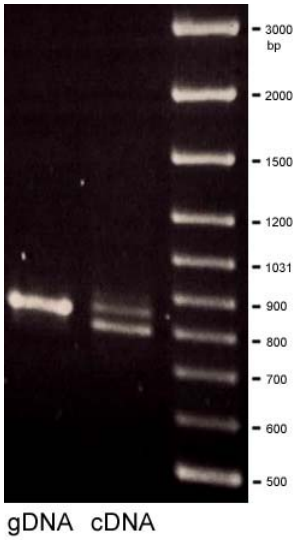


**Figure 3.39: Amplification of *prt1* cDNA with primers which enclose the putative intron of the gene.**

PCR with the prepared cDNA resulted in a fragment of the expected size – about 800 bp – accompanied by a fragment of about 850 bp. RNA that was not submitted to reverse transcription did not show the smaller fragment and the 850 bp amplificate did not appear when DNAse-treated RNA was used for RT-PCR. This indicates that the 800 bp amplificate resulted from cDNA and *prt1* was expressed under the given conditions.

The identity of the obtained cDNA was verified by cloning and sequencing of the 800 bp fragment. The predicted intron was missing in the cDNA, thus proving the mRNA origin. Otherwise the sequence was identical to the genomic sequence. The fragment that was sequenced did not cover the complete predicted coding region, but since no more introns were expected, the remaining 3'-end of the gene was not investigated.

## 3.12 Qualitative expression studies

### 3.12.1 Growth of *Fusarium graminearum* in CM- and PI-medium

Total RNA was isolated from mycelium grown in PI-medium for 8, 27, 31 and 67 hours and in CM-medium for 27 hours past mass inoculation. RT-PCR with the same primers as above showed that *prt1* is expressed throughout the cultivation in PI-medium (see figure 3.40). Even though it is not possible to reliably interpolate the amount of mRNA transcript present from RT-PCR results, the cDNA band obtained at 67 hpi during growth in PI-medium seems to be less intense than in the other samples, which correlates with the decreasing protease activity in the medium. With RNA from mycelium grown in CM-medium for 27 hours, the *prt1* cDNA was not detectible (left lane of the right block in figure 3.40). When the amount of cDNA template used for the PCR was increased three times, a faint band became visible, indicating that *prt1* is also expressed in CM-medium, albeit at only very low levels (right lane).

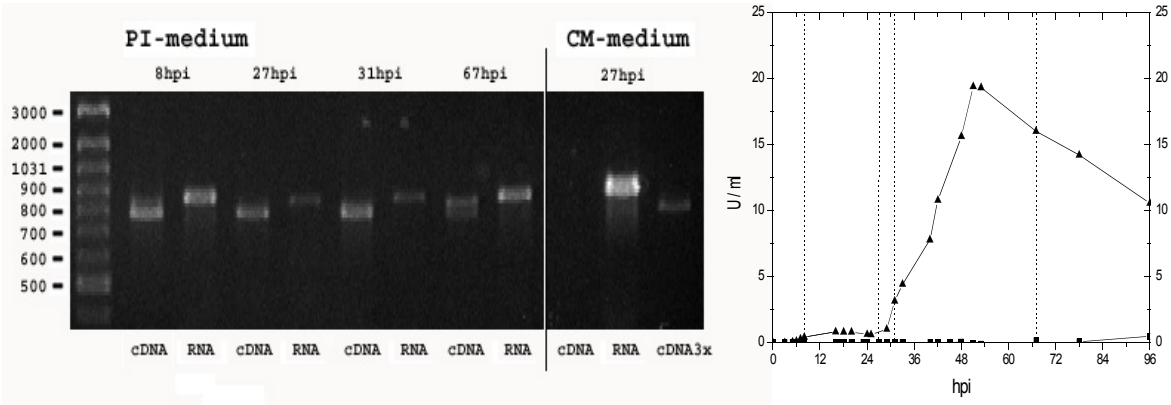


**Figure 3.40: Quantitative investigation of the expression of *prt1* during growth of *Fusarium graminearum* in induction medium.** Samples of mycelium grown in CM- and PI-medium were taken at the given points in time for RNA preparation. Reverse transcription was performed using anchored oligo-dT primers and PCR was carried out with the primer pair **Fg-P Up / FS-19-5 Dw3** which results in fragments of about 800 bp for cDNA and 850 bp for genomic DNA. Shown are the results of RT-PCR together with a control which directly used RNA as template for the PCR step. The RT-PCR samples show bands at 800 bp together with faint bands resulting from residual gDNA while the controls show only the 850 bp genomic DNA band. In the right lane, three times more cDNA template was used for the PCR step. The graph at the right shows the protease activity in the medium and the times at which samples of mycelium were harvested.

### 3.12.2 Growth of *Fusarium graminearum in planta* – detached leaf petri dish assay

To investigate weather *prt1* is not only induced in submerged culture using induction medium, but also during the growth *in planta*, a model system was used. Detached wheat leaves, which were inoculated with a conidia suspension of *F. graminearum* were harvested five days past inoculation. At this time colonization of the plant tissue by the fungus was obvious. The leaves showed brown lesions in addition to the bleaching observed in water-treated controls and mycelium was growing on top of the surface (see figure 3.41).

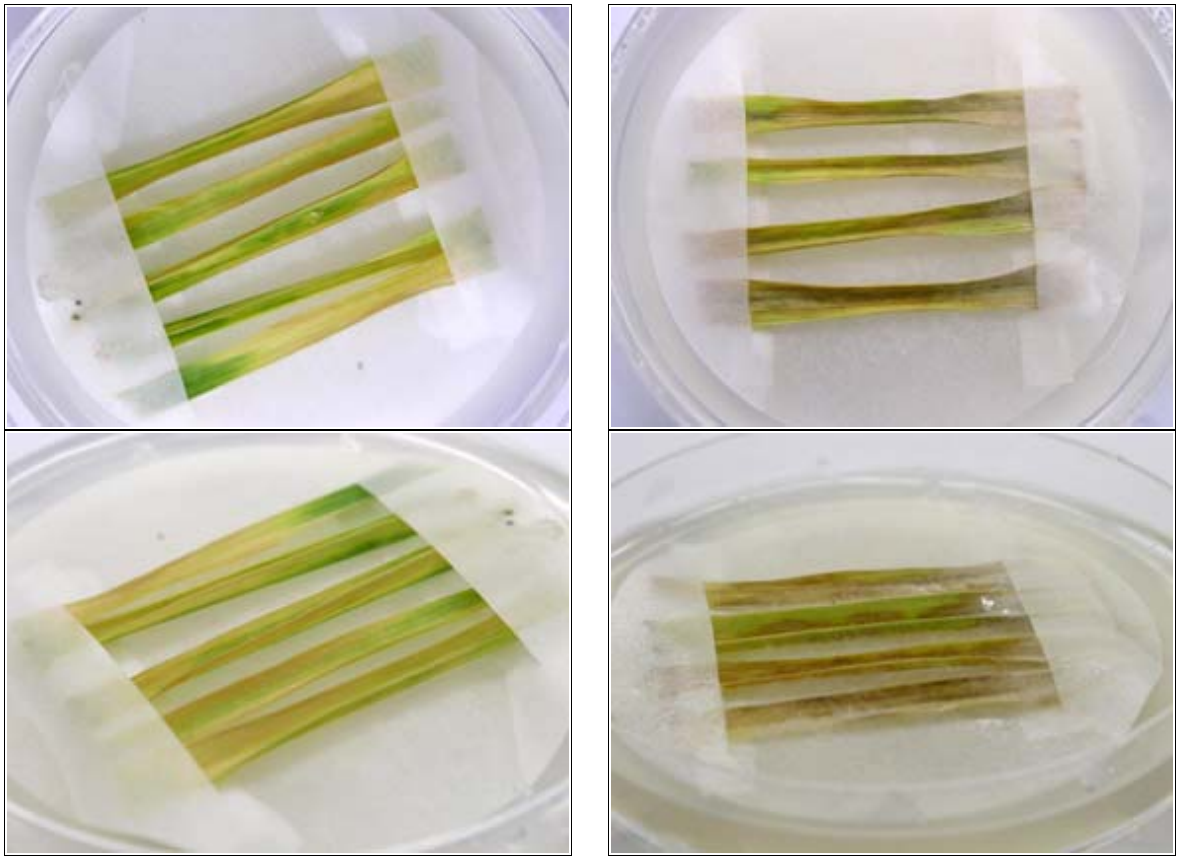

**Figure 3.41: Macroscopic appearance of detached wheat leaves five days past inoculation with conidia of *Fusarium graminearum.*** Brown leasions and abundant growth of mycelium can be seen in the inoculated leaves (right). The controls only show bleached regions but otherwise appear healthy.

In segments of inoculated samples which were stained with calcofluor and inspected under the microscope with UV irradiation, abundant growth of mycelium within the leaf tissue was visible (see figure 3.42). The stomata were often found wide open and hyphae were growing out of the leaf.

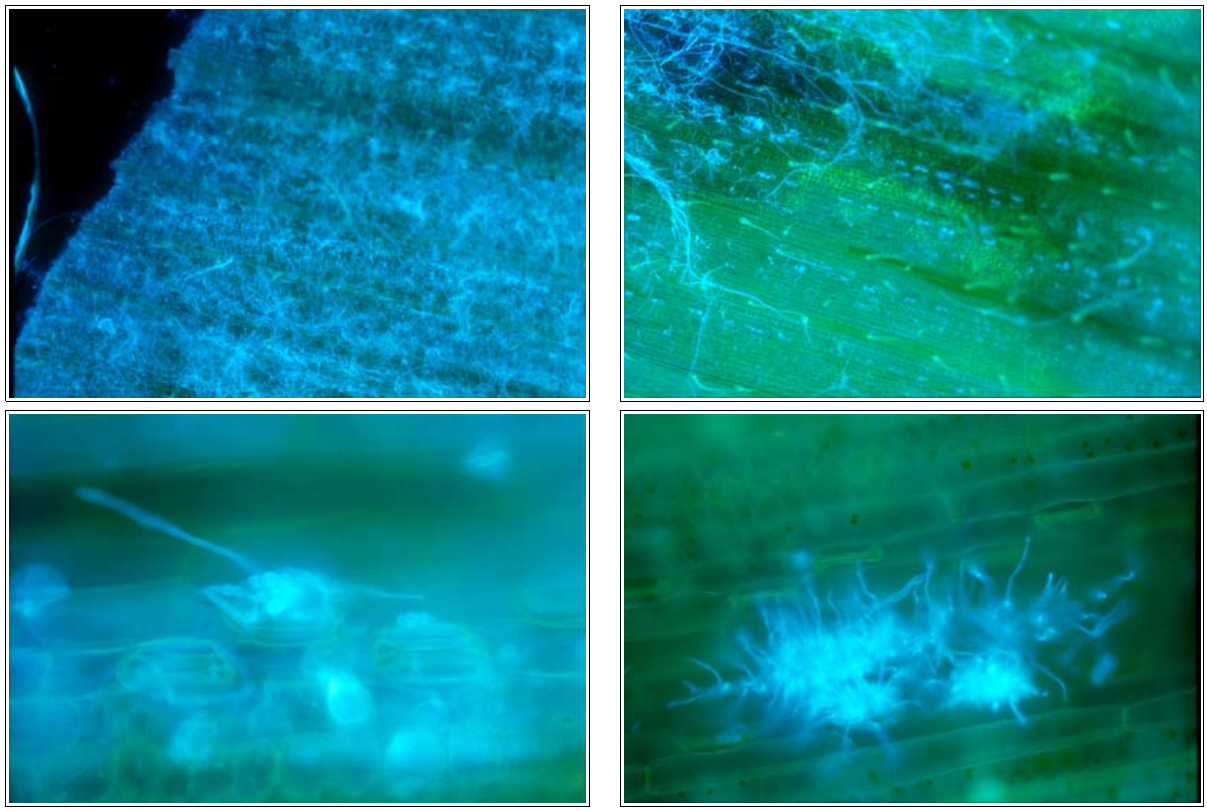

**Figure 3.42: Microscopic appearance of detached wheat leaves five days past inoculation with conidia of *Fusarium graminearum.*** Mycelium was growing within the leaf tissue and on the surface (top row). Hyphae and conidia were observed growing out of stomata (bottom row). Shown are segments of inoculated leaves which were stained with calcofluor. The dye preferrably binds to chitin, which is contained in the fungal cell walls, and causes bright blue fluorescence under UV irradiation. Magnification: 100x (top) and 400x (bottom)

RT-PCR with samples of total RNA prepared from incoculated leaves and from water-treated controls was performed with the same primers as above. In the inoculated samples a faint band at about 800 bp was observed while the RNA control did not show an amplificate. The samples from water-treated leaves gave no fragment at all. This indicates that *prt1* is expressed at least at low levels during the growth of *F. graminearum* in wheat using the detached leaf petri dish assay as a model system.

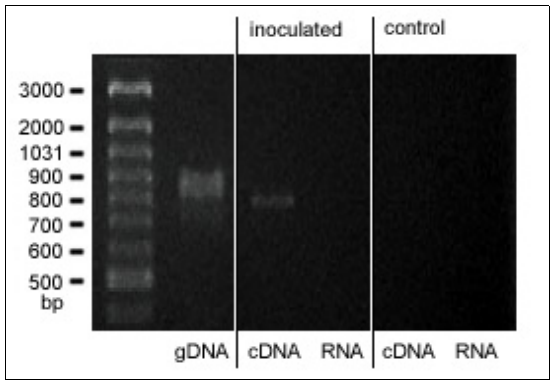


**Figure 3.43: Quantitative examination of the expression of prt1 during growth of *Fusarium graminearum in planta*.** RNA was prepared from inoculated leaves and water-treated controls at 5 dpi. For details on the RT-PCR procedure see the annotations for figure 3.40.

