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Anja Pekkarinen

The serine proteinases of *Fusarium* grown on cereal proteins and in barley grain and their inhibition by barley proteins

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ACADEMIC DISSERTATION

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Abstract

Fusarium head blight (FHB, scab) of wheat and barley is one of the most devastating diseases of cereals. Severe FHB epidemics have occurred all over the world, resulting in major yield and quality losses that cause problems to producers and to various industries that use grain as raw material. Scabby grain processes poorly and the toxins that are produced by the fungi cause potential health risks to humans and animals. The *Fusarium* fungi colonize cereal spikes and utilize the grain components for their own nutrition and reproduction. One of the interesting aspects of the infection mechanism is the question of how important is the hydrolysis of the host plant proteins by the invading fungus. Previous studies have indicated that protein degradation occurs in infected grains, implying that the fungi produce proteinases during the colonization of the kernel tissues. In addition, it has been proposed in the literature that host plants may use various proteinase inhibitors to defend themselves against pathogens. The purpose of this dissertation was to pinpoint and characterize the proteinases that are synthesized by *Fusarium* species to degrade grain proteins during infection and to identify and thoroughly examine any proteins in barley that can inhibit those enzymes.

In this study, it was shown that species that cause FHB, *F. culmorum*, *F. graminearum* and *F. poae,* produced alkaline proteinases when grown in cereal protein media. Two proteinases were purified from a *F. culmorum* culture filtrate by using size-exclusion and ion exchange chromatographies. Both of the enzymes were maximally active at pH ~9 and 40–45 °C, but they were unstable under those conditions. The mechanistic classes of the enzymes were determined by measuring the effects of class-specific proteinase inhibitors on their activities and this indicated that they were subtilisin- and trypsin-like proteinases. In addition, portions of their amino acid sequences were homologous to those of other fungal proteinases that have been categorized into these classes. Both of

the proteinases hydrolyzed C- and D hordeins (barley storage proteins) *in vitro*. The presence of these enzymes in field grown, FHB-infected barley was demonstrated by activity assays using N-succinyl-Ala-Ala-Pro-Phe *p*NA and N-benzoyl-Val-Gly-Arg *p*NA as substrates and by an immunoblotting method. These proteinases were inhibited by several barley proteins, which were then purified and identified. The subtilisin-like proteinase was inhibited by the barley α-amylase/subtilisin inhibitor (BASI) and by the chymotrypsin/subtilisin inhibitors 1A, 1B and 2A (CI-1A, -1B, -2A). The trypsin-like enzyme was only inhibited by the barley Bowman-Birk inhibitor (BBBI). The roles that these proteinases and their inhibitors may play during the *Fusarium*-infection are discussed.

Preface

The first part of this work was conducted at VTT Biotechnology and Food Research in Espoo, Finland during the years 1996–1999. During this time the work was financed by the Tor-Magnus Enari Fund, the Raisio Group Research Foundation, the Kuopio Naturalists' Society, the Finnish Cultural Foundation, the Jenny and Antti Wihuri Foundation, the Finnish Concordia Association, the Kemira Foundation and the Olvi Foundation. The second part was carried out in Madison, Wisconsin, USA at the United States Department of Agriculture, Agricultural Research Service, Cereal Crops Research Unit (USDA–ARS, CCRU) in affiliation with the University of Wisconsin–Madison Department of Agronomy between May 1999 and May 2002. During these years the work was supported by the American Malting Barley Association, Anheuser-Busch, Inc. and the Finnish Food Research Foundation. The thesis was finalized at VTT Biotechnology with financial support from the University of Helsinki and VTT Biotechnology. The support of all of these foundations, organizations and companies is greatly appreciated.

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List of original publications

This thesis is based on the following publications, referred in the text by the Roman numerals given below, and data presented herein:

I Pekkarinen, A., Mannonen, L., Jones, B. L. and Niku-Paavola, M.-L. Production of proteases by *Fusarium* species grown on barley grains and in media containing cereal proteins. J. Cereal Sci. 2000. Vol. 31, pp. 253–261.

II Pekkarinen, A. I., Niku-Paavola, M.-L. and Jones, B. L. Purification and properties of an alkaline proteinase of *Fusarium culmorum*. Eur. J. Biochem. 2002. Vol. 269, pp. 798–807.

III Pekkarinen, A. I. and Jones, B. L. Trypsin-like proteinase produced by *Fusarium culmorum* grown on grain proteins. J. Agric. Food Chem. 2002. Vol. 50, pp. 3849–3855.

IV Pekkarinen, A. I., Sarlin, T. H., Laitila, A. T., Haikara, A. I. and Jones, B. L. *Fusarium* species synthesize alkaline proteinases in infested barley. J. Cereal Sci. In press.

V Pekkarinen, A. I. and Jones, B. L. Purification and identification of barley (*Hordeum vulgare* L.) proteins that inhibit the alkaline serine proteinases of *Fusarium culmorum*. J. Agric. Food Chem. In press.

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Abbreviations

ABA	abscisic acid
*p*APMSF	*p*-amidino phenylmethylsulfonyl fluoride
BASI	barley α-amylase/subtilisin inhibitor
BBBI	barley Bowman-Birk inhibitor
BVGR*p*NA	N-benzoyl-Val-Gly-Arg *p*-nitroanilide
CI	chymotrypsin/subtilisin inhibitor
CMC	carboxymethyl cellulose
CMe	barley endosperm trypsin inhibitor (belongs to CM proteins, see below)
CM-HPLC	carboxymethyl-high pressure liquid chromatography
CM proteins	α-amylase/trypsin inhibitors (chloroform-methanol soluble proteins)
CST	chymostatin
DMSO	dimethylsulfoxide
DON	deoxynivalenol
dpi	days post-inoculation
EDTA	ethylenediaminetetraacetic acid
E-64	trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
FHB	Fusarium head blight
GA	gibberellic acid
GSRPs	glycine- and serine-rich proteins
HR	hypersensitive reaction
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
PMSF	phenylmethylsulfonyl fluoride
PR	pathogenesis-related
QAC	quaternaryammonium cellulose
RP-HPLC	reversed phase-HPLC
SAAPF*p*NA	N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide
SAR	systemic acquired resistance
SBBI	soybean Bowman-Birk inhibitor
SL	subtilisin-like
STI	soybean trypsin inhibitor
TL	trypsin-like

List of EC numbers

α-amylase: 3.2.1.1

aspartic proteinases: 3.4.23

carbohydrolases: 3.2.1

chitinase: 3.2.1.14

chymotrypsin: 3.4.21.1

cutinase: 3.1.1.74

cysteine proteinases: 3.4.22

β-1,3-glucanase: 3.2.1.6

lipase: 3.1.1.3

metalloproteinases: 3.4.24

oryzin: 3.4.21.63

pectate lyase: 4.2.2.2

pectinase: 3.2.1.15

(endo-) pectin lyase: 4.2.2.10

pepsin: 3.4.23.1

peroxidase: 1.11.1.7

polyphenol oxidase: 1.10.3.1 and 1.14.18.1

subtilisin: 3.4.21.62

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trypsin: 3.4.21.4

endo-β-1,4-xylanase: 3.2.1.8

1. Introduction

1.1 Fusarium head blight of wheat and barley

Fusarium species are saprophytic fungi, and many of them are also destructive plant pathogens. Some cause root, foot or crown rots, seedling blight and head blight (FHB, scab) in cereals. Currently, FHB is the most threatening of the cereal diseases that are caused by fusaria. The most aggressive species that cause FHB are *F. graminearum* (teleomorph *Gibberella zeae*) and *F. culmorum*, but *F. avenaceum* (teleomorph *G. avenaceae*), *F. nivale* (synonyms *Microdochium nivale*, *Calonectria nivalis*), *F. sporotrichioides* and *F. poae* have also caused infestations in various countries (Parry *et al.* 1995, Steffenson 2003, Wong *et al.* 1995). These fungi also cause head blight in rye and oats, but this review is limited to the FHB of wheat and barley.

FHB occurs commonly in regions with high humidity or during rainy summers in areas that have been exposed to pathogenic *Fusarium* spp. (Parry *et al.* 1995). The spores survive in debris, and the rain and wind spread the soil-born inoculum to the plants. High humidity favors the fungal growth both in debris and in plants. The optimal temperature for the *F. graminearum* infection is 25 ºC, but infestations also occur in cooler conditions. The timing of infection is an important factor that affects the development and severity of FHB. The fungus can infect plants at any time during the growing season, but the spikelets are most susceptible at anthesis or during the early dough stage of grain development (Bai & Shaner 1994). Tillage and rotating crops with plants other than cereals are recommended practices that can reduce the amount of soil-born inoculum. Also, avoiding the excessive usage of fertilizers may decrease the ability of the fungus to survive in soil. Chemical and biological control methods are being developed to prevent crop losses, but the most efficient way to avoid FHB-problems is to use resistant cultivars (reviews by Bai & Shaner 1994, Gilbert & Tekauz 2000, Steffenson 2003). There are some differences in the resistances of barleys to FHB, but malting barley cultivars are, at best, only moderately resistant. That is why the epidemics are especially harmful for the brewing and malting industries. Wheat is generally more susceptible to *Fusarium* than barley but, on the other hand, there are some highly resistant wheat cultivars. However, no wheat or barley cultivars are totally immune to FHB.