# 4 Discussion

*Fusarium graminearum* is a facultative saprophytic perthotroph. As such, it can grow on a wide variety of substrates ranging from plant tissue of the colonized host and dead plant material in its natural habitat to complex culture media like potato dextrose agar and mung bean liquor medium [Bai and Shaner, 1996] as well as synthetic media like Czapex-Dox medium [Mesterházy et al., 1999] in the laboratory. This makes handling the fungus for experimental purposes relatively easy – in contrast to working with biotrophic pathogens like the rust fungi, which are very demanding with respect to culture media [Staples, 2000]. Furthermore it shows, that *F. graminearum* is equipped with the means to access a wide range of exogenous nutrients and convert them to soluble units which can be absorbed.

Fungal phytopathogens typically secrete a battery of cell wall degrading enzymes (CWDE) which allow the penetration of the plant cell wall and the colonization of the host [Walton, 1994]. Since carbohydrates make up the bulk of plant cell walls, most research has been focused on the ability of pathogens to breach these components. Nevertheless, structural proteins are essential elements of the cell wall and changes in the protein pattern – like oxidative cross-linking of hydroxyproline-rich glycoproteins (HRGPs) and other structural proteins or appearance of PR-proteins – are well-known responses to pathogen attack [Lamb and Dixon, 1997; Muthukrishnan et al., 2001] and have been reported for the wheat / *F. graminearum* pathosystem [Pritsch et al., 2000; El-gendy et al., 2001]. Accumulation of HRGPs in wheat spikes infected with *F. culmorum* has recently been documented with immunocytochemical methods [Kang and Buchenauer, 2003]. Proteolytic activity during the early phase of infection can be seen as a measure to cope with preformed and induced defense reactions of the plant as well as a means to provide basic supply with nutrients.

## 4.1 Selection and evaluation of a suitable proteinacious substrate for protease induction

One objective of the present work was to investigate extracellular proteases of *F. graminearum* with respect to the potential ability to cleave cell wall structural proteins during the colonization of host plants. Since purified structural proteins of wheat cell walls were not available, a model system for the induction of proteases with this potential had to be established.

The production of extracellular proteases by *F. venenatum* (originally published as *F. graminearum* strain A3/5 [O'Donnel et al., 1998]) and *F. culmorum* – both of which are cereal pathogens closely related to *F. graminearum* – has been investigated before [Griffen et al., 1997; Griffen et al., 1998; Pekkarinen et al., 2002; Pekkarinen and Jones, 2002]. As described in these publications, different proteinacious substrates were able to induce the secretion of proteases into the medium, if they were offered as the sole source of nitrogen during cultivation of the fungi in liquid medium.

Griffen et al. used casein as the sole source of nitrogen. Casein is obtained from milk – mostly of bovine origin - and consists of four different phosphorylated proteins which contain hydrophilic and hydrophobic domains. They associate in solution to form the so called casein micelles, much like detergents, but the exact conformation of the casein molecules is subject to controverse discussion [Horne, 2002]. Casein is rich in glutamic acid (about 20%), leucine, aspartic acid, proline, and lysine [Boren et al., 1995].

Pekkarinen et al. demonstrated the induction of protease activity by cultivation of *F. culmorum* in medium containing gluten as the nitrogen source. Wheat gluten consists of at least 50 different components with the high molecular weight (HMW) and low molecular weight (LMW) glutenins accounting for the major part. The glutenins are rich in asparagine, glutamine, arginine and proline and contain extended domains that are formed by repetetive sequences which apparently have a rod-like structure. They form extended networks that are linked by cysteine and tyrosine cross-links [Tilley et al., 2001; Feeney et al., 2003].

While gluten certainly is an important substrate for *F. graminearum* after successful establishment in the host [Boyacioğlu and Hettiarachchy, 1995; Nightingale, 1999], it is not suitable for the modeling of cell wall structural proteins. Some components of gluten can be remotely compared to proline-rich proteins which are part of the plant cell wall, but they lack hydroxyprolin. This amino acid is an essential component of the HRGPs and the prolin-rich proteins which a plant pathogen is likely to encounter during the colonization of the host. Therefore, use of a protein substrate for fungal cultivation which more closely resembles the extensins of the cell wall, can lead to further insight on the cell wall degrading potentials of extracellular proteases of phytopathogenic fungi.

A readily available substrate that is rich in hydroxyproline and exhibits similarities to plant cell wall structural proteins is collagen (or gelatin, which is derived from collagen by partial hydrolysis). The molecular structure of these proteins is characterized by the extended rod-like 'collagen helix' also referred to as the 'polyproline helix'. This is also a major motif of plant HRGPs [Carpita, 1996]. Collagen forms extended networks in the extracellular matrix of animal tissue by cross-linking with other components.

The ability of *F. graminearum* to utilitze gelatin as the sole nitrogen source was investigated in the present work using minimal salt medium containing different concentrations of the protein in addition to a basic supply of carbon in the form of sucrose. As shown in chapter 3.3 the fungus was able to metabolize the offered substrates and readily grew in submerged culture. However, growth was reduced in comparison to complete medium (CM-medium, see table 2.1), which is not surprising, since protein as well as sucrose are non-preferred substrates, which require a fundamental reorganisation of metabolic resources. Furthermore, the restriction of substrate availability not only limited the supply of nitrogen, but also of phosphorus and sulfur.

Extensive studies have been conducted on the nutrient sensing of *Saccharomyces cerevisiae,* the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* during the last decades and a sophisticated network of regulatory mechanisms for repressing / de-repressing single genes and complete metabolic pathways in response to carbon, nitrogen, phosphorus and sulfur availability has emerged [Marzluf, 1993; Marzluf, 1997a; Ruijter and Visser, 1997; Münsterkötter et al., 2000].

If different forms of these macroelements are available, fungi have clear priorities for those that are easily accessible. Preferred sources of nitrogen are ammonia, glutamine and glutamate. In yeast, asparagine is also preferentially utilized. In the absence of these compounds, many secondary nitrogen sources like nitrate, amides, purines, amino acids, peptides and proteins can be readily metabolized. The presence of these secondary compounds was often observed to induce the respective genes required for their acquisition, while primary nitrogen sources repress the expression [Marzluf, 1993; Marzluf, 1997b].

It was clearly shown in the present work, that gelatin is a suitable substrate for the induction of extracellular proteases in *F. graminearum* in submerged culture and increasing concentrations of this substrate induced increasing proteolytic activity in the medium. This observation can in part be accounted to the enhanced growth of the fungus with better substrate availability, but as described in chapter 3.3 there was a pronounced increase in protease activity per fungal biomass at a concentration of 0.2% (w/v) gelatin or more in the medium. This indicates that a certain threshold of the substrate concentration has to be met to induce the enzymes necessary for the degradation. Very low protease activity was detected in both, minimal medium without protein substrate and with 0.1% gelatin respectively. This basic activity could be part of a 'protein-sensing' system, which reports the presence of a suitable substrate by releasing fragments that act as a signal for the up-regulation of extracellular proteases [Drucker, 1973].

During the submerged cultivation of *F. graminearum* in CM-medium, protease activity was usually not detectable or very low. Furthermore, it was not induced by growing the fungus in CM-medium containing 0.5% (w/v) gelatin (data not shown). This matches the aforementioned suppression of enzymes required for nutrient acquisition in the presence of preferred substrates which helps to save up resources.

A gelatin concentration of 0.5% (w/v) was chosen for the protease induction medium (PI-medium, see table 2.1) which was used for most experiments in this work, because it caused the highest overall protease activity from all concentrations tested. However, protease activity per fresh weight was highest in medium containing 0.3% gelatin and did not change significantly at higher concentrations indicating a constant level of protease secretion at these concentrations.

## 4.2 Macroscopic and microscopic characterization of growth

Cultivation of *F. graminearum* on agar plates which were prepared from CM- and PI-medium showed significantly reduced growth on PI-agar compared to CM-agar (see figure 3.8). Nutrient acquisition by the degradation of polymeric extracellular substrates is obviously less efficient in solidified medium. This can be accounted to the limited diffusion of secreted enzymes and the released fragments which only allows the exploitation of the immediate vicinity of the hyphae – a restriction that does not apply to liquid media under agitation. Close investigation of the macroscopic and microscopic appearance of the mycelium (figures 3.8 and 3.9) showed lack of aerial hyphae and very dense, thin and highly branched mycelium on PI-agar which probably serves enhanced nutrient uptake due to their extended surface. Mycelium grown on CM-agar developed abundant aerial hyphae and quickly covered the plate. In liquid medium, the microscopic appearance of the mycelium did not differ significantly between CM- and PI-medium, but on the macroscopic level the spherical colonies that *F. graminearum* forms in submerged culture were more compact in PI-medium, which could be due to the lower growth rate.

The diverse visual appearance of *Fusarium* species on different substrates (presence of aerial mycelium, pigmentation, growth rate, conidia formation) has been described extensively and is traditionally used for taxonomic identification purposes. Media with low carbon content have been listed as reducing the formation of aerial mycelium and stimulating the formation of conidia [Booth, 1971; Burgess et al., 1988]. Since PI-agar contains only 1/10 of the amount of sucrose comprised in CM-agar, this can be an explanation for the differences in aerial hyphae development. The formation of conidia on PI-agar was not investigated. However, conidia were observed after prolonged growth in liquid PI-medium.

Worth mentioning is the change in color that was apparent during cultivation on media containing gelatin. It was common, that CM-plates became dark red to brownish after prolonged cultivation of the fungus when excessive growth had already ceased. However, media containing gelatin – either as an additive to CM-agar (gCM-agar) or as the sole source of nitrogen (PI-agar) – developed a strong red pigmentation during active growth of *F. graminearum* (see figure 3.8).

The shift from yellow / orange to red during growth in liquid PI-medium (see figure 3.23) was accompanied by an alkalization of the medium (see figure 3.24) which directly or indirectly led to the color transition. The alkalization most probably is a side effect of the uptake of solubilized amino acids, since many fungal amino acid transporters work as proton co-transporters [HORAK, 1986; STRUCK ET AL., 2002; WIPF ET AL., 2002].

However, the red pigmentation during growth on gCM-agar was surprising, because the only difference between CM-agar and gCM-agar is the included gelatin (0.5% (w/v)). Since both media include an abundance of nitrogenous nutrients which are readily availabile (peptone, casamino acids, components of yeast extract) degradation of gelatin should not be necessary and actually be repressed (see above). As mentioned above, protease induction was not observed in liquid CM-medium which was complemented with 0.5% gelatin. Hence, an apparent difference during cultivation of *F. graminearum* on gCM-agar compared to CM-agar was not expected. A possible explanation of this phenomenon is the limitation of accessible nutrients to the immediate vicinity of the hyphae. As soon as the primary sources of nitrogen are exploited, cleavage of gelatin and subsequent absorption of the released amino acids becomes necessary which might raise the pH value above the level reached on CM-medium without gelatin and could thereby cause the color transition.

## 4.3 Protease induction - Time course, basic characterization of proteolytic activity and regulation by carbon and nitrogen availability

After the general induction of protease activity with gelatin as the sole nitrogen source using PI-medium was established, further characterization with respect to the time course of protease secretion as well as basic biochemical characteristics and the type of the protease(s) in the medium was performed.

In PI-medium which was inoculated with a small inoculum, proteolytic activity was increasing after one day of cultivation and reached its maximum at three days past inoculation (dpi) (see figure 3.10). This correlates with the exponential growth of the fungus in PI-medium which passed into the stationary phase at day three.

*F. venenatum* was reported to produce extracellular proteases during late exponential growth with casein as the sole nitrogen source [Griffen et al., 1997]. The pH optimum of the proteolytic activity in the medium was 5.0 at 30 hours past inoculation (hpi) and an additional second maximum at pH 8 – 8.5 was determined at later stages of cultivation from 48 to 71 hpi. In chemostat culture – which allows continuous exponential growth by constantly supplying fresh medium – only the acidic protease activity was observed. Inhibitor studies classified this protease as belonging to the aspartic proteases. The alkaline protease activity was not examined further with respect to its type.

Two proteases which are secreted by *F. culmorum* during cultivation with gluten as the sole nitrogen source were described by Pekkarinen et al. [Pekkarinen et al., 2002; Pekkarinen and Jones, 2002]. The authors did not state the growth phase of the fungus at the time of investigation, but the medium was harvested when about 50% of the glucose was used up, indicating that exponential growth had not ceased. Both proteases had pH optima of about 9 and were classified as serine proteases being subtilisin-like and trypsin-like respectively.

In the present work, characterization of the proteolytic activity in PI-medium two days after inoculation with *F. graminearum* – within the exponential growth phase – revealed a broad pH optimum ranging from about 6.5 to 10.5. At this stage acidic protease activity was cleary absent, since a steep decrease of the activity below pH 6.5 was observed. The temperature optimum of proteolytic activity was determined as 60°C. Inhibitor experiments clearly related the activity in the medium to serine proteases (see figure 3.15). Inhibition was about 90% with phenylmethylsulfonylfloride (PMSF) compared to the untreated samples. This indicates that the main activity in the medium can be accounted to subtilisin-like proteases. According to the inhibition by apronitin and leupeptin by 20 and 35% respectively, minor activity results from trypsin-like proteases. The strong inhibition by chymostatin (85%) is surprising at first glance, since chymostatin is generally known as an inhibitor of chymotrypsin – belonging to the trypsin family of serine proteases – and some cystein proteases. However, some members of the subtilisin-like proteases have been reported to be susceptible to inhibition by cysteine protease inhibitors which is accountable to a cysteine residue near the active site histidine that is believed to play a role in the proteolytic activity of these enzymes [Betzel et al., 1986; Barrett, 1991; Rawlings, 1994].