The most apparent visual symptoms of FHB are shrivelled, discolored 'tombstone' kernels that may contain dark perithecia and pinkish mycelia (Parry *et al.* 1995). The fungi can invade the kernel and colonize the endosperm (Bechtel *et al.* 1985, Jackowiak *et al.* 2002, Narziß *et al.* 1990, Nightingale *et al.* 1999) and parts of the embryo (Chełkowski *et al.* 1990). They can destroy the developing kernel by either interfering with the synthesis of the storage reserves or by using up the stored proteins and carbohydrates for their own growth and reproduction (reviewed by Schwarz 2003). The infection may affect the seed physiology so that the developing kernels start sprouting before maturation or the seed becomes unviable. In addition, even if the the seed remains viable, it is unsuitable for sowing, because it is a source of inoculum for seedling blight (Steffenson 2003). Thus, FHB affects the crop yield by reducing the grain weight and subsequently decreasing the number of viable seedlings in the following year.

The poor quality of FHB-diseased grain poses restrictions on its usage and thus lowers its value. The FHB infection affects the grain composition, which can be detected as alterations in certain enzyme activities and in the proportions of storage proteins and carbohydrates (Boyacıoğlu & Hettiarachchy 1995, Narziß *et al.* 1990, Nightingale *et al.* 1999, Schwarz *et al.* 2002). During malting and mashing the grain storage reserves are modified into forms that can be used by the fermentation yeast to yield ethanol and flavors. Because good malting and brewing quality requires very well regulated enzyme synthesis and function, the altered enzyme compositions of the FHB-infected malts often render them unsuitable for brewing (reviewed by Schwarz 2003). In addition to decreasing these quality parameters, FHB has been associated with the overfoaming (gushing) of beer. FHB also lowers the pasta- and baking quality of wheat (in review by Gilbert & Tekauz 2000). In addition, certain metabolites of some *Fusarium* species, such as the trichothecenes, estrogen-like compounds and fumonisins are toxic to humans and animals and the occurrence of these toxins may render the grain unsafe for consumption (Peraica *et al.* 1999, Placinta *et al.* 1999).

In the past, FHB outbreaks have been sporadic and the disease was not considered as a major threat. However, because severe epidemics have occurred frequently during the last ten years, research on this disease has become very important in the Americas, Asia and Europe. Recent studies on the infection

pathways used by the fusaria when attacking barley and wheat heads and on the defense mechanisms of the cereal spikes that may contribute to the disease resistance are reviewed in the following sections. To indicate the complexity of the methods that these fungi may use to attack the spikes, various aspects of infection mechanism(s) are discussed. Because the focus of this work is on *Fusarium* proteinases and their inhibitors in cereal grain, the research related to fungal proteinases in various plants is reviewed to indicate how these enzymes may participate in development of FHB. The last section of this literature review covers four different types of serine proteinase inhibitors that presumably hinder microbial growth in cereal grain.

1.2 Some aspects of the infection mechanisms of FHB

Various phytopathogens use similar strategies to attack their hosts, ie. they may produce toxins or hydrolytic enzymes, or form appressoria and special penetration hyphae with which to infect the plant (Knogge 1996, Kolattukudy *et al.* 1995, Oliver & Osbourn 1995, Walton 1994). However, because most of the pathogens are specific to certain plants or plant organs, their interactions with the various hosts often vary. By understanding both the fungal infection mechanism(s) and the host defense responses that are involved, it should be possible to develop more specific strategies for combating *Fusarium* infestations. These interactions between fusaria and the spikes are not yet well-understood, but some of the factors that may be associated with the initial infection or spreading of the disease in the developing kernels are discussed in this section.

1.2.1 Infection of cereal spikes by *Fusarium*

The spikes are most vulnerable to *Fusarium* infection during anthesis, but the fungus can also attack later in the growing season if the weather is humid or rainy. Electron microscopy studies on wheat florets that were inoculated with *F. culmorum* or *F. graminearum* have indicated that the fungus can colonize the glumes, lemma and palea within 2 days post-inoculation (dpi) (Kang & Buchenauer 2000a, Pritsch *et al.* 2000). Kang & Buchenauer (2000a) showed that *F. culmorum* hyphae attacked the host via the stomatal openings and

through the inner surfaces of the glumes, lemma and palea, but the fungus was not able to penetrate the thick waxy layer on the outer surfaces of the glumes. Similar results with barley were reported by Skadsen *et al.* (2000a) who used an *F. graminearum* strain that was expressing green fluorescent protein to detect the mycelia. In this study, the preferred infection site of *F. graminearum* was the tip of the kernel, where the extruded ovary epithelial hairs provided good support for fungal proliferation and the fungus had colonized the pericarp within 2 dpi. In wheat florets, the fungus grew both intra- and intercellularly into the ovary and rachis within 6 dpi (Kang & Buchenauer 2000a). Under favorable conditions, the infection can also spread from the point-inoculated spikelet to the adjacent kernels via the rachis (Bai & Shaner 1996, Pritsch *et al.* 2001). The mycelial growth into the rachis may block the vascular system of infected heads and cause a wilting of the spikelets above the infection point (Bai & Shaner 1996). The fungus invaded the wheat floret in the same manner in both susceptible and resistant cultivars, but the hyphal growth was slower in the resistant cultivar, indicating that the resistant plants were probably reacting more efficiently to the infection and could hinder the rate of fungal invasion (Kang & Buchenauer 2000b).

The studies discussed above were carried out under optimal temperatures and humidities for fungal growth, so the kernels were probably destroyed by the fungus before the wheat endosperms were fully developed. However, the fungus does not normally grow as fast under natural conditions (dryer climate, less aggressive pathogen strain, competing microbes) and the infection may occur after anthesis. Hence, the endosperm may have time to develop more or less normally. Skadsen *et al.* (2000b) mentioned that *F. graminearum* did not invade the aleurone layer of barley kernels within 6 dpi when the heads were inoculated at the milky stage. However, the fungus can apparently grow into the endosperm. The presence of hyphae in, and destruction of, the aleurone and starchy endosperm have been described in several studies (Bechtel *et al.* 1985, Jackowiak *et al.* 2002, Narziß *et al.* 1990, Nightingale *et al.* 1999). These light- and electron microscopy studies have indicated that during the infestation of the starchy endosperm of wheat kernels by *F. graminearum or F. culmorum* the cell walls were macerated, the protein matrix disappeared and the starch granule structures were changed.

1.2.2 The role of fungal toxins in FHB

The most intensively studied aspect of the infection mechanism(s) of *Fusarium* species that cause FHB is the impact of toxins on pathogenicity or virulence. The accumulation of various trichothecenes and zearalenone in infested grain is well documented, but their occurrence in grain apparently depends both on the host and fungal genotypes, and on the environment (Evans *et al.* 2000, Perkowski *et al.* 1996, Snijders 1990).

The accumulation of toxins in infected host plants does not necessarily indicate that the toxins play an important role in pathogenesis, because their presence in the grain might just as well be a consequence of the fungal colonization. However, there are several indications that trichothecenes such as deoxynivalenol and its derivatives affect the virulence of the fusaria, even though they are not necessary for the infection and fungal growth in the grain (Atanassov *et al.* 1994, Desjardins & Hohn 1997). Firstly, two trichothecene-producing fungi, *F. graminearum* and *F. culmorum*, are generally the most pathogenic species that cause FHB (Wong *et al.* 1995). The pathogenicities of different strains of these species vary, but they are normally positively correlated with the abilities of the fungi to synthesize toxins (Atanassov *et al.* 1994, O'Donnell *et al.* 2000). Secondly, immunomicroscopy studies by Kang & Buchenauer (1999) established the presence of deoxynivalenol (DON) and some of its derivatives in wheat spikelets during the early stages of infection. Their results showed that most of the DON occurred in the proximity of the invading mycelia, but that it could diffuse into the surrounding tissues and spread through the xylem vessels and phloem sieve tubes to the neighbouring spikelets. This implied that the toxin may facilitate the invasion by weakening the host cells. Thirdly and most importantly, *F. graminearum* mutants (*Tri5*$^{-}$) that did not synthesize trichodiene synthase (EC 4.2.3.6), the first enzyme in the trichothecene synthesis pathway, caused less severe disease symptoms on infected wheat than the wild type fungus did (Desjardins *et al.* 1996). Since these mutants did not contain trichothecenes, but still caused some disease, the fungus must have other tools, in addition to these toxins that it uses to attack the host.

According to Kang & Buchenauer (2000a), *F. culmorum* seldom invaded living cells. The authors proposed that toxins produced by the *Fusarium* killed the host

plant cells, which could then be invaded. Alternatively, the infection may have caused hypersensitive reaction(s) in the host cells that led to the cell disruption. Nevertheless, weakened or dead plant cells provide readily usable nutrients for the fungus that the pest can thrive on, unless the host contains antifungal compounds or pathogenesis-related (PR) proteins in strategically proper locations. Some of the PR-proteins are induced subsequently to the infection. It is possible that the defense responses do not function properly in the presence of DON, because this toxin is able to inhibit protein synthesis (discussed in Kang & Buchenauer 2000a and 2000b). If the translation of the defense protein mRNAs were decreased, this could partially explain the results of Yu & Muehlbauer 2001, who showed that the induction of the defense response genes did not stop fungi from colonizing spikelets. This might also contribute to the aggressive appearance of the DON-producing strains, because the hyphae would be confronting only cells with weakened defenses and the invasion could proceed faster than it would against the full strength defense that would be present in the absence of DON. Kang and Buchenauer (2002) showed that the amount of PR-2 and PR-3 proteins increased in infected spikelets, so the protein synthesis does not cease totally. Still, it is not clear whether the accumulation of these and/or other PR-proteins would be faster or more abundant if DON was not present. Studying the expression and localization of PR-proteins in spikes that are inoculated with a pathogen strain that does not produce trichothecenes might help to understand the role of these toxins in fungal-plant interactions.

1.2.3 Fungal hydrolytic enzymes that degrade spike tissues

The fungi have to penetrate the cutin layer and cell walls before entering the host cells, so they probably need to produce various hydrolase enzymes to degrade these structures. The hydrolases also presumably digest the grain nutrients into forms that the fungus can utilize for its own growth. It is very difficult to study the roles of enzymes by disrupting their genes, because the fungi can often compensate for the lack or dysfunction of one enzyme with the presence or synthesis of another, similar enzyme. This section reviews some of the hydrolases that may help the *Fusarium* fungi to attack and metabolize cereal spikes.

1.2.3.1 Cutinase and lipases

Plant epidermal cells are covered by a cuticular layer that protects the cells from dehydration and against microbes. Cutin is formed of lipid polymers that are cross-linked by ester bonds. Several fungi synthesize esterase enzymes that can hydrolyze cutin (Kolattukudy 1984). It has been suggested by several researchers (Dantzig *et al*. 1986, see review by Urbanek 1989) that cutinase (EC 3.1.1.74) plays an important role in the infection of pea stems by *F. solani* f. sp. *pisi* (pea foot rot, teleomorph *Nectria hematococca*). However, Stahl and Schäfer (1992) presented contradictory results which showed that a virulent *F. solani* strain that did not produce cutinase was able to cause pea foot rot just as well as the wild type strain did. The role of cutinases in FHB have not been studied, but it has been found that the cuticle of the palea tissues of developing wheat kernel was lysed beneath the hyphae of infecting *F. culmorum*, but not elsewhere**,** indicating that cutinases may have been produced to facilitate the infection (Kang & Buchenauer 2000a).

Some lipases (EC 3.1.1.3) were produced by *Fusarium* grown in culture media that contained oils (Maia *et al.* 2001, Mase *et al*. 1995), but Boyacıoğlu & Hettiarachchy (1995) did not observe any significant differences between the fatty acid compositions of normal wheat and that of grain that was infected with *F. graminearum*.

1.2.3.2 Carbohydrate degrading enzymes

The cell walls of cereal grains are composed mainly of carbohydrates such as glucans and xylans. Kang & Buchenauer (2000c) showed, using immunomicroscopy, that cellulose, pectin and xylan components of the lemma, ovary and rachis cell walls were degraded in *F. culmorum*-infected wheat spikes. This implies that the fungus had synthesized enzymes (carbohydrolases EC 3.2.1) to degrade these components, even though the authors did not measure enzyme activities. Alternatively, the host cells may have contained hydrolytic enzymes that were released when the cells died or fungal hormone-like compounds may have induced the synthesis of host endogenous enzymes that are normally synthesized during germination. *Fusarium* spp. are known to produce various enzymes that can degrade plant cell wall polysaccharides such

as cellulose, hemicelluloses and pectin (Cheilas *et al*. 2000, Christakopoulos *et al.* 2000, Saha 2001, Urbanek 1989). *F. moniliforme* and *F. proliferatum* produced various polysaccharide hydrolyzing enzymes in maize grain (Marín *et al*. 1998), *F. graminearum* produced xylanases and cellulases in infected wheat leaves (Klechkovskaya *et al.* 1998), and increased β-glucanase and/or xylanase activities have been detected in *Fusarium*-infested barley grain (Narziß *et al.* 1990, Schwarz *et al*. 2002). The roles that the cell-wall degrading enzymes play in promoting pathogenicity in infected spikes have not been studied, but the disruption of two pectate lyase (EC 4.2.2.2) genes, *pelA* and *pelD*, in *F. solani* f. sp. *pisi* drastically reduced the virulence of that fungus in peas (*Pisum sativum*) (Rogers *et al*. 2000). The *pelA*$^{-}$/*pelD*$^{-}$ *F. solani* did not synthesize detectable amounts of lyases, but when only one or the other of the two enzymes was absent, the fungus was able to cause disease symptoms that were similar to those of the wild type fungus. On the other hand, the disruption of two endo-β-1,4-xylanase (EC 3.2.1.8) genes, *xyl3* and *xyl4*, from the tomato vascular wilt fungus, *F. oxysporum* f. sp. *lycopersici*, did not affect its pathogenicity (Gómez-Gómez *et al*. 2002). However, it appeared that the mutated fungus was still able to synthesize some other xylanases. These observations indicate that the fungi apparently need hydrolases to attack the plants, but it is difficult to prove their contributions to the infection process because the fungus can often compensate for the lack of one enzyme by utilizing another, similar, enzyme.

The starch granules in the endosperms of wheat grains that were infected by *F. culmorum* or *F. graminearum* contained grooves and cavities that implied that they had been attacked by amylolytic enzymes (EC 3.2.1) (Bechtel *et al*. 1985, Jackowiak *et al.* 2002, Nightingale *et al.* 1999). It is likely that the fungus had produced the amylases, although no one has ever detected *Fusarium* amylase activities in such situations.

1.2.3.3 Proteinases and fungal-plant interactions

Many fungi synthesize proteinases in infected plant tissues, but very little is known about their influence on pathogenesis. Some proteinases are determining factors for pathogenesis. An aspartic proteinase (EC 3.4.23.x) of *Botrytis cinerea* was not important in macerating its host's cell walls, but treatment of the fungal spores with an aspartic proteinase inhibitor, pepstatin, reduced the virulence of

the fungus in carrot roots and in many other host plants (Movahedi & Heale 1990). In this case, the proteinase caused the carrot cell death indirectly, because it functioned in conjunction with an endo-pectin lyase (EC 4.2.2.10) to release a cytotoxic compound from the host cell wall. In another case, a cysteine proteinase (EC 3.4.22.x) of *Pyrenopeziza brassicae* was closely associated with the pathogenicity of the fungus, because a proteinase-deficient mutant could not cause its associated disease, light leaf spot, in oilseed rape (Ball *et al.* 1991).

A subtilisin-like proteinase produced by the endophyte *Acremonium typhinum* has been detected in the leaves of *Poa ampla* (big bluegrass) (Reddy *et al.* 1996). Since *A. typhinum* does not cause a disease, the proteinase was not a pathogenicity factor, but it may have played an important role in allowing the fungus to survive in the tissue, eg. perhaps by suppressing plant defense responses. When roots of *Poa pratensis* (Kentucky bluegrass) were infected with the pathogen *Magnaporthe poae*, the severity of the disease symptoms correlated with the fungal subtilisin-like proteinase activity (Sreedhar *et al*. 1999). However, it was not determined whether the presence of the proteinase was a consequence or a requirement for the invasion. So, similar fungal proteinases have been observed both in symbiotic and pathogenic fungal-plant interactions. It is possible that the roles of the proteinases depend on the plant organ where the fungus attacks. Also, the substrate specificities of these fungal enzymes may vary, so that the pathogen proteinase can more readily hydrolyze proteins that, for example, are crucial for the structural integrity of the cells. However, it seems more likely that in these instances the presence of these subtilisin-like enzymes in the host tissues was not directly associated with the pathogenicity of the fungus.

Both histological and biochemical studies have indicated that grain proteins are degraded in FHB-diseased wheat kernels (Boyacıoğlu & Hettiarachchy 1995, Nightingale *et al*. 1999). *F. culmorum, F. avenaceum and F. oxysporum* all produced acid proteinase(s) in infected maize seedlings (Urbanek & Yirdaw 1978), neutral proteinase activity occurs in *F. graminearum*-infected wheat leaves (Klechkovskaya *et al*. 1998), and the degradation of storage proteins in *F. graminearum*-infected wheat was associated with an increase in the alkaline proteinase activities (Nightingale *et al*. 1999). Alkaline proteinase activities had also increased more than acid proteinase activities in *F. graminearum*-infested barley (Schwarz *et al*. 2002). The role played by the alkaline serine proteinases

in cereal grain pathogenesis has not been studied, but the disruption of a subtilisin-like enzyme gene from *F. oxysporum* f. sp. *lycopersici* did not affect its pathogenicity in tomato stems (Di Pietro *et al.* 2001). Similarly, the removal of a trypsin-like proteinase did not affect the pathogenicity of *Cochliobolus carbonum* on maize leaves (Murphy & Walton 1996). In this case, the pathogenicity of the mutant may have remained unchanged because other, compensating, proteinases were present.

The fungi may synthesize proteinases just so they can more readily digest the host proteins for the nutrition of its growing hyphae, but they may also produce the proteinases to assist the carbohydrate-degrading enzymes by hydrolyzing some of the protein components of cell walls. For example, Carlile *et al.* (2000) detected a trypsin-like proteinase in wheat leaves that were infected with *Stagonospora nodorum* (leaf and glume blotch of wheat) and this enzyme released hydroxyproline from wheat cell walls, indicating that it could participate in host cell wall degradation. It was not confirmed, however, that this function was important to the pathogenicity of the fungus.