'Mass inoculation' of PI-medium with a large amount of mycelium, which was grown in a preculture with CM-medium, allowed to investigate the initial phase of protease induction. The increased fungal biomass resulted in a higher concentration of the released proteases. With this method it was demonstrated that *F. graminearum* does in fact react

very fast to the change in medium. A first increase in protease activity was observed after only a few hours (see chapter 3.5.3). The exact time course of the change in activity varied between individual experiments, but a general scheme was identified that consists of two distinct phases: An early 'plateau phase' of low but constant protease activity, which lasts for 12 to 24 hours, and a 'peak phase' characterized by a pronounced transient increase of activity reaching 10 to 20 times that of the plateau phase (see figure 3.18). The peak phase most probably corresponds to the observed time course of protease activity in PI-medium that was inoculated with a small inoculum (see above), even though fungal growth entered the stationary phase at the time the peak phase started (figures 3.16 and 3.22).

The two phases were subject to differential regulation by nitrogen and carbon availability. The nitrogen dependence was shown by comparing the protease induction during cultivation of *F. graminearum* in PI-medium (containing gelatin) and pPI-medium, which is similar to PI-medium, but contains predigested gelatin. The latter medium provides readily available nitrogen sources, which resulted in the repression of the plateau phase (see figure 4.1). However, the plateau phase was also not present if sucrose was omitted from PI-medium, in which this phase usually was observed. This indicates an antagonistic effect of nitrogen and carbon on the initial protease induction after inoculation. Similar regulation was found for a subtilisin-like protease of the tomato pathogen *Fusarium oxysporum* f.sp. *lycopersici*, which is expressed *in planta* during the infection process, and reportedly is strongly induced in medium containing collagen and glucose but not on either substrate on its own [Di Pietro et al., 2001].

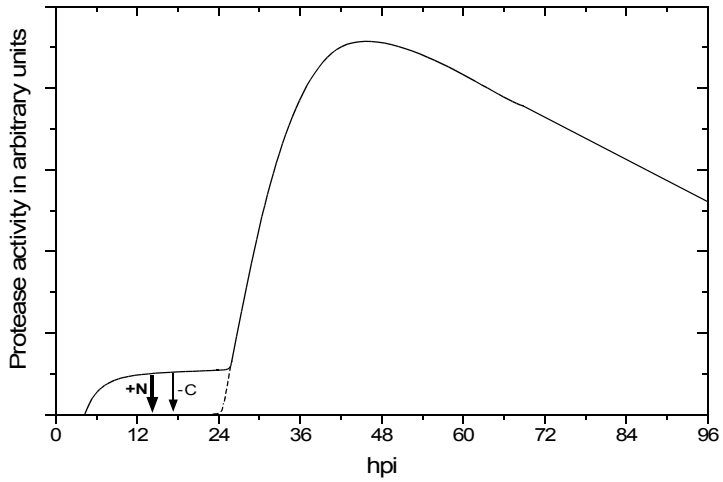


**Figure 4.1: Schematic representation of the effect of nitrogen and carbon availability on the 'plateau phase' of protease activity in the growth medium.** Readily available nitrogen from predigested gelatin suppressed the early increase of activity regardless of the sucrose concentration (bold arrow), while a basic supply of carbon was necessary for its presence (light arrow).

With respect to the suppression of the plateau phase in pPI-medium it has to be kept in mind that this medium contains only residual amounts of long-chained gelatin in addition to small peptides and amino acids. It is possible that the rapid induction of protease activity during the plateau phase is not only repressed by primary nitrogen sources, but also induced by polymeric substrate [DRUCKER, 1973; MARZLUF, 1997; DA SILVA ET AL., 2001]. The small amounts of residual protein substrate in pPI-medium might not be sufficient for protease induction, as discussed above for the evaluation of induction media. A way to verify this would be the complementation of PI-medium with primary nitrogen sources like ammonia, glutamine or glutamate to see whether this suppresses the plateau phase in the presence of undigested gelatin – an experiment that was not conducted in the present work.

The strong increase of protease activity in the peak phase was shown to be carbon repressed. Addition of increasing concentrations of sucrose to PI- and pPI-medium respectively resulted in a delay of the peak phase regardless of the presence of predigested or long-chained gelatin. This suggests that the prominent proteolytic activity in the medium during the peak phase primarily serves the acquisition of carbon from protein substrate and can be interpreted as a reaction to nutrient depletion. This is in accordance to the widespread recognition of extracellular subtilisin-like proteases of saprophytic fungi [GUNKEL AND GASSEN, 1989; ST LEGER ET AL., 1997] and bacteria [YANG ET AL., 1984] as scavenger enzymes that serve the general provision with nutrients.

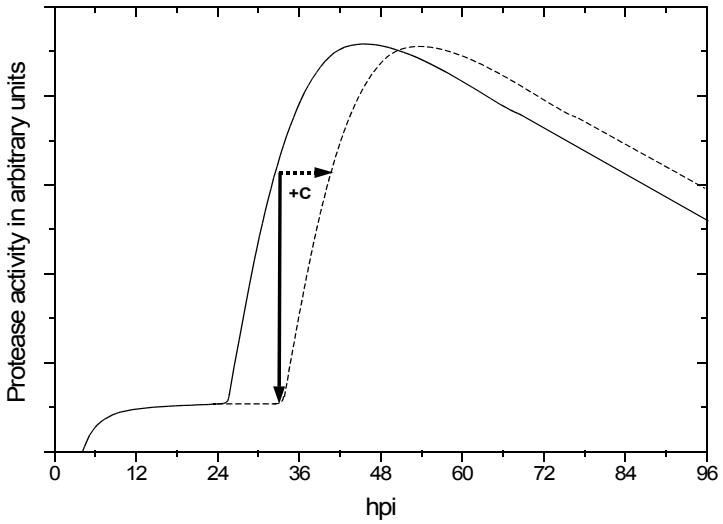


**Figure 4.2: Schematic representation of the effect of carbon availability on the 'peak phase' of protease activity in the growth medium.** Increasing sucrose concentration in PI- and pPI-medium resulted in a delay of the peak phase regardless of the presence of predigested or long-chained gelatin. Shown is the effect of carbon availability in PI-medium which is characterized by the presence of the initial plateau phase.

## 4.4 Protease induction – Electrophoretic analysis of substrate digestion and identification of induced proteases

Electrophoretic analysis of changes in PI-medium after mass inoculation showed a rapid digestion of the gelatin substrate (see figure 3.26). At 21.5 hours past inoculation only fragments of low molecular weight were visible. Since the peak phase in this experiment started at about 18 hpi, the cleavage of gelatin can be mainly accounted to the protease activity which makes up the plateau phase. This also correlates with the onset of a shift from high molecular weight protein in the samples to smaller fragments which was observed as soon as the plateau phase started. The peak phase of protease activity apparently started at a time when the bulk of protein substrate was already cleaved. This supports the aforementioned classification of this phase as mainly caused by nutrient depletion.

Analysis of the protein pattern in crude media samples which were harvested at different points in time revealed the presence of a very prominent band at about 71 kDa which was only observed in samples taken during the peak phase of protease activity. Samples taken during the plateau phase showed two bands of 74 and 30 kDa, which were not visible before or after this phase. The disappearance of the 74 kDa band correlated with the appearance of the band at 71 kDa which suggests processing of this protein by removing a 3 kDa fragment. This observation will be discussed in further detail below together with possible involvement of the pH value of the medium.

Samples of medium which were harvested during the maximum of the peak phase were submitted to cation exchange chromatography to partially purify the protease(s) responsible for the observed activity in the medium. This proved to be a very efficient method to separate the secreted proteins in the medium from the proteinaceous substrate (see chapter 3.7.1). Furthermore it was possible to concentrate components of the medium with proteolytic activity by injecting multiple samples and eluting the amassed binding fraction.

Blotting zymography (see chapter 2.2.4.9) of partially purified protease showed strong activity in a band of about 72 kDa and low activity at about 27 kDa (see figure 3.30). The molecular weight determination in the receiving gel after blotting zymography is not very exact, because the size markers are often just barely visible in stained substrate-containing gels. Hence, is likely that the observed activity corresponds to the 71 and 30 kDa bands mentioned above. No activity was observed after preincubation of the sample with PMSF which indicates that both bands result from subtilisin-like serine proteases. The protease activity at the larger molecular weight corresponded well to a very prominent band in a silver stained gel showing the partially purified FPLC sample and

to the 71 kDa band in crude media samples taken during the peak phase. It can therefore be assumed, that the strong proteolytic activity observed during the peak phase is caused by this protein.

As mentioned above for the investigation of crude medium, a band at 30 kDa was only visible in samples taken during the plateau phase and apparently disappeared during the peak phase. The faint band observed at about 27 kDa in blotting zymography could correlate to this 30 kDa protein band. In the investigated sample – which was taken during the peak phase – this protease is apparently only present at a low concentration and therefore is not visible in SDS-PAGE. Possibly the observed activity represents a remainder of the protease which makes up the plateau phase. The disappearance of the 30 kDa band after the plateau phase can explained by progressively suppressed production of this protease after the gelatin substrate has been digested. It is also possible that the 30 kDa protein is still present in the medium during the peak phase, but gets cleaved by the strongly active 71 kDa protease during sample preparation.

However, verification of these assumptions requires the purification of crude media samples taken during the early phases of protease induction, which was not conducted in the present work. A possible way to check for the presence of the 30 kDa band during the peak phase would be immediate inactivation of the protease activity in harvested media samples by addition of PMSF followed by SDS-PAGE analysis of the protein pattern.

As described in chapter 3.7.2, zymography of protease samples was not sucessful using standard procedures. When the active fractions obtained from cation exchange chromatography were submitted to zymography, only a cleared lane from the top of the gel down to a blurred band at 174 kDa was observed. Though this provided information about the insensitivity of the protease activity to SDS, which is not uncommon amongst subtilisin-like proteases from various microorganisms [Eberling et al., 1974; von der Osten, 1993; Lindstrom et al., 1993; Wolff, 1996; Rao et al., 1998], the size determination was not satisfactory.

Interactions between enzyme and substrate during the electrophoresis have already been mentioned as a possible reason for misleading size determination by zymography with substrate copolymerized with the acrylamide gel (see chapter 3.7.2). However, the extreme difference in the apparent molecular weight observed between standard zymography and blotting zymography (174 kDa to 72 kDa) is unlikely to result from subtrate interactions alone.

Anomalous behavior during electrophoresis has been reported for other proteases belonging to the subtilisin family [Owers and Arakwa, 1989; Lindstrom et al., 1993; Olivieri, 2002]. Glycosylation, aggregation due to SDS treatment and strong hydrophobicity of the protein were discussed as possible reasons for the high apparent molecular weight. Owers and Arakwa as well as Olivieri found significantly farther migration of the purified proteases in SDS-PAGE using boiled samples compared to the native protein. This indicates that the denaturing effect of SDS is not always sufficient to provide the compact shape of the protein which is required for reliable size determination in SDS-PAGE. However, the problems with standard zymography encountered in the present work cannot be related to a generally slow mobility of the proteases in the gel since blotting zymography and silver staining yielded consistent results that differed significantly from those obtained with standard zymograms. Since the blotting method gave satisfactory results, the reasons for the anomalous mobility in standard zymography were not further investigated.

As shown in chapter 3.5.6, the strong increase in proteolytic activity at the start of the peak phase correlated with an increasing pH value of the medium. During the cultivation of *F. graminearum* in PI-medium the peak phase started as the pH reached a value of 6.0 (see figure 3.24). It was described above for the pH dependence of proteolytic activity within the early peak phase that it showed optimal activity above pH 6.5 and significantly decreasing activity towards lower pH values. At first glance, this suggests that the onset of the peak phase simply reflects the protease activity at changing ambient pH. It has to be kept in mind though, that all protease assays were performed at pH 7.8 and showed the maximum activity of the subtilisin-like proteases at the time the sample was taken. Together with the observed shift of the 74 kDa protein band visible during the plateau phase to 71 kDa the sudden increase in activity suggests another explanation:

Many extracellular proteases, including subtilisin-like proteases, are known to be secreted as zymogens containing a propeptide that is cleaved during or after the secretion to give the mature, active protease [Barrett et al., 1998]. This is seen as an efficient way to regulate the activity of extracellular proteases and prevent the cleavage of intracellular proteins before secretion is completed. Propeptides are also discussed as intramolecular chaperones that mediate the correct folding of proteins [Ikemura et al., 1987; Beggah et al., 2000]. The shift of the 74 kDa band to 71 kDa could reflect the cleavage of a short propeptide, releasing the mature protease and resulting in the rapid increase of proteolytic activity at the start of the peak phase. Since at this time the pH value of the medium reaches the pH optimum of the proteolytic activity in the peak phase, the processing of the zymogen could be performed by autocatalyis which was for example described for Subtilisin E [Ikemura and Inouye, 1988].

Since the pH value of the medium is below 6.0 during the plateau phase, it would be expected that acidic proteases are responsible for the digestion of the gelatin substrate. The observed relatively low but constant protease activity at this stage could be accounted to an acidic protease which works suboptimal under the alkaline conditions employed in the protease assay. An explicit pH profile of the protease activity during the early plateau phase was not determined in this work. However, no indication of protease activity with an acidic optimum was observed in the pH profile during the early onset of the peak phase – at a time when at least residual activity from the plateau phase should be detectable. If the 30 kDa subtilisin-like protease is in fact responsible for the activity observed during the plateau phase, as assumed above, it would be confronted with a suboptimal pH for its activity in the medium. Subtilisin-like proteases are generally most active in neutral to alkaline environments [BARRETT ET AL., 1998]. However, the pH-profile of the protease activity described above indicates that about 40% of the maximum activity would be remaining at pH 5, which was the initial pH value of PI-medium.

The data for the two phases of protease induction during incubation of *F. graminearum* in PI-medium which was collected from biochemical experiments is summarized in figure 4.3 together with the hypothetical involvement of the two proteases which were observed in botting zymography.