Fusarium proteinases may also degrade host PR-proteins, and thus reduce the effectiveness of the plant's defense responses. It has been shown recently that two PR-proteins, a potato chitinase (EC 3.2.1.14) and a β-1,3-glucanase (EC 3.2.1.6), were degraded by a subtilisin-like proteinase from *F. solani* f. sp. *eumartii* (Olivieri *et al*. 2002). Also, a bean chitinase was hydrolyzed in bean roots that were infected with a virulent race of *F. solani* f. sp. *phaseoli* (Lange *et al.* 1996). In the latter case, however, it was not confirmed whether the proteinase was produced by the fungus or by the plant. It is not known whether or not similar hydrolyses can happen in cereal grain. Despite the efforts that have been expended to determine the roles of various fungal serine proteinases during infection, there is still neither proof or disproof of their importance for the development of the diseases.

1.2.4 Other factors that possibly affect FHB pathogenesis

Hydrophobins are small hydrophobic proteins that are involved in the formation of aerial mycelia and sporulation structures in fungi (Kershaw & Talbot 1998, Wessels 1997). They are pathogenicity factors in certain plant-microbe

interactions, because they facilitate the attachment of the fungus onto the waxy surface of the host plant. *Fusarium* fungi do produce hydrophobins (Kleemola *et al.* 2001), but their impact on infection has not been studied.

F. graminearum and *F. moniliforme* (whose teleomorphs are *Gibberella zeae* and *G. fujikuroi*, respectively) produce gibberellin-like compounds (Artemenko *et al.* 1999, Candau *et al.* 1992). The virulence of *F. moniliforme* in rice was positively correlated with its ability to produce gibberellin (Sunder & Satyavir 1998). Fungal gibberellins probably affect spore germination, but since they are similar to the plant hormone, gibberellic acid (GA), they may also induce the synthesis of host plant enzymes that are normally produced during seed germination to degrade endosperm cell walls, starch and proteins. Also, like plant GA (Bethke *et al.* 1999), they may induce the programmed cell death of aleurone cells. However, there is very little evidence about the roles the fungal gibberellins may play in pathogenesis (Artemenko *et al.* 1999).

1.3 The resistance of host plants to *Fusarium* attack

The resistance of a host plant to *Fusarium* depends on its morphological and biochemical traits, including various constitutive and inducible defense mechanisms. No single trait confers resistance to FHB, but a combination of various properties probably act synergistically to inhibit fungal invasions. Even though some barley and wheat cultivars show differences in their resistances to this disase, no totally immune varieties are known. The defense mechanisms used by cereals against FHB are probably complex and may differ in their various organs and during the different growth stages of the host (Mesterházy 1995, Miedaner 1997). Because breeding studies have shown that the resistance of wheat and rye to FHB is different from that to foot or crown rot (Miedaner 1997), this section will concentrate on discussing factors that are associated with the defense mechanisms that occur in cereals spikes.

1.3.1 Morphological traits and resistance

Various observations on the effects of plant morphology on disease resistance have been reviewed by Miedaner (1997) and Steffenson (2003). The spike angle

(upright or downward) and kernel density influences how rain water is retained in the spikelet and, thus, the conditions for sporulation and the attachment of mycelia to the seed surface. Two-rowed barley cultivars are generally more resistant to fusaria than six-rowed, but it is not known whether this is because of the kernel density differences in the spikelets or whether the defense mechanisms of the two-rowed cultivars are more efficient. The height, thickness and strength of a plant stem may indirectly affect its resistance to FHB, because the soil-born spores can more easily reach the heads of short or lodged stems.

1.3.2 Biochemical traits that may confer resistance to FHB

Pathogen infections, wounding or other stresses can initiate very complex chains of reactions in plants that lead to various defense responses, such as local cell death (hypersensitive reaction, HR) and/or systemic aquired resistance (SAR). These reactions can involve the production of nitric oxide, reactive oxygen species, ion fluxes, pathogenesis-related (PR)-proteins and/or of secondary metabolites such as phytoalexins and phenolic compounds. The PR-proteins are divided into families and subsequently into classes according to their biological functions and homologies (reviewed in Bowles 1990, Dixon & Lamb 1990, Kombrink *et al.* 1992, Linthorst 1991, Muthukrishnan *et al.* 2001, Osborn & Broekaert 1999, Shewry & Lucas 1997, Stintzi *et al.* 1993). The members of different PR-protein classes are often expressed in different plant organs. Many of these antimicrobial proteins are synthesized in healthy plants to serve as a primary protection against diseases and pests and are probably also needed for the normal development of the plant. These are sometimes called 'PR-like proteins' as opposed to the true PR-proteins, whose syntheses are induced by pathogens. Several antimicrobial proteins from cereal grains have been characterized, but how each of them contributes to the *Fusarium*-resistance and the occurrence of HR or SAR reactions in cereal heads has hardly been studied.

1.3.2.1 Defense proteins in barley and wheat grains

Among the defense proteins that occur in cereal spikes are β-1,3-glucanases (PR-2) and chitinases (PR-3), which hydrolyze glucans and chitin, which are major fungal cell wall components (Leah *et al*. 1991). These enzymes can also

contribute to the induction of defense responses in the host plants because they release compounds that can act as elicitors, which initiate various defense pathways. Cereal seeds also contain chitin-binding proteins (PR-4), called barwins, wheatwins and lectins (Caruso *et al.* 2001, Chrispeels & Raikhel 1991, Hejgaard *et al.* 1992, Svensson *et al.* 1992). These are antifungal and enhance the effects of other PR-proteins, but they do not have enzymatic activities. Ribosome-inactivating proteins (Coleman & Roberts 1982, Leah *et al.* 1991), defensins (formerly 'γ-thionins', Broekaert *et al.* 1995, Mendez *et al.* 1990) and possibly thionins (García-Olmedo *et al.* 1992) apparently hinder fungal growth by inhibiting protein synthesis. Like the thaumatin-like (PR-5) proteins (Hejgaard *et al.* 1991, Skadsen *et al.* 2000b), the thionins can affect the permeabilities of fungal cell membranes, but the mechanisms they use to do this are still not known. The thaumatin-like proteins from barley seeds can also bind to insoluble microbial β-1,3-glucans (Trudel *et al.* 1998). Peroxidases (PR-9, EC 1.11.1.7) participate in the lignification of plant cell walls, which helps to restrict the movement of the invading fungi (Mohammadi & Kazemi 2002). Various enzyme inhibitors, such as those that affect xylanases (McLauchlan *et al.* 1999), amylases and proteinases (García-Olmedo *et al.* 1987, García-Olmedo *et al.* 1992, Valueva & Mosolov 1999a) also may play an important role in protecting seeds. Barley seeds also contain lipid transfer proteins (LTPs), which are homologous to the antimicrobial LTPs of barley leaves, but the antifungal properties of the seed LTPs have not yet been studied (reviewed by Svendsen 1996 and Osborn & Broekaert 1999). Many of the defense proteins operate synergistically (Leah *et al.* 1991, Hejgaard *et al.* 1992, Terras *et al.* 1993). It seems likely that the accumulation of thaumatin-like proteins, chitinases, proteinase inhibitors and other defense proteins in the aleurone layers of seeds during their development is one reason why maturing grain is not as susceptible to fungal invasion as the young spikelet is.

1.3.2.2 The induction of defense responses in infected spikes

The expression of PR-1 (function not known), PR-2 (β-1,3-glucanase), PR-3 (chitinase), PR-4 (chitin-binding protein), PR-5 (thaumatin-like protein) and PR-9 (peroxidase) genes was enhanced in wheat spikelets that were inoculated by *F. graminearum* (Pritsch *et al.* 2000, Pritsch *et al.* 2001, Yu & Muehlbauer 2001). The mRNA levels of the mentioned genes were increased both in susceptible

and resistant cultivars (Wheaton and Sumai 3, respectively), but the responses of the PR-4 and PR-5 genes were stronger and faster in the resistant cultivar (Pritsch *et al.* 2000). Acidic β-1,3-glucanase (PR-2) and chitinase (PR-3) proteins accumulated in the cell walls of the lemma, ovary and rachis tissues of *F. culmorum*-infected wheat spikelets and their concentrations were higher in the resistant cv. Arina than in the susceptible cv. Agent (Kang & Buchenauer 2002). Accordingly, Li *et al.* (2001) have observed that the expression of the genes encoding (probably) these proteins was induced more rapidly in the spikes of a resistant wheat than they were in a susceptible wheat. These various results indicate that the efficient expession of these defense proteins can retard fungal growth. However, the invasion was not totally arrested in any of these cases.

The gene expression of the thaumatin-like protein called permatin (PR-5) was also enhanced in barley spikelets that were inoculated at the milky stage with *F. graminearum* (Skadsen *et al.* 2000b). Permatin is present in the aleurone layer and ventral furrow of healthy barley seeds, where it probably inhibits penetration by the fungus, but it was not determined in which tissue the permatin gene expression was affected. A susceptible wheat that was transformed with a rice thaumatin-like protein gene, and that was expressing this gene constitutively, did show increased resistance to FHB (Chen, W.P. *et al.* 1999).