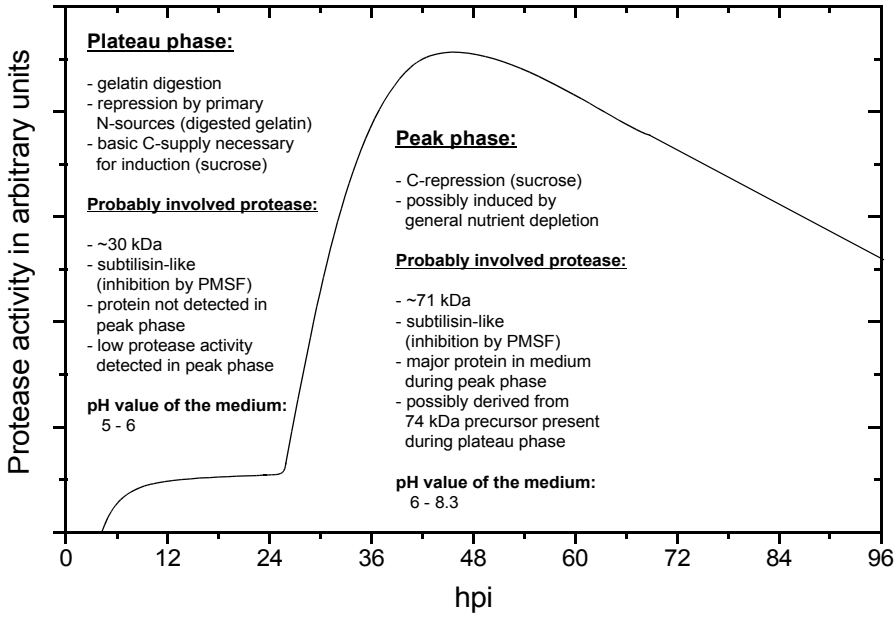


**Figure 4.3: Overview of the data collected for the indution of protease activity in PI-medium after mass inoculation with *F. graminearum* including the putative involvement of two observed proteases.**

## 4.5 Protease induction during cultivation with cell wall material?

During the cultivation of *F. graminearum* in minimal medium containing 0.5% (w/v) wheat cell wall material no significant protease activity was observed (see chapter 3.5.7). However, the fungus was able to grow on this substrate at low levels, indicating that nutrients were obtained from the cell wall preparation. The fast increase in the pH value of the medium was similar to that observed during the cultivation in pPI-medium containing predigested gelatin. This suggests that nutrient uptake involving proton symport could be responsible for the fast alkalization as discussed in chapter 4.2.

For some pathogenic fungi like the wheat pathogen *Stagonospora (= Septoria) nodorum* [Carlile et al., 2000], the insect pathogen *Metarhizium anisopliae* [St Leger et al., 1986] and the mycoparasite *Trichoderma harzianum* [Geremia et al., 1993] protease induction during the cultivation with host cell wall material has been reported. However, in the present work, incubation with wheat cell wall material failed to induce protease activity in liquid culture. This might be due to the requirement of a certain amount of inducing proteinacious substrate as described in chapter 4.1. Possibly the structural proteins contained in the added amount of cell wall material were not sufficient to induce detectable protease activity in this cultivation system. Carlile et al. used 1% (w/v) of wheat cell wall preparation in the medium which induced protease secretion in *S. nodorum*. A major difference in the experimental setup of their cultivation methods was a 12 hour period of 'starvation' of the mycelium in basal salts medium before transferring it to the induction medium. Whether this treatment is necessary for the induction is not clear. It was described that protease induction in *Neurospora crassa* grown in the presence of bovine serum albumin was reduced after prolonged periods of starvation [Cohen and Drucker, 1977] – an effect that the authors accounted to exhausted nitrogen reserves which reduce the capacity of protease synthesis.

The quality of the cell wall material used in the present work was not checked with respect to the integrity of carbohydrate polymers and structural proteins (e.g. by chromatographic methods that are able to visualize small fragments). Hence it cannot be excluded that soluble fragments which can serve as preferred sources of nitrogen and carbon were released during the preparation of the medium. The availability of preferred nutrients could explain the results obtained in this experiment since they were shown to repress the secretion of proteases by *F. graminearum* in PI-medium (see above).

Optimization of the conditions which influence the expected induction of extracellular proteases includes a number of factors like the amount of substrate in the medium, amount of mycelium used for inoculation, incubation time, incubation temperature, pH of the medium, and starvation of the mycelium prior to inoculation. Since the described experiment was conducted at the final phase of the present work, and said factors could not be elucidated in detail, the observations made should be regarded as a basis for further research.

However, the data gathered from the gelatin based model system for digestion of structural proteins and which was summarized in figure 4.3 provided valuable information on the regulation and nature of proteases that might play a role during the pathogenic growth of *F. graminearum*. Further insight was gained using a genetic approach to the characterization of proteases of the fungus.

## 4.6 *Prt1* – a gene encoding a subtilisin-like protease

Only two genomic sequences of proteases from members of the genus *Fusarium* were available in the online resources of the European Molecular Biology Laboratory (EMBL) at the beginning of the present work (see chapter 3.8). Based on these sequences a gene encoding a subtilisin-like protease from *F. graminearum* was cloned and sequenced. It was named *prt1* after a subtilase which was described for the tomato pathogen *F. oxysporum* f.sp. *lycopersici* [Di Pietro et al., 2001] because of the high homology found for the deduced amino acid sequences of the two proteases. However, the two genes are not identical (see chapter 3.9) and differ significantly in their exon / intron structure.

The *prt1* gene of *F. graminearum* exhibits high homology to a variety of subtilisin-like proteases from fungi that also belong to the phylum ascomycota. It closely groups together with a number of proteases of plant and animal pathogens (see figure 3.37) suggesting that *prt1* could be involved in pathogenesis. Amongs the closely related genes are members of the PR1 proteases of the insect pathogen *Metarhizium anisopliae* that are virulence factors during the penetration of the cuticle of its host. However some proteases of saprophytic fungi are also related to *prt1*. The highest homology was found with *F. oxysporum prt1*.

*F. oxysporum* f.sp. *lycopersici* infects its host by direct penetration of the roots and colonizes the cortex by intracellular and intercellular growth. It has been shown to secrete a wide array of enzymes with the ability to attack the cell walls of the plant. Amongst these are polygalacturonases, xylanases, a pectate lyase and the protease *prt1* [Di Pietro and Roncero, 1996a; Di Pietro and Roncero, 1996b; Di Pietro and Roncero, 1998; Huertas-González et al., 1999; Ruíz-Roldan, 1997; Ruíz-Roldan, 1999; García-Maciera et al., 2000; Di

PIETRO ET AL., 2001]. Expression studies using RT-PCR have shown that some of these enzymes are differentially expressed at specific stages during the process of infection and colonization while others – including *prt1* - are expressed constitutively at low levels during *in planta* growth [RONCERO ET AL., 2000]. However, targeted disruption of the *prt1* gene did not result in reduced pathogenicity on tomato plants [Di Pietro, 2001]. As mentioned above in chapter 4.3 this protease gene was shown to be strongly induced during the cultivation of *F. oxysporum* in medium containing glucose and collagen, but was only expressed at low levels during cultivation in glucose or collagen alone.

This perfectly matches the observed regulation of the proteolytic activity during the plateau phase when cultivating *F. graminearum* in PI-medium with or without sucrose. It suggests that the observed activity could be resulting from the gene product encoded by the *F. graminearum prt1* gene which was isolated in the present work. The inducing effect of carbon sources on a protease gene is unusual and suggests that *prt1* is not involved in nutrient acquisition. The positively carbon regulated induction of the protease can be interpreted as related to environmental signals generated during the *in planta* degradation of cell wall carbohydrate polymers by cell wall degrading enzymes.

The available sequence of the 5'-flanking region of *prt1* – though very short – was analyzed for putative binding sites for regulatory elements. The results are listed in appendix VIII together with those obtained for the investigation of the upstream region of *F. oxysporum prt1*. Two putative targets for transcription factors of the GATA-family were identified for the *F. graminearum* sequence. A degenerative third one was found by alignment with the sequence from *F. oxysporum*. It showed a mismatch in the consensus sequence for GATA-factor binding (5'-CCATAG-3' instead of 5'-CGATAG-3'), which might be due to a sequencing error. The GATA trancription factors comprise a diverse group of zinc finger DNA-binding proteins which recognize the consensus sequence 5'-HGATAR-3' (H = A/C/T, R = A/G). They are widespread among eukaryotes and serve manifold regulatory purposes like the repression of genes encoding iron chelators, blue light-regulated photomorphogenesis and circadian regulation [LOWRY AND ATCHLEY, 2000]. In fungi some GATA transcription factors play an important role as global positive acting transcription factors which activate many different co-regulated genes in response to nitrogen depletion. *AreA* from *Aspergillus nidulans* or its homolog *nit2* from *Neurospora crassa* are well described examples for such globally acting regulators [MARZLUF, 1993; MARZLUF, 1997].

Only one occurrence of the consensus sequence was reported for the available sequence of the *F. oxysporum prt1* upstream region [DI PIETRO ET AL., 2001]. This was based on the consensus sequence 5'-WGATAR-3' (W = A/T) as the target for the transcriptional

activator. However the target sequence for AreA is described as 5'-HGATAR-3' [Ravagniani et al., 1997; Wilson and Arst Jr., 1998]. If this motif is employed, a total of five putative GATA-factor binding sites can be identified in the *F. oxysporum* sequence.

Furthermore three putative target sites for the transcription factor CreA which mediates carbon catabolite repression in many fungi were detected. This zinc finger transcription factor which recognizes the sequence 5'-SYGGRG-3' (S = C/G) was first described for *A. nidulans* and a homolog was also identified in *Gibberella fujikuroi* (anamorph: *F. moniliforme)* [Tudzynski et al., 2000]. Homologs from other fungi like *Cre1* of *Trichoderma reesei* have been described [Strauss et al., 1995]. CreA is a globally acting negative regulator which represses a variety of structural genes of carbon metabolism if preferred carbon sources like glucose are available [Ruijter and Visser, 1997].

A third global regulator for which two putative binding sites were detected in the *F. oxysporum* sequence is the zinc finger transcription factor PacC that mediates gene expression with respect to ambient pH in filamentous fungi and yeast [Tilburn et al., 1995; Peñalva and Arst Jr., 2002]. It recognizes the consensus sequence 5'-GCCAAG-3'.

Since the 5'-flanking regions of the two *prt1* genes showed some homologies and the three GATA binding sites which were observed correlated well, it is possible that at least some of the consensus sequences found in the *F. oxysporum* sequence are also present in *F. graminearum*. Based on this assumption a complex regulation of *prt1* with respect to nitrogen and carbon availability and ambient pH can be expected. While this seems to point towards a nutritional role of *prt1*, the regulation of various genes that are seen as virulence factors in pathogenic fungi has been shown to be influenced by the global acting transcription factors mentioned above. Nutrient depletion – particularly nitrogen depletion – has been discussed as an environmental cue for disease development in fungal pathogens [van den Ackerveken et al., 1994; Talbot et al., 1997].

Well documented is the regulation of enzymes that are capable of degrading cell wall carbohydrate polymers like endoxylanases and pectinolytic enzymes from *Aspergillus* species by CreA [De Vries and Visser, 2001; De Vries, 2003]. A wide array of cuticle-degrading proteases from the insect pathogen *Metarhizium anisopliae* is differentially regulated by nitrogen induction and carbon repression in combination with substrate induction [Clarkson and Charnely, 1996] and by ambient pH [St Leger et al., 1998]. Furthermore, regulation of mycotoxin production in *Gibberella fujikuroi* has been related to AreA regulation [Shim and Woloshuk, 1999]. A nitrogen-activated protein kinase belonging to the MAPK / YERK1 extracellular signal-regulated kinase subfamily was reported to be

important for the host root penetration and pathogenesis of *F. oxysporum* [DI PIETRO ET AL., 2001b]. Mutants lacking the respective kinase show significantly reduced expression of a pectate lyase gene which plays a major role in the degradation of plant cell walls but they exhibit normal vegetative growth and conidiation in culture.

These examples illustrate the importance of extracellular signals, which primarily indicate the ambient nutrient availability for the expression of genes that are involved in the pathogenicity of fungi.

*In silico* analysis of the putative gene product of *prt1* (see chapter 3.9) predicted a pre-pro-enzyme of 38 to 39 kDa that contains a signal peptide of 15 amino acids at the N-terminus – tagging the protein for secretion – and a 93 amino acid propeptide. The putative mature protein is expected to have a molecular weight of 28 to 29 kDa.

These results support the correlation between the *prt1* gene and the 30 kDa protease observed in zymogram analysis of partially purified media samples which was discussed above as the protease which makes up the plateau phase. Further evidence was gathered by RT-PCR experiments which were performed with RNA from mycelium sampled at different points in time during cultivation in PI-medium. It was shown that *prt1* is clearly expressed throughout the plateau phase and the early peak phase of protease activity. The mRNA is also present during the decrease of activity in the late peak phase, but – as far as this can be deduced from RT-PCR experiments – apparently at lower levels. During the cultivation of *F. graminearum* in CM-medium, *prt1* was barely detectable.

Using the inoculation of detached wheat leaves in a petri dish assay as a model system for *in planta* growth of *F. graminearum* (see chapter 3.12.2) the expression of *prt1* during the colonization of the leaf tissue was demonstrated as well, indicating a role of the protease in the infection process. Under natural circumstances *F. graminearum* is not primarily associated with leaf diseases, but the fungus was shown to readily colonize wheat leaves in laboratory experiments [NOLTE, 2001]. Whether the gene is also expressed during the infection of wheat heads remains to be investigated, but judged by brief macroscopic and microscopic assessment of the infection process in detached leaves (see chapter 3.12.2) fungal growth within the leaf tissue is comparable to that reported in the lemma and palea of wheat florets [WANJIRU ET AL., 2002].