In both resistant and susceptible wheats, the elevated expression of the PR-1, PR-3, PR-5 and PR-9 genes was strongest in the middle area of the spikes, where the infection had occurred, but the levels of the mRNAs coded by these genes had also increased in the upper and lower regions of the spikelets, where no fungal growth had been detected (Pritsch *et al.* 2001). This indicated that the infection could cause systemic responses in both susceptible and resistant wheat cultivars, but again the gene expression was more enhanced in the resistant cultivar. This induction mechanism was different from that obtained with a chemical, benzothiadiazole (BTH), that is known to induce the systemic expression of defense proteins (Yu & Muehlbauer 2001). BTH was able to induce the expression of defense response genes, but not the same genes that were transcribed due to the *F. graminearum* infection, and BTH treatment did not enhance the resistance of wheat to FHB. The increased levels of the PR-protein mRNAs that occurred in response to infection with *Fusarium* did not prevent the development of FHB in the infected spikelets, but the spread of the

disease proceeded more slowly in the resistant cv., implying that the proteins synthesized from these mRNAs hindered the fungal growth.

Another sign of the induction of defense responses during fungal invasion was the observation that the lignification of cell walls and formation of callose (β-1,3-glucan) was enhanced in *F. culmorum*-infected wheat spikes of the resistant cvs Arina and Frontana, but not in the susceptible cv. Agent (Kang & Buchenauer 2000b). Lignin is a polyphenolic compound whose synthesis involves peroxidases (EC 1.11.1.7) and polyphenol oxidases (EC 1.14.18.1, EC 1.10.3.1). Increased activities of these enzymes have been detected in *Fusarium*-infected wheat spikes (Mohammadi & Kazemi 2002). Also, El-Gendy *et al.* (2001) have observed the deposition of certain small (4–9 kD), acidic, glycine- and serine-rich proteins (GSRPs) into the cell walls of a wheat callus culture soon after it was treated with filtered and sterilized *F. graminearum* culture medium. This reaction was also probably associated with the expression of peroxidase activity, because the presence of H_2O_2 and peroxidase was required for the deposition of the GSRPs (El-Gendy *et al.* 2001).

1.3.2.3 The detoxification of DON in cereal kernels

Deoxynivalenol (DON) and its derivatives are toxic to plant cells and the DON-producing fusaria are generally more aggressive than the strains that do not synthesize the toxin. Any mechanisms that can reduce the effects of DON in the spikes, such as detoxification by the degradation or binding of the DON, or by increasing the tolerance of the host cell to it, should increase the resistance (Miedaner 1997). This presumption is supported by the finding that DON was metabolized more efficiently by the cultured cells of the *Fusarium*-resistant wheat cv. Frontana than by those of the susceptible cv. Casavant (Miller & Arnison 1986). In addition, immunomicroscopy has shown that infected spikelets of the resistant cvs. Frontana and Arina contained lower concentrations of DON than those of the susceptible cv. Agent (Kang & Buchenauer 2000b). The authors proposed that the increased lignification and callose formation that is seen in the cell walls in resistant wheat cultivars may reduce the diffusion of DON in the tissues.

1.3.3 Problems in developing FHB-resistant cultivars

The resistance of cereals to FHB depends on several traits that are not necessarily genetically linked (Miedaner 1997). Some potential resistance factors have been identified, but their roles in the complete defense system in spikes are not well understood (section 1.3.2). The fact that it is not totally clear which traits in plants would be most effective in combating *Fusarium* infections and that there are problems associated with the breeding methods make it very challenging to develop *Fusarium*-resistant cereal cultivars. There are sources of resistance to FHB in both cultivated cereal species and in land races, but the problem is how to combine this resistance with good agronomic properties in the plant (Bai & Shaner 1994, de la Pena *et al.* 1999, Gagkaeva *et al.* 2002, Gilbert & Tekauz 2000, Mesterházy 2002, Miedaner 1997, Steffenson 2003). In conventional breeding, the poor agronomic and quality attributes of the resistant plant are usually inherited by the progeny and the resistance genes may be lost in back-crossings. In theory, genes can be transferred specifically by molecular breeding, but as discussed earlier, multiple defense properties are needed to effectively combat the FHB. Even if it was known which genes needed to be transformed in order to increase the resistance, it is still very hard and time-consuming to transform genes into an agronomically good variety and to get the stable expression of the gene products (Dahleen *et al.* 2001). Thus, methods in both conventional breeding and genetic modification need to be developed, so that it will be possible to combine the resistance traits into agronomically profitable cultivars that are suitable for end product processing, eg. malting and brewing.

1.4 The serine proteinase inhibitors of barley

Cereal grains contain certain proteins that inhibit various exogenous proteinases from mammals, insects and microbes. The main serine proteinase inhibitors that are present in barley seeds are the chymotrypsin/subtilisin inhibitors 1 and 2 (CI-1 and 2), a bifunctional barley α-amylase/subtilisin inhibitor (BASI), a barley Bowman-Birk type trypsin inhibitor (BBBI), some trypsin/α-amylase inhibitors (CM proteins) and serpins (Shewry 1999, Carbonero & García-Olmedo 1999). These inhibitors are categorized into families (Table 1) according to their amino acid and nucleotide sequence homologies, but their inhibitory properties are

determined by their reactive site amino acids. Hence, each inhibitor family may contain members that have different enzyme specificities. The CI, BASI and BBBI proteins strongly inhibit microbial proteinases, but the CM proteins, CMe and CMc, that have been shown to inhibit proteinases only affected trypsin (EC 3.4.21.4) molecules from bovines and from some insects, not those from microbes (Shewry 1999, Carbonero & García-Olmedo 1999). Even though barley CM proteins apparently do not inhibit microbial proteinases, they have been shown to inhibit the growth of fungi (Terras *et al.* 1993), which is why they are briefly discussed in this section. Serpins inhibit bovine, but not microbial, serine proteinases (Shewry 1999), and have never been shown to posess antifungal activity, so they have not been considered further in this thesis. BASI inhibits the endogenous α-amylase 2 (EC 3.2.1.1) of barley, but only very recently has it been demonstrated that any of these inhibitors (some of the CM proteins and CI-2A) can inhibit endogenous barley proteinases (personal communication, Dr. Berne L. Jones).

All of these inhibitors occur abundantly in various tissues of the barley grain and it has been proposed that they may defend the seeds against pathogens (Ryan 1990, Svendsen 1996, Valueva & Mosolov 1999b). However, their inhibitory properties have generally only been characterized by testing their abilities to curb the activities of commercial bovine or microbial enzymes such as subtilisin (EC 3.4.21.62) or oryzin (EC 3.4.21.63). Little or no research has been done to determine how they interact with and affect the proteinases that occur in relevant plant pathogens. Even though most of these inhibitors do not seem to inhibit endogenous barley proteinases, they may have functions that have not been discovered yet. A few serine proteinases (EC 3.4.21) have been observed in germinating barley (Zhang & Jones 1995). More of these enzymes might be synthesized in barley seeds, but like the cysteine proteinases (Jones 2001), their activities might not all be detectable if they bind to endogenous inhibitors as soon as the ground grain is suspended in buffer. The main characteristics of the serine proteinase inhibitors are reviewed in the next few sections. Also, similar inhibitors from other *Triticeae* (rye, wheat, triticale) and, in some cases, examples from other cereals (oat, sorghum, rice and maize) are briefly discussed. A summary of the barley grain inhibitors is presented in Table 1.

Table 1. The major serine proteinase inhibitors in barley seeds.

Inhibitor (Family)	Location[a]	Enzyme blocked	Reference
CI-1 (Potato inhibitor I)	SE, AL	Chymotrypsin, oryzin, subtilisin, alkaline proteinases of *Alternaria tenuissima* and *Streptomyces griseus*, neutrophil elastase	Mikola & Suolinna 1971, Greagg *et al.* 1994 Mundy *et al.* 1986, Jakobsen *et al.* 1991
CI-2 (Potato inhibitor I)	SE, AL	Chymotrypsin, subtilisin Pancreatic and neutrophil elastase	Hejgaard 1981, Longstaff *et al.* 1990 Greagg *et al.* 1994 Rasmussen 1985, Mundy *et al.* 1986
BASI (Kunitz)	SE, AL, EM	Subtilisin (strongly), some *Aspergillus* proteinases (weakly) Endogenous barley α-amylase 2	Yoshikawa *et al.* 1976 Mundy *et al.* 1983, Weselake *et al.* 1983 Jensen 1994, Hill *et al.* 1995
BBBI (Bowman-Birk)	AL, EM	Bovine and bacterial trypsins	Boisen & Djurtoft 1982
CMe (Cereal proteinase/ α-amylase)	endosperm	Bovine trypsin Some insect trypsins and α-amylases	Mikola & Suolinna 1969 Alfonso *et al.* 1997 Mikola & Kirsi 1972
BZ4, BZ7 (Serpin)	endosperm	Bovine chymotrypsin and trypsin	reviewed by Shewry 1999

[a] SE: starchy endosperm, AL: aleurone, EM: embryo, endosperm (SE+AL)

1.4.1 Chymotrypsin/subtilisin inhibitors (CI-1 and -2)

CI-1 and CI-2 are closely related proteins that belong to the potato inhibitor I family (Svendsen *et al.* 1980, Svendsen *et al.* 1982). The molecular masses of the intact forms of the CI proteins are approximately 9 kDa but, at least *in vitro*, each of these inhibitors may be N-terminally cleaved, which results in their having lower molecular masses, and they may form dimers or trimers (Boisen *et al.* 1981, Williamson *et al.* 1987, Williamson *et al.* 1988). When subjected to isoelectric focusing they separate into two groups that have pI values of 4.5–5.5 and 6–8 (Hejgaard 1981). The slightly acidic group is comprised of three highly homologous inhibitors, called CI-1A, -1B and -1C, and the neutral group contains various size forms of CI-2A (Boisen *et al.* 1981, Svendsen *et al.* 1980, Svendsen *et al.* 1982). The mRNA of CI-2B has been isolated, but its protein product has never been purified or characterized (Williamson *et al.* 1987). Unlike many other proteinase inhibitors, but like many other of the potato inhibitor 1 family, CI-1 and -2 do not contain any cysteine (Williamson *et al.* 1987, Williamson *et al.* 1988). Still, CI-2A is relatively heat stable, even though CI-1 is inactivated quickly at 100 ºC (Boisen *et al.* 1981). Despite the similarities of their amino acid sequences, the acidic and neutral CI groups have different immunochemical properties (Boisen *et al.* 1981, Hejgaard 1981).