## 4.7 Comparative overview and outlook

In recent years an increasing number of extracellular proteases which are produced by phytopathogenic fungi have been described. This includes metalloproteases like those produced by the rust fungus *Uromyces viciae-fabae* [Rauscher et al., 1995] or *Xanthomonas campestris* [Dow et al., 1990], aspartic proteases like AspS of *Sclerotinia sclerotiorum* [Poussereau, 2001] and cysteine proteases like that of *Pyrenopeziza brassicae* [Ball et al., 1991]. However the majority of described proteases belongs to the serine proteases. Trypsin-like and subtilisin-like enzymes are both widespread and a role in the pathogenic growth of fungi has been proposed.

Extracellular subtilisin-like proteases of a wide variety of plant pathogens like *Pyrenopeziza brassicae* [Ball et al., 1991; Keniry et al., 2002], *Ophiostoma piceae* [Abraham and Breuil, 1996], *Cochliobolus carbonum* [Murphy and Walton, 1996], *F. venenatum* (originally published as *F. graminearum*) [Griffen et al., 1997], *Magnaporthe poae* [Sreedhar et al., 1999], *F. oxysporum* [Di Pietro et al., 2001], *F. culmorum* [Pekkarinen et al., 2002], *F. eumartii* [Olivieri et al., 2002], and *Stagonospora nodorum* [Bindschedler et al., 2003] have been reported. Where tested – either indirectly by expression studies or biochemically - the respective proteases were found to be present during the colonization of the host plant.

Involvement of the proteases in the degradation of cell wall structural proteins and PR-proteins was discussed, but only for the protease produced by the potato pathogen *F. eumartii* this was verified *in vitro* [Olivieri et al., 2002]. Proteases showing the ability to degrade plant cell wall proteins have also been described amongst the metalloproteases [Rauscher et al., 1995; Dow et al., 1998] and the trypsin-like serine proteases [Carlile et al., 2000]. However, it is not easy to assess their importance as virulence factors and so far only one report on clearly impaired virulence of a phytopathogenic fungus after the inactivation of an extracellular protease is available [Ball et al., 1991]. As described for the cell wall carbohydrate degrading enzymes of pathogens it is assumed that the targeted deletion of a single gene encoding a cell wall degrading enzyme can be complemented by other genes with similar functions [Annis and Goodwin, 1997]. Furthermore it is likely that the variety of cell wall degrading enzymes degrade the different components of cell walls in synergistic cooperation.

Nevertheless, the important role of proteases in phytopathology can be inferred indirectly based on the widespread occurrence of protease inhibitors as a reaction to animal feed and pathogen attack [Joshi et al., 1998; Vernekar et al., 1999; Tamayo et al., 2000;

LAWRENCE, 2002]. An interesting bifunctional inhibitor of α-amylase and subtilisin-like proteases has been described for wheat [MUNDY ET AL., 1984] which can be regarded as an effective defense against pathogens of cereal grains.

Cell wall degrading enzymes of *F. graminearum* and *F. culmorum* have received considerable attention in the recent years due to the increasing threat of these fungi as pathogens of economically important crops. Significant degradation of the host cell wall components and direct penetration of cell walls was demonstrated [WANJIRU ET AL., 2002; KANG AND BUCHENAUER, 2002; KANG AND BUCHENAUER, 2003]. Since the accumulation of PR-proteins and cell wall enforcement by oxidative cross-linking of structural proteins and the formation of papillae have been documented in the interaction between *F. graminearum* and wheat [PRITSCH ET AL., 2000; PRITSCH ET AL., 2001; EL-GENDY ET AL., 2001; KANG AND BUCHENAUER, 2003], fungal proteases are almost certainly part of the interaction between the pathogen and the host.

It was shown in the present work that *F. graminearum* produces significant amounts of extracellular proteases during the cultivation with gelatin as the sole nitrogen source. Two extracellular subtilisin-like proteases with a molecular weight of about 71 and 30 kDa respectively were identified. The 71 kDa protease is thought to be involved in general nutrient acquisition based on the timing of its appearance and the observed carbon repressability. The 30 kDa protease is thought to be correlated with the digestion of the gelatin substrate. The protease activity during the digestion of the substrate was nitrogen repressable and showed an unusual requirement for a basic carbon supply instead of being carbon repressed.

Furthermore a gene encoding a subtilisin-like protease (named *prt1*) was identified and characterized. Based on the predicted size of its product, quantitative expression analysis by RT-PCR and informations published for a close homolog in *F. oxysporum* f.sp. *lycopersici,* it was correlated to the 30 kDa protease.

Since the gelatin employed as the inductor of the extracellular protease exhibits similarities to plant cell wall structural proteins with respect to amino acid composition as well as molecular structure and the *prt1* gene was found to be expressed *in planta*, a putative role of the 30 kDa subtilisin-like protease in the degradation of plant cell wall components during the pathogenic growth of the fungus in the host is proposed.

The present work has provided a basis for further research on the role of extracellular proteases of *F. graminearum* in pathogenicity. Cultivation methods for the induction of extracellular proteases were developed and a model system for *in planta* growth was applied. Genetic and biochemical evidence for the importance of subtilisin-like proteases in *F. graminearum* has been presented. Even though it was shown that the loss of the *prt1* gene in *F. oxysporum* did not result in impaired virulence on tomato plants [DI PIETRO ET AL., 2001], *prt1* and its product might be involved in the degradation of the host cell wall in the *F. graminearum* / wheat interaction. Investigation of the expression pattern during the cultivation of the fungus with cell wall material as well as *in planta* growth on wheat heads and in the detached leaf assay could provide valuable information about this proposed function. Biochemical methods for the partial purification and concentration of the described proteases have been established. These could be employed on crude extracts or intracellular washing fluids of infected wheat leaves to further investigate the role of the enzymes *in planta*. Furthermore, the genetic approach to the investigation of fungal proteases should be extended to members of metallo-, aspartic and cysteine proteases to gather an overview of the proteolytic enzymes that are available to the pathogen.

# 5 Abstract

In the present work extracellular proteases of the phytopathogen *Fusarium graminearum* that causes the disease *Fusarium head blight* (FHB) in cereals were investigated with biochemical and genetic methods. Cultivation of the fungus in liquid medium with gelatin as the sole source of nitrogen was chosen as model system for protease induction because of the similarities in amino acid composition and molecular structure between gelatin and structural proteins in the plant cell wall. Since the cell wall is one of the major structural barriers that protect plants against pathogens, fungal enzymes that aid the degradation of cell wall components can be regarded as factors involved in pathogenicity.

It was shown that the secretion of extracellular alkaline proteases can be induced under the employed conditions and protease activity in the growth medium is detectable as soon as three hours past inoculation. The time course of changes in protease activity in the medium is characterized by two distinct phases consisting of an early 'plateau phase' with low, constant activity that is nitrogen repressible and requires a basic supply of carbon, and a subsequent 'peak phase' with a strong transient increase in protease activity that is subject to carbon repression. The two phases could be correlated with two subtilisin-like serine proteases with molecular weights of about 30 and 71 kDa respectively.

The 71 kDa protease was shown to be responsible for the major part of the detected protease activity during the 'peak phase' and is discussed as a scavenger enzyme that plays a role in general nutrient acquisition. The 30 kDa protease is discussed in relation to the induced degradation of the gelatin substrate. A putative role of this protease in the cleavage of structural proteins of the cell wall during the infection of wheat is proposed.

Furthermore a gene encoding a subtilisin-like protease was identified in *F. graminearum* which was correlated to the 30 kDa protease. This assignment is based on the molecular characterization of the gene, qualitative expression studies during cultivation of the fungus in protease inducing medium and an *in planta* model system as well as on information published for a close homologue in the tomato pathogen *F. oxysporum* f.sp. *lycopersici.* The identified gene was named *prt1* after the *F. oxysporum* homologue. Putative regulatory elements in the 5'-upstream region of both genes are discussed.

# 6 Zusammenfassung

In der vorliegenden Arbeit wurden extrazelluläre Proteasen des Phytopathogens *Fusarium graminearum*, dass bei Getreide Ährenfusariosen verursacht, mit biochemischen und molekularbiologischen Methoden untersucht. Die Kultivierung des Pilzes in Flüssigmedium unter Zugabe von Gelatine als einziger Stickstoffquelle wurde als Modellsystem zur Proteaseinduktion ausgewählt, da Gelatine sowohl hinsichtlich der Aminosäurezusammensetzung als auch der molekularen Struktur Ähnlichkeiten mit Strukturproteinen der pflanzlichen Zellwand besitzt. Da die Zellwand eine der Hauptbarrieren ist, die Pflanzen gegen Pathogene schützen, können pilzliche Enzyme, die am Abbau von Zellwandkomponenten beteiligt sind, als Pathogenitätsfaktoren betrachtet werden.

Es konnte gezeigt werden, dass unter den angewandten Bedingungen die Sekretion von extrazellulären alkalischen Proteasen induziert wird und dass Proteaseaktivität schon drei Stunden nach der Inokulation im Medium nachweisbar ist. Der zeitliche Verlauf der Proteaseaktivität im Medium ist durch zwei Phasen gekennzeichnet. Zunächst tritt eine frühe ”Plateau-Phase” niedriger, konstanter Aktivität auf, die einer Hemmung durch Stickstoffquellen unterliegt und die einer grundlegenden Kohlenstoffversorgung bedarf. Darauf folgt eine ”Peak-Phase” mit starkem, transienten Anstieg der Proteaseaktivität, die Kohlenstoffhemmung unterliegt. Die beiden Phasen konnten mit zwei subtilisinähnlichen Proteasen mit Größen von 30 bzw. 71 kDa in Verbindung gebracht werden.

Es wurde gezeigt, dass die 71-kDa-Protease für den Hauptanteil der Proteaseaktivität während der ”Peak-Phase” verantwortlich ist. Sie wird als ”Scavenger-Enzym” diskutiert, dessen Aufgabe in der allgemeinen Bereitstellung von Nährstoffen liegt. Die 30-kDa-Protease wird im Zusammenhang mit dem induzierten Abbau des Gelatine-Substrats betrachtet. Eine mögliche Rolle dieser Protease bei der Spaltung von Strukturproteinen der Zellwand während der Infektion von Weizen wird vorgeschlagen.

Darüber hinaus wurde ein Gen bei *F. graminearum* identifiziert, dass mit der 30-kDa-Protease korrelierbar ist. Diese Zuordnung basiert einerseits auf der molekularen Charakterisierung des Gens, qualitativen Untersuchungen zur Expression während der Kultivierung des Pilzes in Protease induzierendem Medium und einem *In-planta*-Modellsystem, andererseits auf veröffentlichten Daten zu einem nahen Homologen aus dem Tomatenpathogen *F. oxysporum* f.sp. *lycopersici.* Das identifizierte Gen wurde entsprechend dem homologen Gen aus *F. oxysporum* ebenfalls mit *prt1* bezeichnet. Mögliche regulatorische Elemente der beiden Gene in der 5‘-upstream-Region werden diskutiert.