Both CI-1 and 2 are slow, tight-binding type inhibitors of bacterial subtilisin and have K_i values at the nano- and picomolar levels, respectively (Greagg *et al.* 1994, Longstaff *et al.* 1990). CI-2 has one reactive site, Met^{59}-Glu^{60}, for both chymotrypsin (EC 3.4.21.1) and subtilisin, but it binds to chymotrypsin about 10^3 times more weakly than to subtilisin (Jonassen & Svendsen 1982, Longstaff *et al.* 1990). X-ray crystallographic studies have shown that CI-2 is a wedge-shaped disk with its reactive site loop on its narrow side (McPhalen *et al.* 1985). It binds to the active site cleft of subtilisin as a substrate, but either the peptide bond cleavage and the dissociation of the reaction products from the enzyme is very slow, or else cleavage does not occur at all (Jonassen & Svendsen 1982, McPhalen *et al.* 1985). The fact that there is a loop structure at the reactive site of CI-2 is important for the inhibition, because a cyclic peptide comprised of the 18 amino acids that correspond to its reactive site loop bound to subtilisin as tightly as the intact inhibitor, while a linear peptide of the same amino acids was not inhibitory (Leatherbarrow & Salacinski 1991). CI-1 has one reactive site at the peptide bond Leu^{59}-Asp^{60}, where chymotrypsin binds, but subtilisin can also

bind at Met^{30}-Ser^{31} (Jonassen & Svendsen 1982). Surprisingly, despite the fact that CI-1 has two reactive sites for subtilisin, CI-2 still inhibits it more strongly than CI-1. The explanation for this is revealed from their molecular structures. Two of the Arg residues of CI-2 (Arg^{65} and Arg^{67}) apparently stabilize the inhibitor-enzyme complex by forming hydrogen bonds with the reactive site residues of the enzyme (McPhalen *et al.* 1985). In CI-1, however, the Arg^{67} is replaced by a hydrophobic Phe-residue which possibly weakens its binding properties. Like CI-2, CI-1 inhibits chymotrypsin more weakly than it does subtilisin (Greagg *et al.* 1994). It has been shown that CI-1 does inhibit the alkaline proteinase of a plant pathogen, *Alternaria tenuissima* (Mikola & Suolinna 1971).

The CIs are synthesized in the aleurone layer and starchy endosperm tissues (Jakobsen *et al.* 1991, Mundy *et al.* 1986, Rasmussen 1985). Barley embryos, young rootlets and shoots also contain inhibitors of chymotrypsin and microbial proteinases, but their isoelectric focusing elution patterns indicated that they were different from the endospermal forms (Kirsi & Mikola 1977). This was supported by mRNA transcription studies that revealed that a homologous, but larger, gene than that of CI-2 was expressed in shoots and young leaves (Williamson *et al.* 1987). The presence of CI-1 in embryos has not been studied, but immunofluorescent studies have shown that there was no CI-2 in embryos (Rasmussen 1985). Neither CI-1 nor CI-2 has a signal peptide sequence at its N-terminus and their locations in cell organelles have been disputed (Williamson *et al.* 1987, Williamson *et al.* 1988). However, the deposition of CI-2 in the vacuoles of endosperm cells has been shown by immunomicroscopy (Rasmussen *et al.* 1990). This synthesis starts approximately two weeks after anthesis in the endosperm and continues for one to two weeks, depending on the barley cultivar or line (Kirsi 1973, Rasmussen *et al.* 1988, Williamson *et al.* 1988). The inhibitory activity disappeared during germination (Kirsi & Mikola 1971). The inhibitors may have been degraded by proteinases that were synthesized by barley during germination, but it is possible that they bound to serine proteinases during the extraction process in the same way the cysteine proteinase inhibitors do (Jones 2001).

Because these inhibitors contain more lysine than other storage proteins, it has been proposed that by increasing their concentrations in cereals, the nutritional quality of grain could be improved (reviewed by Shewry *et al.* 1994). On the

other hand, high concentrations of the inhibitors may interfere with the digestion of proteins by mammals, because they also inhibit chymotrypsin. In Hiproly barley, their expression has been increased 15- to 20-fold (mutation in the *lys 1* gene) and in the Bomi mutants Risø 1508 and 56, it has been boosted by 2.5- to 4-fold (mutation in *lys 3a*) (Boisen *et al.* 1981, Jakobsen *et al.* 1991, Rasmussen *et al.* 1988, Williamson *et al.* 1987, Williamson *et al.* 1988). Whether or not the increased expression of the CIs in these mutated cultivars has increased their resistance to FHB has not been studied; possibly because their agronomic characteristics are so poor.

Other cereals contain subtilisin inhibitors that may be related to the barley CI-proteins (Hejgaard 1981), but very little is known about them. A chymotrypsin/subtilisin inhibitor with a Met residue in its reactive site has been purified from wheat endosperm tissues (Khludnev *et al.* 1992). This inhibitor may be related to CI-2, because an antibody raised against barley CI-2, but not that prepared against CI-1, recognized two proteins in wheat (Hejgaard 1981). Three inhibitors of bovine chymotrypsin have been isolated from sorghum but, unlike the CI-proteins, they also inhibited trypsin (Kumar *et al.* 1978). Several chymotrypsin inhibitors have been detected in extracts prepared from oats (Mikola & Mikkonen 1999). However, either these do not inhibit subtilisin or they inhibit it only weakly, because in another study only a very weak inhibition of subtilisin was observed in oats (Hejgaard 1981).

1.4.2 Barley α-amylase/subtilisin inhibitor (BASI)

Yoshikawa *et al.* (1976) purified a protein from barley grain that strongly inhibited the activity of subtilisin BPN', a *Bacillus* proteinase, but which, unlike CI-1 and -2, did not inhibit bovine chymotrypsin. It also weakly inhibited proteinases from *Aspergillus sulphureus*, *A. sydowi* and *Streptomyces griseus* (Yoshikawa *et al.* 1976). The same protein was later purified and characterized by other reseachers because of its ability to inhibit barley α-amylase 2 (Mundy *et al.* 1983, Weselake *et al.* 1983) and was named barley α-amylase/subtilisin inhibitor (BASI) (Hejgaard *et al.* 1983, Mundy *et al.* 1983). BASI also inhibits some of the α-amylases of wheat, rye and oats, but not those of sorghum and rice (Mundy *et al.* 1984). The primary structure of BASI shows homology to that of soybean trypsin inhibitor, so it is categorized into the Kunitz inhibitor family

(Hejgaard *et al.* 1983, Svendsen *et al.* 1986). However, it did not inhibit bovine trypsin (Yoshikawa *et al.* 1976). Unlike the trypsin/α-amylase inhibitors, BASI does not inhibit α-amylases from mammals, insects or microbes (Mundy *et al.* 1983, Mundy *et al.* 1984).

BASI synthesis begins at an early stage of seed development. Detectable levels of BASI mRNA or protein are present approximately two weeks post-anthesis in the starchy endosperm and embryo tissues (Hill *et al.* 1995, Leah & Mundy 1989, Robertson & Hill 1989). The BASI concentration of the grain increases rapidly until four weeks after pollination, when it reaches a maximum (Munck *et al.* 1985, Robertson & Hill 1989). It is stored in protein bodies, from which it is released and degraded during germination (Hill *et al.* 1995). Contradictory results have been obtained about the presence of BASI in the aleurone layer, depending on the experimental methods that have been used. BASI mRNA has been detected in the aleurone layer of cv. Bomi, but not in that of its mutant, Risø 1508, (Jakobsen *et al.* 1991) or in other cultivars (Leah & Mundy 1989, Mundy & Rogers 1986, Mundy *et al.* 1986). However, the presence of BASI in the aleurone layers of resting and germinating seeds of three different barley cultivars has been shown with immunomicroscopy (Jensen 1994). In a second immunomicroscopic study anti-BASI immunolabel was visible in barley aleurone layers, although this was not specifically discussed (Hill *et al.* 1995, personal communication, Dr. Robert. D. Hill). Hence, it seems that BASI is produced in, or transported into, the aleurone cells during seed development. It is possible that the BASI mRNA concentrations drop below detectable levels while the aleurone layers are being collected for analysis, since BASI mRNA was present in cultured aleurone cells (Leah & Mundy 1989, Mundy & Rogers 1986, Mundy *et al.* 1986). Possibly, only certain cvs express BASI in their aleurone layers (Jakobsen *et al.* 1991). BASI has not been detected in the vegetative tissues of barley, except in seedlings that are undergoing dehydration stress (Leah & Mundy 1989, Robertson & Hill 1989, Robertson *et al.* 1989).

The addition of exogenous absisic acid (ABA) increased the BASI contents of cultured embryo (Liu & Hill 1995, Robertson *et al.* 1989) and aleurone cells (Leah & Mundy 1989, Mundy & Rogers 1986, Mundy *et al.* 1986). The effect of ABA on BASI synthesis in embryos is apparently due to an increase in the stability of the mRNA (Liu & Hill 1995). BASI synthesis in cultured aleurone cells was reduced by a gibberellic acid (GA_3) in the absence of ABA (Leah &

Mundy 1989, Mundy 1984, Mundy & Rogers 1986, Mundy *et al.* 1986). This is in agreement with immunohistochemical studies that showed that the BASI content of the aleurone layer decreased at the beginning (1–3 d) of germination (Jensen 1994). Possibly the endogenous BASI was degraded or bound by α-amylase 2 and *de novo* synthesis was prevented by the presence of GA. The amount of anti-BASI immunolabelling increased during the later stages of germination (Jensen 1994), but it is unclear whether this was due to *de novo* synthesis (Leah & Mundy 1989) or to the release of BASI from protein vacuoles (Hill *et al.* 1995). Because of its hormonal regulation and its location in the grain near the starch granules, it has been proposed that BASI participates in the controlling of germination and possibly prevents preharvest sprouting (Hill *et al.* 1995, Leah & Mundy 1989, Liu & Hill 1995, Mundy 1984, Mundy *et al.* 1983, Robertson & Hill 1989). However, Munck *et al.* (1985) did not find any correlation between the tendency for precocious germination and the BASI contents of grains.

BASI may also protect the seed proteins from hydrolysis by microbial proteinases (Leah & Mundy 1989, Mundy 1984, Mundy *et al.* 1983). It would be intriguing to study whether BASI synthesis is induced in the aleurone layer when the fungus invades the grain in the same way it is induced in seedlings by dehydration stress. The induction of BASI in dehydrated seedlings is mediated by ABA (Robertson *et al.* 1989). However, some other factor is probably needed to trigger the BASI mRNA synthesis, because the addition of ABA does not increase the BASI mRNA transcription rate, but only its stability (Liu & Hill 1995). The BASI contents of different cultivars varied by two- to five fold in studies that were carried out in Denmark (Munck *et al.* 1985), Canada (Masojc *et al.* 1993) and Australia (Jarrett *et al.* 1997). In these three different studies the BASI concentrations varied between 50 and 500 µg per g of grain. The BASI gene expression in the high-lysine barleys Risø 1508 and Piggy (*lys 3a* mutants) and Hiproly (*lys1* mutant) was not affected as much as was that of CI-1 and -2 (Leah & Mundy 1989, Rasmussen *et al.* 1988). Whether the BASI concentrations of barleys correlates with their resistances to microbes has not been studied.

Proteins that inhibit both subtilisin and endogenous α-amylases are present in wheat, triticale and rye (Mundy *et al.* 1984, Zawistowska *et al.* 1989, Weselake *et al.* 1985, Mosolov & Shul'gin 1986). They are all, like BASI, heat-labile,

approximately 20-kDa proteins, with isoelectric points (pI) close to neutrality (Mosolov & Shul'gin 1986, Mundy *et al.* 1984, Weselake *et al.* 1983, Zawistowska *et al.* 1989) and they cross-reacted with an antibody that was raised against BASI (Weselake *et al.* 1985). Rice contains a subtilisin inhibitor (RASI) with a pI of 9, but it only inhibits the endogenous rice α-amylase weakly (Yamagata *et al.* 1998). Neither RASI nor potentially similar inhibitors in other cereals responded to a BASI antibody (Weselake *et al.* 1985). The inhibitors from barley, wheat, rye and triticale all inhibited fungal proteinases, but not as strongly as they affected the bacterial subtilisins (Mosolov & Shul'gin 1986, Mundy *et al.* 1984, Yoshikawa *et al.* 1976). A protein that bound to bacterial subtilisin and that was slightly larger than BASI was present in oat grain extracts (Hejgaard & Hauge 2002).

1.4.3 Barley Bowman-Birk inhibitor (BBBI)

The barley Bowman-Birk inhibitor (BBBI) contains amino acid sequences that are homologous with those of the double-headed trypsin-chymotrypsin inhibitors that occur in various legumes and grasses (Nagasue *et al.* 1988, Prakash *et al.* 1996). Like the 8-kDa soybean BBI (SBBI), which also inhibited several microbial proteinases (Marchetti *et al.* 1995), BBBI strongly inhibited both bovine and bacterial trypsins (Boisen & Djurtoft 1982). BBBI is a single chain protein that contains two homologous domains that probably resulted from the duplication of an ancestral gene (Nagasue *et al.* 1988, Prakash *et al.* 1996). The two domains can simultaneously bind two trypsin molecules (Nagasue *et al.* 1988, Song *et al.* 1999). Although some of the duplicated BBIs have lost their reactive sites that bind chymotrypsin (Prakash *et al.* 1996), the BBBI inhibited bovine chymotrypsin weakly (Boisen & Djurtoft 1982).

BBBI is present in barley embryos, aleurone layers, rootlets and possibly in the coleoptiles, but not in the older vegetative tissues (Boisen & Djurtoft 1982, Nagasue *et al.* 1988, Kirsi & Mikola 1971). Boisen & Djurtoft (1982) purified two BBBI isoforms with molecular masses of about 16 kDa from barley embryos. The molecular mass that was calculated from the amino acid sequence of the rootlet inhibitor was approximately 13.8 kDa (Nagasue *et al.* 1988). These masses probably differ because they were determined using different analysis methods and the embryonal and rootlet inhibitors are apparently the same

protein (Nagasue *et al.* 1988, Kirsi 1974). BBBI contains ten disulfide bridges that are highly conserved among the various Bowman-Birk type inhibitors and they probably confer to the protein its resistance to heat and to pepsin (EC 3.4.23.1) treatments at pH 2 (Boisen & Djurtoft 1982, Nagasue *et al.* 1988, Prakash *et al.* 1996, Song *et al.* 1999).

Trypsin inhibitors have been purified from wheat and rye embryos (Hochstrasser & Werle 1969), but only the wheat embryo proteins have had their amino acid sequences determined (Odani *et al.* 1986). However, the wheat and rye embryonal inhibitors are probably both very similar to the barley inhibitor, because an antibody raised against BBBI cross-reacted with proteins extracted from the wheat and rye embryos (Boisen & Djurtoft 1982). Only the double-domain inhibitor has been detected in barley, but wheat embryos contain both single- and double-domain inhibitors, which have molecular masses of 7 and 14 kDa, respectively (Odani *et al.* 1986). Boisen & Djurtoft (1982) reported that BBBI was present in aleurone cells, but no trypsin-chymotrypsin inhibitor has ever been detected in barley starchy endosperm tissues. An 8.5-kDa inhibitor from wheat endosperm, whose N-terminal amino acid sequence was about 50% identical with that of several other Bowman-Birk type inhibitors from various plants, inhibited only trypsins from various sources (Chilosi *et al.* 2000, Poerio *et al.* 1989). Both rye and wheat endosperms contained trypsin inhibitors that also affected chymotrypsin (Boisen & Djurtoft 1981a and 1981b), but these have previously been categorized into the trypsin/α-amylase inhibitor group and will be discussed later.

In addition to their abilities to strongly inhibit microbial proteinases, the wheat and barley BBIs can reduce the growth rates of various plant pathogenic fungi (Chilosi *et al.* 2000, Terras *et al.* 1993). High trypsin inhibitor levels in wheat grain have been correlated with resistance to diseases that are caused by *Fusarium* spp. (Klechkovskaya *et al.* 1998) and the loose smut fungi, *Ustilago* spp. (Yamaleev & Ibragimov 1986), but it is not known whether these were Bowman-Birk or trypsin/α-amylase inhibitor types, or both. The Bomi mutant Risø 1508 contains from two to four times more BBBI than normal barleys (Boisen & Djurtoft 1982), but it is not known if the mutant line is more resistant to *Fusarium* infestation than the motherline. Even if the BBBI could potentially protect barley against microbes, there might be a disadvantage in using it for

improving the disease resistance of grains, because high concentrations of BBBI, or of the similar inhibitors from other cereals, may cause nutritional problems. This is exacerbated by the fact that they are very resistant to denaturation by heating and by pepsin (Boisen & Djurtoft 1982).

1.4.4 Trypsin/α-amylase inhibitors (CM proteins)

CM proteins belong to the cereal proteinase/α-amylase inhibitor family. The general physical, chemical and genetic properties of these inhibitors have been reviewed previously (García-Olmedo *et al.* 1987 and 1992, Carbonero & García-Olmedo 1999), so only those details that relate to the possibility of their serving to defend plants against microbes and insects are discussed in this review. These 10-15 kDa proteins, which can occur as either monomers or multimers in solution, are synthesized in the grain endosperm during the grain filling period. Generally, they inhibit exogenous α-amylases from various sources, and a few of them also inhibit bovine and insect trypsins. However, not all of the inhibition properties of the various forms have been determined in detail and their inhibitory properties cannot be generalized on the basis of the homologies that occur among their amino acid sequences. The interactions of these inhibitors with enzymes from various sources sometimes are very specific (Feng *et al.* 1991, Moralejo *et al.* 1993). The strongest trypsin inhibitor amongst the barley CM proteins is the 13-kDa monomer CMe. Its mobility during size exclusion chromatography differs from that of the embryo BBBI and antibodies against BBBI do not cross-react with the trypsin inhibitor(s) from barley endosperm, and vice versa (Boisen & Djurtoft 1982, Mikola and Kirsi 1972). No inhibition of microbial proteinases by CMe has ever been detected (Mikola & Suolinna 1969).