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```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        10         20         30         40         50         60         70         80
A**CTATCT**CGG ACGATGGCGA TCATCGGAGC AGAG***CACATG*** TCTTGTTACC ATAGGCCTAG GTGCCGAACG ACTACCAACA
 -273 ┘                          -245 ┘
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        90        100        110        120        130        140        150        160
ACCGAACATA GACGATGGAC CAAGATTAAA GGAGATGAAG CAACG**AGATA A**ATAGATCCC TTCGTCTCCA GAAACTCAAT
                                           -154 ┘
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       170        180        190        200        210        220        230        240
CTCATCTCAT CTCATCACAC TCAGCTCCTC GGTCTTTACA GCAAAGTCAA CAACTACATT CACTCACTCA CAACCACTCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       250        260        270        280        290        300        310        320
CTTTTTCAAA ACTCCAACTT TAACGTTCAC TATATCAAGA TGCGTTCCGC TACTCTTCTC GCTCTTTTGC CCTTTGCGCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       330        340        350        360        370        380        390        400
GGCCGCTCCT TCAGCCTCAG TCTCTCGACG AACTGAGCCC GCGCCTGTCA TCCGCCCTCG CGGTGTCAAG CTTGTCGATG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       410        420        430        440        450        460        470        480
GCAAGTACAT CGTCAAGATG AAGGCCGGTG TCCGAGCTGC TTCTGTTGAC AGCGCTGTTT CGACCATCCA GGCTGATGCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       490        500        510        520        530        540        550        560
GATTACACCT ACACCAAGAG CTTCAGTGGT TTCGCTGCTA GTCTCAAGGA TGAGGAGATT GAGACCCTCA AGCACGACCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       570        580        590        600        610        620        630        640
CAATGTCAGT CATGTAGATC CCAATCGTTC AGGGTAGTAC TAACAAATCC CAGGTCGAGT ACATTGAGCA GGATGCTGTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       650        660        670        680        690        700        710        720
ATCACCATCA AGGCTACTGT TGACCAGGAC AATGCTCCTT GGGGTATCGC CCGTCTTTCC AGCTCCAAGC CCGGTAGCAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       730        740        750        760        770        780        790        800
GACTTACACC TACGACGAGA GCGCTGGTGA GGGTACTTGC TCTTACGTCA TCGACACCGG CATTGATGTT GAGCATCCCG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       810        820        830        840        850        860        870        880
ACTTTGATGG ACGTGCCAAG TTCCTCAAGA ACTTTGCTGG TGGCCGTGAT GGTGACGGCC AAGGTCACGG CACCCACGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       890        900        910        920        930        940        950        960
GCTGGAACTA TCGGCTCCAC CACCTACGGT GTCGCCAAGA AGACCACCCT CTACGCCGTC AAGGTCCTGG GTGATGACGG
```

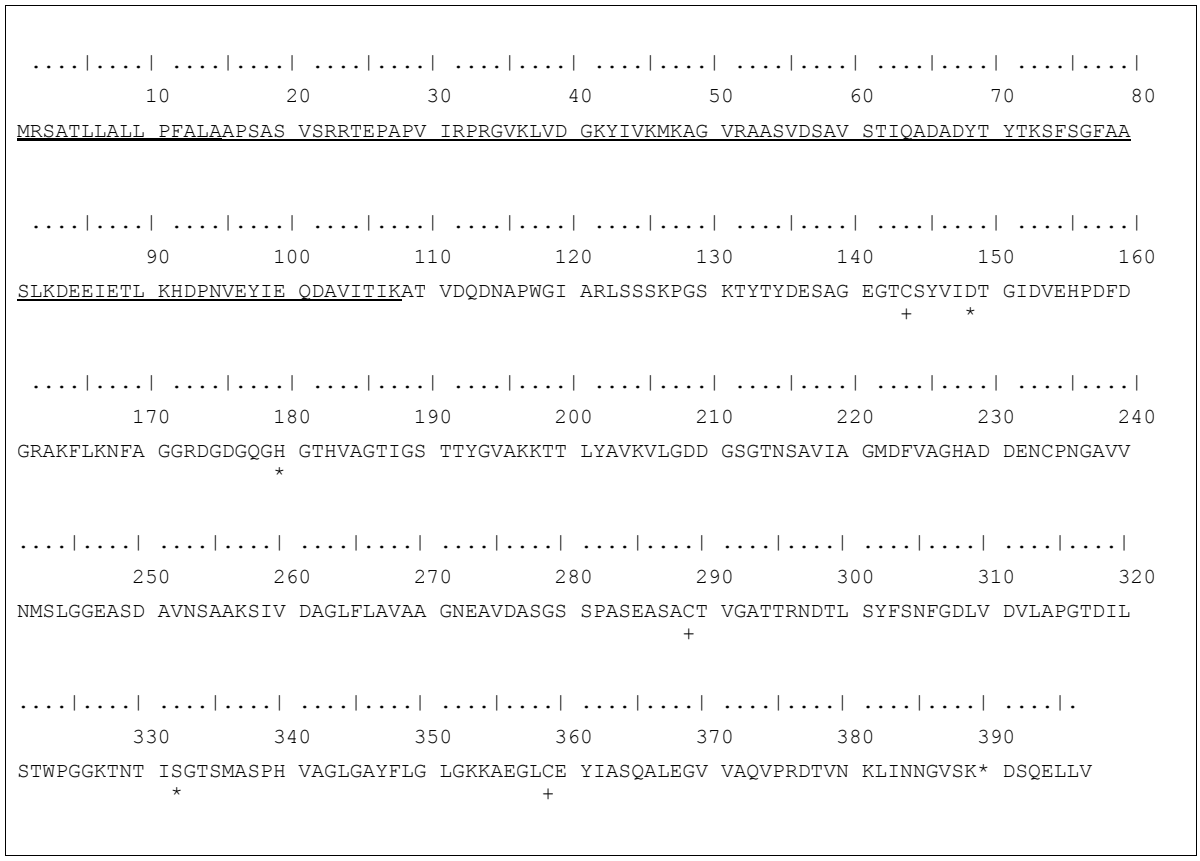
**Genomic sequence comprising the *prt1* gene of *Fusarium graminearum strain* K59**

The sequenced cDNA is underlined with a bold line. Two potential binding sites for transcription factors of the GATA-family upstream of the start codon are indicated by **bold** letters and arrows oriented in 5'-3' direction. A potential binding site for bHLH type transcription factors is marked by ***bold italic*** letters and an arrow. Continued on next page

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        970        980        990        1000       1010       1020       1030       1040
TTCCGGTACC AACTCTGCTG TCATTGCTGG CATGGACTTT GTCGCTGGCC ACGCCGATGA CGAGAACTGC CCTAACGGTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1050       1060       1070       1080       1090       1100       1110       1120
CCGTTGTCAA CATGTCTCTT GGTGGCGAAG CCTCGGATGC TGTCAACAGC GCTGCTAAGA GCATTGTTGA TGCTGGTCTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1130       1140       1150       1160       1170       1180       1190       1200
TTCCTTGCCG TCGCAGCTGG CAACGAAGCC GTCGACGCTT CCGGCTCATC CCCTGCTTCC GAGGCCAGCG CCTGCACCGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1210       1220       1230       1240       1250       1260       1270       1280
TGGCGCAACC ACTCGAAACG ACACCCTTTC CTACTTCTCC AACTTTGGCG ACCTCGTCGA TGTTCTTGCT CCTGGTACCG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1290       1300       1310       1320       1330       1340       1350       1360
ACATCCTGAG CACCTGGCCC GGCGGCAAGA CCAACACCAT CTCCGGAACT TCCATGGCTT CGCCCCATGT CGCTGGTCTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1370       1380       1390       1400       1410       1420       1430       1440
GGCGCTTACT TCCTTGGACT TGGAAAGAAG GCCGAGGGTC TCTGCGAGTA CATTGCTTCT CAGGCTCTTG AGGGAGTTGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1450       1460       1470       1480       1490       1500       1510       1520
GGCCCAGGTG CCTCGCGACA CTGTTAACAA GCTCATCAAC AACGGTGTTA GCAAGTAAGA CAGCCAAGAG CTGCTTGTTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1530       1540       1550       1560       1570       1580       1590       1600
AGATACAGCT GTATCAGTTT CCTAGATGTA TGATTAACGT ATGGAATAAT ATTATTCGCT TGACCTTTGA TTCGCTGTCA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        1610       1620       1630       1640       1650       1660       1670       1680
GATTCGTGCA AAAAGTGATG TAGATATGTG AGATGTATCC CACTCAAAAT GGGGAAGATG GTGTTTGTTG GACTCAGTCG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
        1690       1700       1710       1720       1730       1740       1750
GGTMCGAGCC ACCAAGATTG ATCAAAGTCA CATTGGCTTC AATTGACACT ATGCTGTTCA TATCACTCGA CA
```

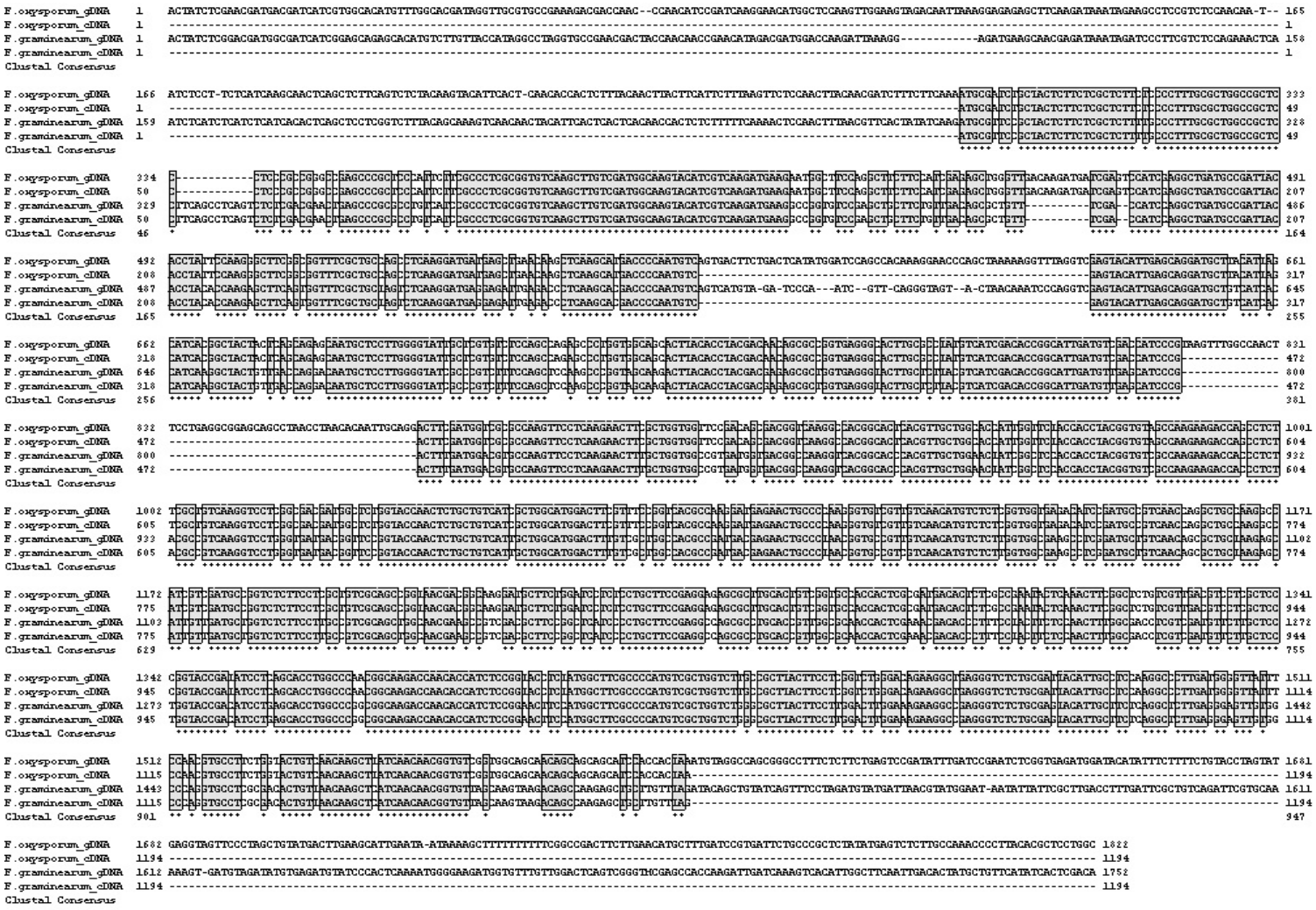
**Genomic sequence comprising the *prt1* gene of *Fusarium graminearum strain* K59**

The sequenced cDNA is underlined with a bold line. The putative 3'-end of the cDNA deduced from the gDNA sequence is underlined. Continued from previous page



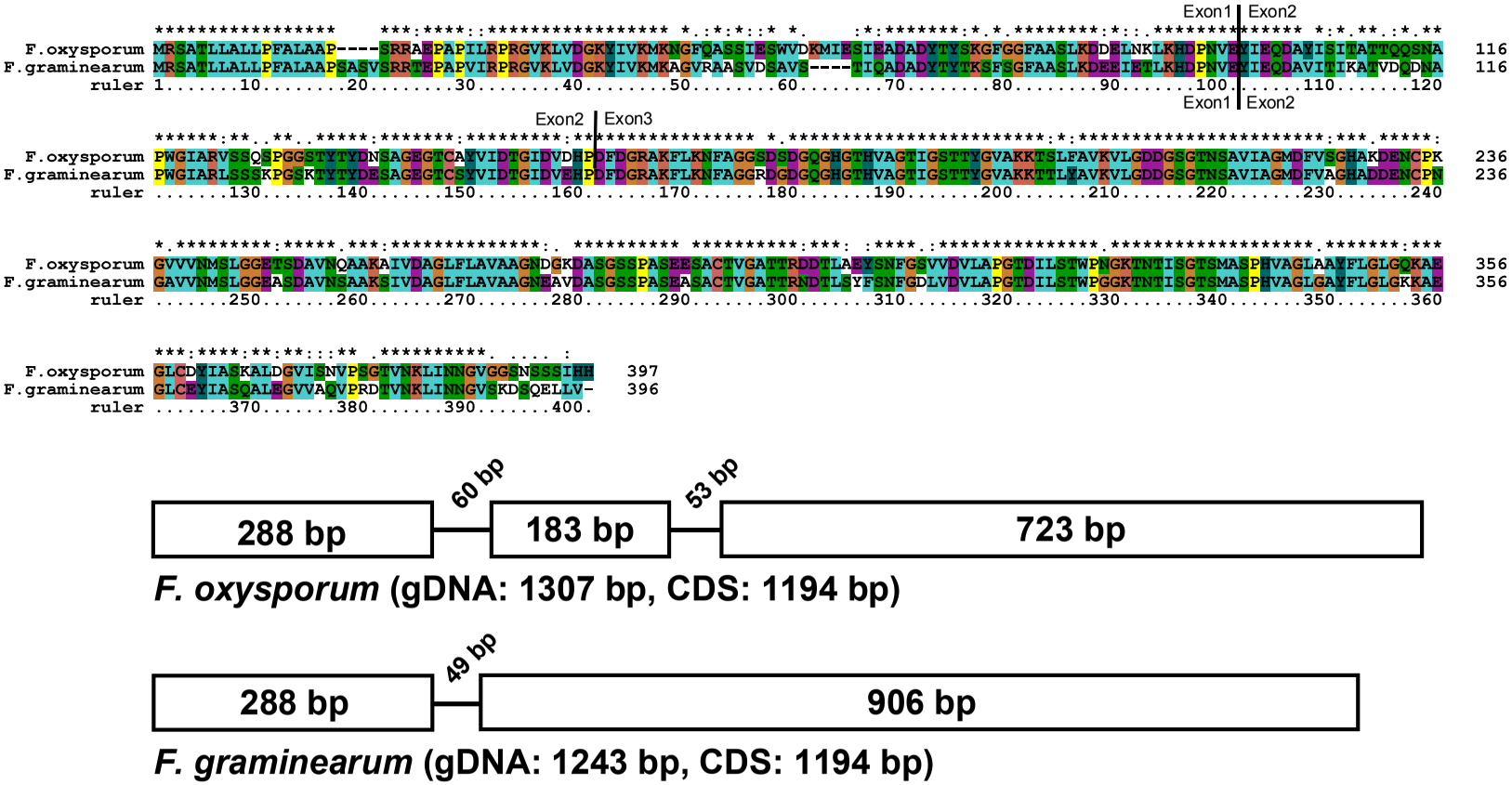

**Deduced amino acid sequence of the *prt1* gene of *Fusarium graminearum strain* K59**

The putative signal sequence is underlined with a bold line. The putative propeptide is underlined. The asterisks mark the amino acids that make up the catalytic triad characteristic for the members of the subtilisin family (Asp, His and Ser). Conserved cystein residues that are potentially involved in the formation of disulfide bridges are marked with a cross.




**Sequence alignment of *Fusarium oxysporum* and *Fusarium graminearum prt1* genes**

The genomic and cDNA sequences of *F. oxysporum* f. sp. lycopersici and *F. graminearum* strain K59 respectively are shown grouped together. The two introns contained in the *F. oxysporum* sequence are obvious. The *F. graminearum* sequence contains only one intron.



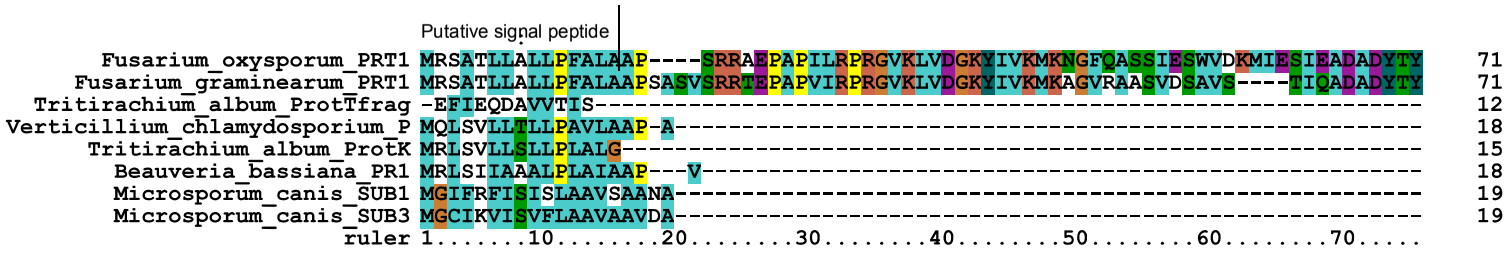

**Top: Sequence alignment of the deduced amino acid sequence of *Fusarium oxysporum* and *Fusarium graminearum prt1* genes**

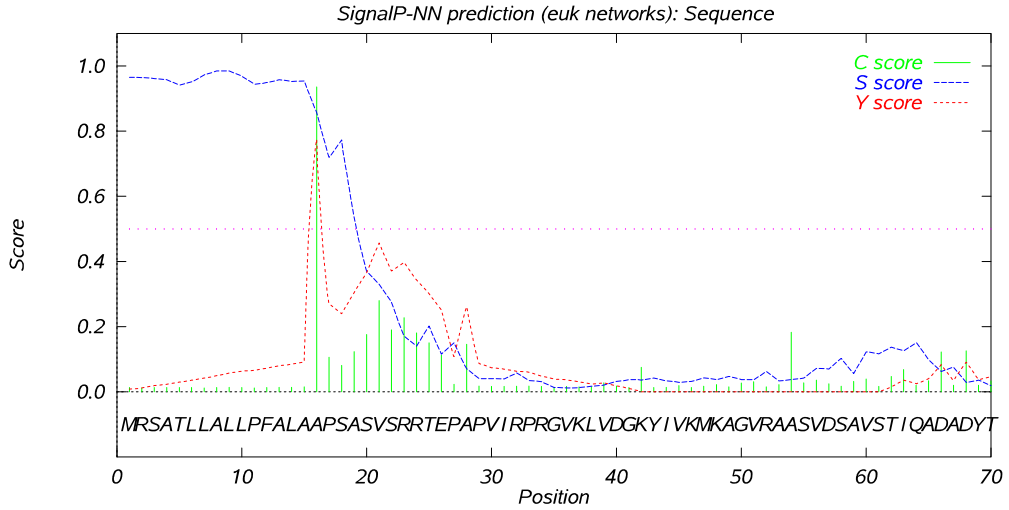
The annotations show the position of the putative exons 1-3 for *F. oxysporum* and exons 1 and 2 for *F. graminearum*.

**Bottom: Schematic diagram of the exon / intron structure of the two genes**

Open boxes indicate exons while introns are represented by lines. The annotations give the length of the respective regions in basepairs.

gDNA: length of the genomic DNA from start to stop codon including the introns. CDS: coding sequence excluding the introns.




**Target of *prt1* as predicted by the program 'PSORT II' (see chapter 2.3.5)**

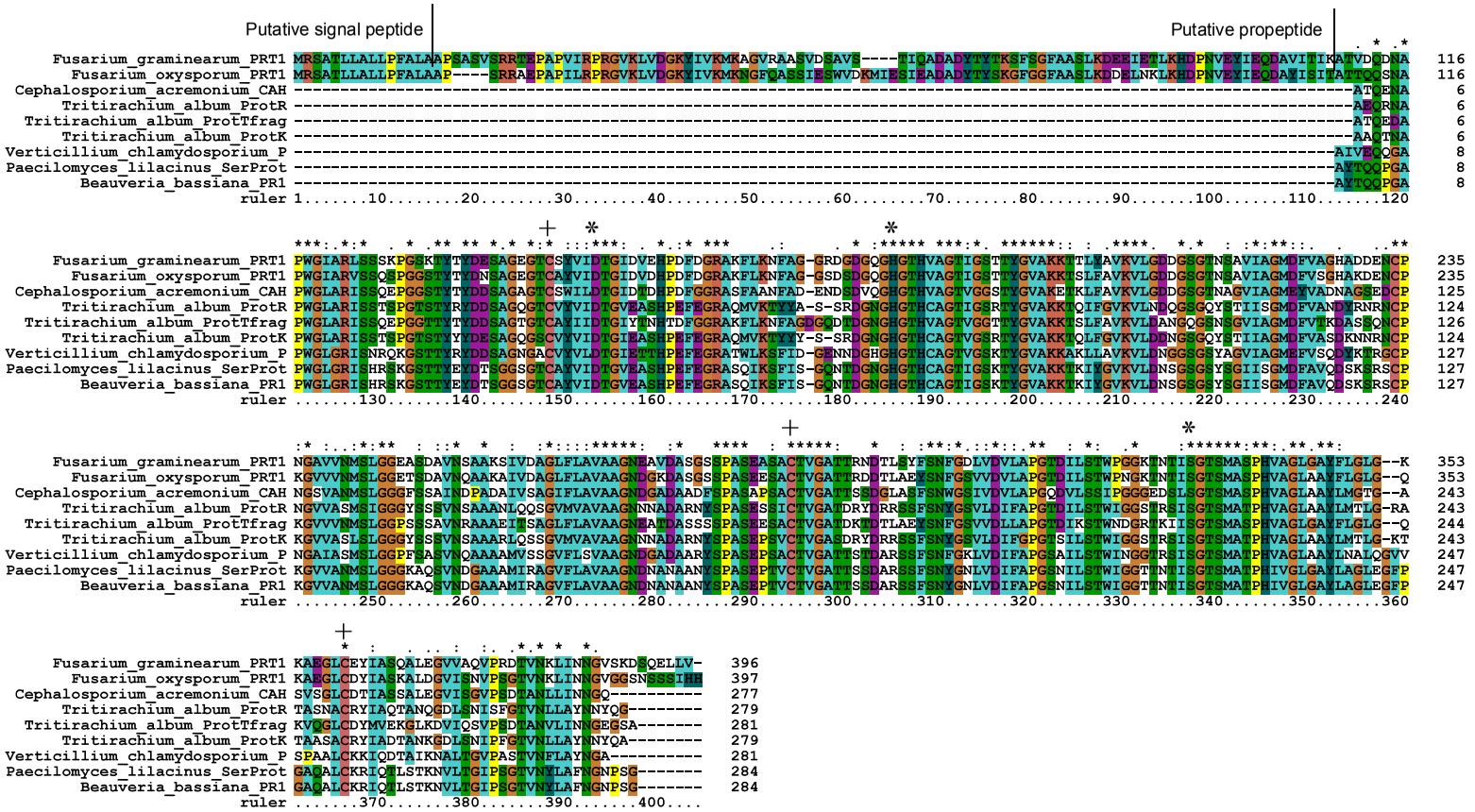
| | |
|---|---|
| extracellular incl. cell wall | 55.6% |
| cytoplasmic | 11.1% |
| mitochondrial | 11.1% |
| Golgi apparatus | 11.1% |
| vacuolar | 11.1% |

**Top: Sequence alignment of the deduced amino acid sequence of *Fusarium oxysporum* and *Fusarium graminearum prt1* genes with published sequences of signal peptides of subtilisin-like proteases.**

The annotation marks the putative cleavage site of the signal peptide in the *prt1* genes.

**Bottom: Signal peptide prediction using 'SignalP' (see chapter 2.3.5)**

Green: 'C-Score' gives the probability of a cleavage site at this position. Blue: 'S-Score' gives the probability of a signal peptide at this position. Red: 'Y-Score' = combined combination of the two scores. The peak between the positions 15 and 16 marks the putative cleavage site of the signal peptide.

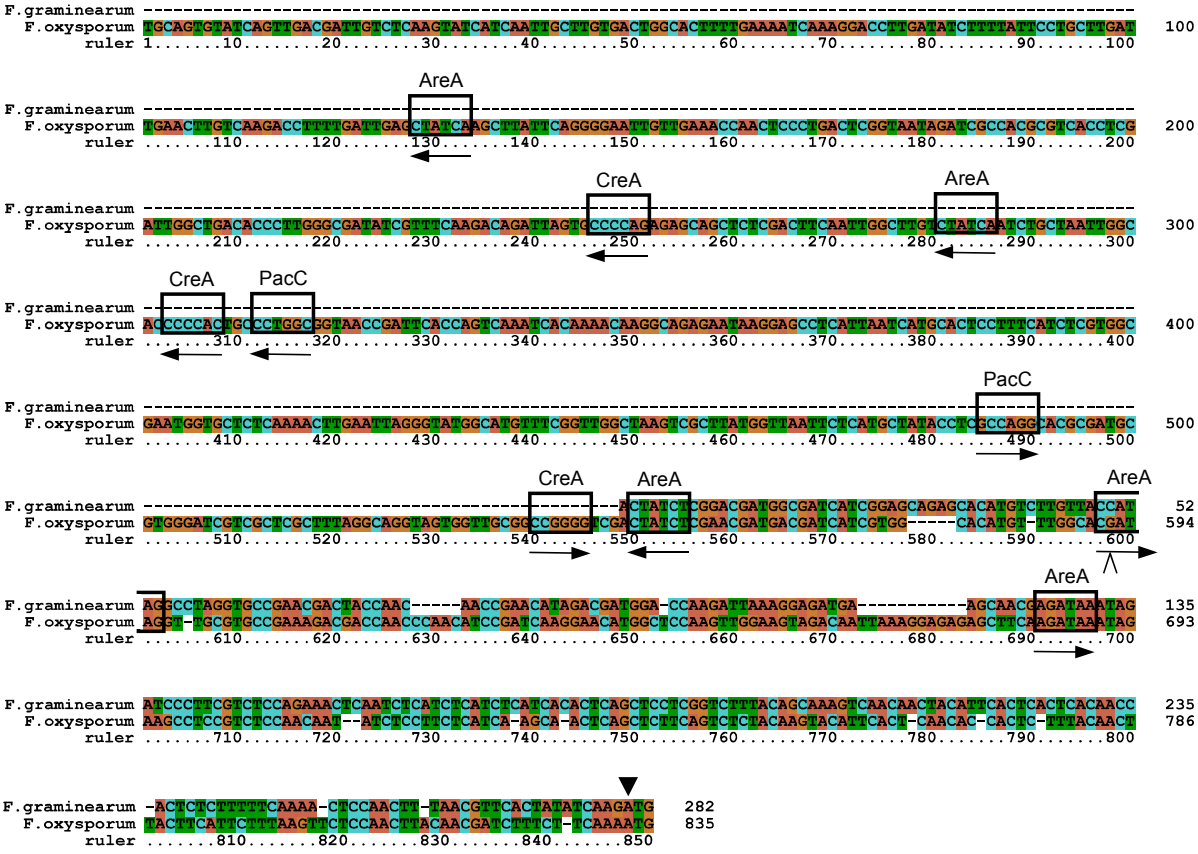



**Sequence alignment of the deduced amino acid sequence of *Fusarium oxysporum* and *Fusarium graminearum prt1* genes with selected sequences of mature subtilisin-like proteases after the cleavage of the propeptide.**

The annotation marks the putative cleavage sites of the signal peptide and the propeptide in the *prt1* genes.

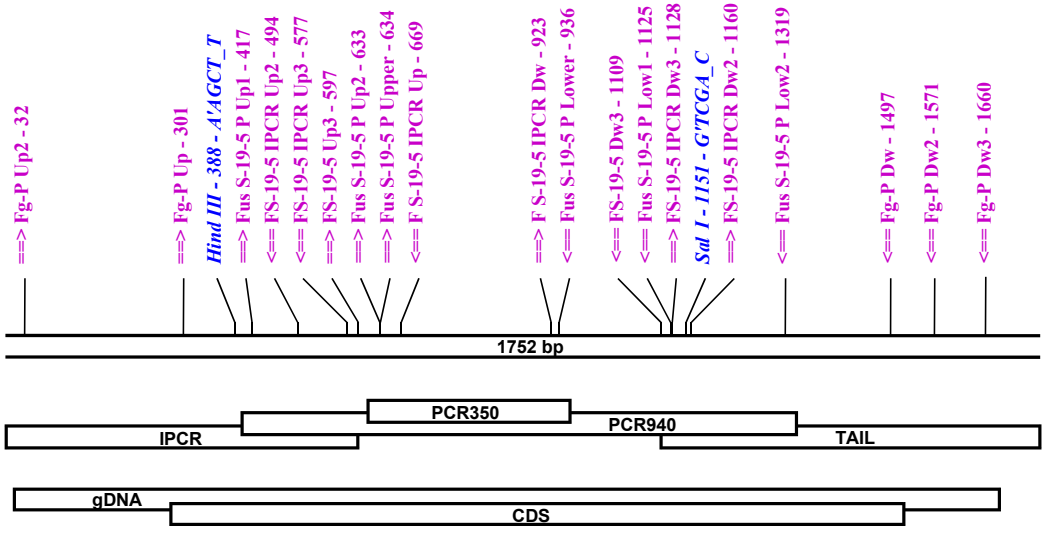
The amino acids that make up the catalytic triad characteristic for the members of the subtilisin family are marked with a big asterisk.

Conserved cystein residues that are potentially involved in the formation of disulfid bridges are marked with a cross.




**Genomic sequences of the 5'-flanking regions of *prt1* from *F. graminearum* and *F.oxysporum* f.sp. *lycopersici*.** Boxes indicate putative binding sites of transcription factors which mediate repression / derepression by nitrogen (AreA), carbon (CreA) and ambient pH (PacC). The arrows indicate the orientation of the consensus sequences in 5'-3' direction. The open arrowhead indicates a mismatch within a putative AreA binding site in the sequence from *F. graminearum*. The filled arrowhead indicates the start codons of the *prt1* genes.

| Name | Sequence (5' --> 3') | Length [bp] | GC-content [%] | Tm [°C] |
|---|---|---|---|---|
| Fus S-19-5 P Upper | GGT CGA GT(5=GATC) (5=GATC)AT TGA GCA GGA T | 22 | 45.5 | 58.4 |
| Fus S-19-5 P Lower | GGC AGC CTG GTT (5=GATC)AC GG | 17 | 64.7 | 57.6 |
| Fus S-19-5 P Up1 | GGC AAG TAC ATT GTC AAG | 18 | 44.4 | 51.4 |
| Fus S-19-5 P Up2 | TGT CGA (R=AG)T(H=ACT) CAT TGA GCA GGA | 21 | 46.8 | 57.5 |
| Fus S-19-5 P Low1 | TCG TT(R=AG) CC(M=AC) GC(K=GT) GCG AC(R=AG) GCG A | 22 | 68.2 | 67.7 |
| Fus S-19-5 P Low2 | GCC AT(R=AG) GA(S=GC) GT(D=AGT) CC(V=AGC) GAG AT | 20 | 57.5 | 60.4 |
| F S-19-5 IPCR Up/Up Sq | ACC CCA AGG AGC ATT GT | 17 | 52.9 | 52.8 |
| F S-19-5 IPCR Dw/Dw Sq | TAC GGT GTC GCC AAG AAG A | 19 | 52.6 | 56.7 |
| FS-19-5 IPCR Up2 | GCG AAA CAC TGA AGC TCT TGG | 21 | 52.4 | 59.8 |
| FS-19-5 IPCR Dw2 | AAC GAA GCC GTC GAC GCT T | 19 | 57.9 | 58.8 |
| FS-19-5 Up3 | GAT CCC AAT CGT TCA GGG TA | 20 | 50.0 | 57.3 |
| FS-19-5 Dw3 | GCA AGG AAG AGA CCA GCA TC | 20 | 55.0 | 59.4 |
| FS-19-5 IPCR Up3 | TAC CCT GAA CGA TTG GGA TC | 20 | 50.0 | 57.3 |
| FS-19-5 IPCR Dw3 | GAT GCT GGT CTC TTC CTT GC | 20 | 55.0 | 59.4 |
| Fg-P Up | ATG CGT TCC GCT ACT CTT CTC G | 22 | 54.5 | 62.1 |
| Fg-P Dw | CTA AAC AAG CAG CTC TTG GCT GTC | 24 | 50.0 | 62.7 |
| Fg-P Dw2 | GCG AAT CAA AGG TCA AGC GAA TA | 23 | 43.0 | 61.0 |
| Fg-P Up2 | TGG CGA TCA TCG GAG CAG | 18 | 61.1 | 62.2 |
| Fg-P Dw3 | ACC CGA CTG AGT CCA ACA AAC A | 22 | 50.0 | 62.7 |
| | | | | |
| DP1 | (W=AT)AG TG(N=GATC) AG(W=AT) A(N=GATC)C A(N=GATC)A GA | | | 47.0 |
| DP2 | (N=GATC)GT CGA (S=GC)(W=AT)G A(N=GATC)A (W=AT)GA A | | | 47.0 |
| DP3 | (N=GATC)CA GCT (W=AT)(S=GC)C T(N=GATC)T (S=GC)CT T | | | 49.0 |
| Anchored oligo dT | xTTT TTT TTT TTT TTT TTT TTT TT (x = A/C/G/T) | 24 | | |
| M13 universal | ACG ACG TTG TAA AAC GAC GGC CAG | 24 | | |
| M13 reverse | TTC ACA CAG GAA ACA GCT ATC ACC | 24 | | |




**Primers used during cloning and sequencing of *prt1* and for cDNA studies.** The lower diagram gives a graphic representation of the positions of the primers and the DNA fragments that were compiled to obtain the complete genomic sequence of *prt1*. Abbreviations: PCR350 = Fragment obtained with touch down PCR, PCR940 = Fragment obtained with standard PCR, IPCR = Fragment obtained with inverse PCR, TAIL = Fragment obtained with TAIL PCR, gDNA = resequenced range of genomic DNA comprising the putative coding sequence (CDS). The location of the restriction sites of Hind III and Sal I within the sequence are also given. The positions listed for the primers are the respective 3'-ends.

| Forward primer | Reverse primer | Annealing temperature [°C] | Elongation time | Number of cycles | Expected fragment [bp] |
|---|---|---|---|---|---|
| Fus S-19-5 Upper | Fus S-19-5 Lower | 65 – 60 touch down | 2 min | 25 - 30 | ~350 |
| Fus S-19-5 Up1 / Up2 | Fus S-19-5 Low1 / Low2 | 44 tested | 2 min | 25 - 30 | ? |
| Fus S-19-5 Up | Fus S-19-5 Low2 | 44 | 2 min | 25 - 30 | ~940 |
| FS-19-5 IPCR Up | FS-19-5 IPCR Dw | 42 – 55 tested | 2 min | 25 - 30 | ? |
| FS-19-5 IPCR Up2 | FS-19-5 IPCR Dw2 | 42 – 55 tested | 2 min | 25 - 30 | ? |
| FS-19-5 IPCR Up3 | FS-19-5 IPCR Dw3 | 60 | 2 min | 25 - 30 | ~600 |
| Fg-P Up | FS-19-5 IPCR Up3 | 53 | 1 min | 25 - 30 | ~275 |
| Fg-P Up | Fg-P Dw | 60 | 2 min | 25 - 30 | ~1230 |
| Fg-P Up | Fg-P Dw2 | 60 | 2 min | 25 - 30 | ~1270 |
| Fg-P Up | Fg-P Dw3 | 60 | 2 min | 25 - 30 | ~1360 |
| Fg-P Up2 | FS-19-5 IPCR Up3 | 53 | 1 min | 25 - 30 | ~550 |
| Fg-P Up2 | Fg-P Dw3 | 61 | 2 min | 25 - 30 | ~1670 |
| Fg-P Up | FS-19-5 Dw3 | 61 | 1 min | 25 - 30 | ~850 gDNA<br>~800 cDNA |

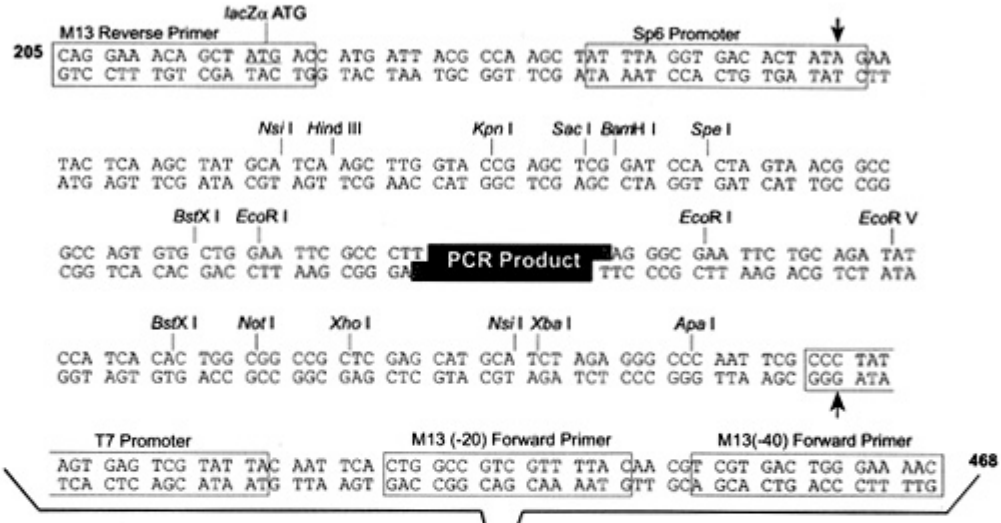
**Conditions for PCR used during cloning and sequencing of *prt1* and for cDNA studies.**

The conditions used for TAIL PCR are given in chapter 2.2.5.5

# Map of pCR®II-TOPO®

**pCR®II-TOPO® Map**

The map below shows the features of pCR®II-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrows indicate the start of transcription for Sp6 and T7 polymerases. For the full sequence of the vector, you may download it from our web site or call Technical Service (page 19).




**Comments for pCR®II-TOPO®**
**3950 nucleotides**

LacZα gene: bases 1-588
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-399
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
M13 (-40) Forward priming site: bases 453-468
f1 origin: bases 590-1004
Kanamycin resistance ORF: bases 1338-2132
Ampicillin resistance ORF: bases 2150-3010
pUC origin: bases 3155-3828

**Map of the pCR®II-TOPO® vector (INVITROGEN, Karlsruhe) that was used for cloning and sequencing of DNA fragments.**
Taken from the manufacturers user manual.

Und zu guter Letzt:

# Danke...

**...Prof. Bruno Moerschbacher für die herzliche Aufnahme in seinen Arbeitskreis, seine unkomplizierte offene Art und seine unerschütterliche Motivationsfähigkeit**

**...Prof. Walter Stöcker für die hilfsbereite Unterstützung eines 'Protease-Neulings' die bereitwillige Übernahme des Koreferates und die Teilfinanzierung als Computer-Hiwi**

**...Allen aktuellen und ehemaligen Moerschis, Bocks und ex-Barzis für das unschlagbare Arbeitsklima. Ich hab mich bei euch echt wohl gefühlt!**

**...Der Autoklavenrunde! Es lebe das Feierabend-Bierchen! Lasst die Tradition nicht aussterben!**

**...Den alten Säcken Holger, Markus, Nico und Nour-Eddine, die mich den ganzen Weg zum Doc begleitet haben.**

**...Den jungen Hüpfern (oder denen die sich dafür halten ;-), die das IBBP auch weiterhin als gutes Team bewahren.**

**...ganz besonders herzlich an Nico und Holger, die als Power-Korrektoren unter Zeitdruck zum Gelingen dieser Arbeit beigetragen haben.**

**...Dem Biologenpack, das ich leider in der letzten Zeit viel zu selten gesehen habe. Ohne euch wäre das ganze Studium nix gewesen!**

**...ganz zum Schluss Danke an meine Eltern und an Gerhard,
die immer für mich da waren!**

# Lebenslauf

## Matthias Hellweg

geboren am *10. Mai 1972* in Bremen

| | |
|---|---|
| **Familienstand:** | ledig |
| **Eltern:** | Ursula Hellweg, geb. Singbartl,<br>Gerhard Walter Hellweg |
| **Schulbildung:** | *1978 – 1982* Grundschule Luttum<br>*1982 – 1984* Orientierungsstufe Kirchlinteln<br>*1984 – 1991* Domgymnasium Verden (Aller) |
| **Allgemeine Hochschulreife:** | *29. Mai 1991* am Domgymnasium Verden (Aller) |
| **Studium:** | **Biologie – Diplomstudiengang**<br>*von Oktober 1991 bis April 1998*<br>an der Westfälischen Wilhelms-Universität Münster<br><br>**Informatik – Bachelorstudiengang**<br>*seit Oktober 2000* an der FernUniversität Hagen |
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| **Tätigkeiten an der Universität:** | *Oktober 1992 – März 1998*<br>Mitglied der Fachschaftsvertretung Biologie<br>Oktober 1993 – September 1994<br>Studentische Hilfskraft: Kursbetreuungen<br>April 1995 – April 1998<br>Studentische Hilfskraft: Betreuung der Rechner im Computer-Pool des Fachbereichs Biologie<br>Mai 1998 – Juni 2000<br>Wissenschaftliche Hilfskraft: Administration des fachbereichs-internen Netzwerks des FB Biologie<br>Februar 1999 – März 2003<br>Wissenschaftliche Hilfskraft / Wissenschaftlicher Mitarbeiter am Institut für Biochemie und Biotechnologie der Pflanzen<br>April 2001 – März 2002 sowie seit April 2003<br>Einrichtung und Pflege eines Web-Servers sowie einer Website zu einem EU geförderten Forschungsprojekt als freier Mitarbeiter |

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| **Außeruniversitäre Tätigkeiten:** | *Juni/Juli 1991*<br>Aushilfskraft bei der Effem GmbH, Verden<br>*März/April 1995*<br>Aushilfskraft bei der Deutschen Post AG, Münster<br>*seit 1998*<br>freie Mitarbeit als Patentübersetzer für die Patentanwaltskanzleien Türck, Gille, Hrabal & Leifert, Düsseldorf sowie Leifert & Steffan, Düsseldorf |
| **Auslandsaufenthalt:** | *Sommer 1995*<br>Achtwöchiger 'Summer-Session'-Kurs an der University of California, Berkeley, USA |
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