A trypsin inhibitor whose amino acid sequence was 76% identical to that of barley CMe has been purified from rye endosperm tissue (Lyons *et al.* 1987). There is a trypsin inhibitor in wheat endosperm that has properties that are very similar to those of a rye trypsin inhibitor, eg. both proteins also weakly inhibit chymotrypsin (Boisen & Djurtoft 1981b). However, the wheat endosperm trypsin inhibitor was immunochemically different from those in barley or rye. Even though antibodies raised against the wheat endosperm trypsin inhibitor did not cross react with CMe, three wheat genes that were homologous to barley

CMe were expressed in developing wheat endosperms (Sanchez de la Hoz *et al.* 1994). The proteins coded by these genes were not expected to inhibit trypsin, because their reactive site amino acids were different from those of CMe.

Terras *et al.* (1993) showed that CMe and two Bowman-Birk type inhibitors all have some antifungal properties, but that these properties were probably due to the proteins rendering the hyphal plasmalemma permeable. A 14-kDa trypsin inhibitor from maize, whose N-terminal amino acid sequence was homologous to that of the barley CMe, was associated with resistance to the fungus *Aspergillus flavus* (Chen, Z.-Y. *et al.* 1998). It also inhibited the germination of spores and mycelial growth of various plant pathogens that were grown in potato dextrose broth (Chen, Z.-Y. *et al.* 1999a). However, the inhibitor did not affect the fungal growth if bacterial α-amylase was added to the potato dextrose broth or when the fungus was grown on a protein-containing (gelatin) medium, implying that the effect was probably due to its ability to interfere with the fungal α-amylase activity (Chen, Z.-Y. *et al.* 1999b). Thus, CMe and similar proteins in other cereals may have a role in plant defense, but it seems unlikely that their effect on fungi is related to proteinase inhibition. On the other hand, the function played by CMe in defending plants from insects may be due to the inhibition of insect proteinase(s) or of α-amylase, or both, because CMe inhibited both the trypsin-like proteinase and the α-amylase of *Spodoptera frugiperda* (fall armyworm) (Alfonso *et al.* 1997).

The prospect of using trypsin/α-amylase inhibitors to increase the resistance of plants to insects seems promising, because the resistance of wheat seeds to moth larvae (*Sitotroga cerealella*) has been slightly increased by expressing the barley CMe protein in wheat plants (Altpeter *et al.* 1999). However, some of the larvae developed a resistance to the inhibitor. There are no examples of the resistance of cereals to fungal diseases being increased by the genetic modification of CMe or of any other CM-proteins. Caution should be exercised in increasing the expression of these inhibitors in foods, because some of them are potent allergens that may cause baker's asthma (Sanchez-Monge *et al.* 1992).

1.5 The aims of this study

There are two hypotheses that have formed the background for this study. The first presumption was that *Fusarium* spp. produce proteinases in infected grains and that these enzymes actively participate in helping the fungus to penetrate the host plant tissues (as a pathogenicity or virulence factor) and/or that they degrade the host grain proteins so they can be used for the nutrition of the growing mycelium (a saprophytic role). The second hypothesis was that the inhibitors of microbial proteinases that occur in cereal grains may protect the seed from microbial attack by preventing the hydrolysis of cell walls and/or of other grain proteins (Ryan 1990, Svendsen 1996, Valueva & Mosolov 1999b). The aims of this study were: 1) to identify and characterize the proteinases that are synthesized by *Fusarium* fungi *in vitro* when the fungi are grown in media that contain cereal proteins, 2) to determine whether similar proteinases are produced in *Fusarium*-infected field-grown barley kernels and 3) to detect, purify and identify any barley proteins that inhibited the *Fusarium* proteinases *in vitro.*

2. Materials and methods

Fungi:
All of the *Fusarium* species, *F. culmorum* (strain VTT-D-80148), *F. graminearum* (VTT-D-95470) and *F. poae* (VTT-D-82182), that were used throughout the project were obtained from the Culture Collection of VTT (VTT Biotechnology, Espoo, Finland). The fungi were maintained on potato dextrose agar (PDA, Difco) as described in article I.

Barley:
A two-rowed Finnish malting barley (*Hordeum vulgare* L.) cultivar Kymppi was used for preparing grain medium that was used to study *Fusarium* proteinase production, two-rowed malting cvs Mentor (Swedish) and Saana (Finnish) were used for the field experiments and the proteinase inhibitors were purified from the U.S. six-rowed malting cv. Morex.

Table 2. The main experimental procedures that were used in the studies.

Procedure	Description	Article
Culture media for testing proteinase production	- Mineral-glucose medium, (starting pH 4.5) - Gluten-glucose medium, (starting pH 5.0) - Grain medium (autoclaved and moistened barley cv. Kymppi, starting pH 5.7) - 21 ºC, 12 h light/dark periods	I
Glucose measurement Proteinase activity assays	- Glucose oxidase kit (Boehringer-Mannheim) - Hemoglobin, pH 2.2–5.5, 40 ºC - Azogelatin, pH 5.0–10.5, 40 ºC - SAAPF*p*NA, pH 8.0, 37 ºC	
Fusarium culture used for the proteinase production	Gluten-glucose medium, pH maintained at ~5, 21 ºC	II
Proteinase purification by chromatography	- Size exclusion (Bio-Gel P30) and CMC, pH 5 - CM-HPLC, pH 8	II, III
Activity assays during the proteinase purification	- Azogelatin and SAAPF*p*NA*, pH 9	II*
Molecular size and mass analyses	- SDS-PAGE - MALDI-TOF (University of Wisconsin, Madison, WI, USA)	II, III, V

N-terminal amino acid sequence analyses	Edman degradation (University of Texas Medical Branch, Galveston, TX, USA) and comparison of the results to the NCBI (http://www.ncbi.nlm.nih.gov) and SWISSPROT/TrEMBL protein databases (http://ca.expasy.org)	II, III, V
Determination of the mechanistic classes and families of the proteinases	- PMSF, *p*APMSF, CST, STI, SBBI, E-64, EDTA, *o*-phenanthroline, pepstatin A; azogelatin substrate, pH 6, 40 ºC - CST, STI; SAAPF*p*NA or BVGR*p*NA substrate, pH 6, 28 ºC	II, III
Substrate 'specificities' using synthetic substrates	Various *p*-nitroanilide peptide derivatives, pH 9, 28 ºC	II, III
Measuring the kinetics of the proteinases	SAAPF*p*NA or BVGR*p*NA at 28 ºC and azogelatin at 40 ºC, pH 6 and 9	II, III
Substrate specificity analysis using purothionin as substrate	Reduced and pyridylethylated β-purothionin digestion at pH 6 and 40 ºC, partial purification of the products by RP-HPLC and product identification by MALDI-TOF	III
Hydrolysis of barley protein classes by the fungal proteinases	- Albumins and globulins extracted from barley cv. Mentor - Hordeins extracted from cv. Morex - Digestion of proteins at pH 6 and separation of products by SDS-PAGE	III, IV
Detection of the proteinases from inoculated, field-grown barley kernels	- Western blotting: rabbit antibodies raised against purified *Fusarium* proteinases - Activities: SAAPF*p*NA or BVGR*p*NA at 25 or 28 ºC, azogelatin at 40 ºC, pH 9	IV
Purifying the barley grain inhibitors by chromatography	- Size-exlusion (Bio-Gel P30) - CMC, pH 5 - QAC, pH 8 - RP-HPLC	V
Assays for detecting the *Fusarium* proteinase inhibitors in barley extracts	Measured inhibition of *Fusarium* proteinases; SAAPF*p*NA and BVGR*p*NA substrates, pH 6	V
Quantifying the inhibitors	Absorbance at 280 nm, calculated ε	V

3. Results and discussion

3.1 The *in vitro* production of *Fusarium* endoproteinases (I)

In general, the production of proteinases by fungi that are grown in artificial culture media has been studied in order to produce enzymes for industrial purposes (food processing, cleaning agents, etc.) or to investigate how various pathogens synthesize their proteinases. The advantage of studying the production of enzymes *in vitro* is that other microbes and/or host endogenous enzymes that might be present in the living host plant will not disturb the enzyme analyses or interfere with the identification of the fungal enzymes. However, the conditions in the culture medium are obviously going to differ from those in the natural environment. This means that if one uses such methods to study the enzymes of pathogens in plant hosts, the presence of any pathogen enzymes detected *in vitro* must be confirmed in infected plants and their absence from healthy plants should also be demonstrated.

To investigate which types of proteinases fusaria synthesize during their invasion of barley grains, *F. culmorum*, *F. graminearum* and *F. poae* were grown in two media that contained cereal proteins. One was a suspension medium that contained glucose, minerals and wheat gluten ('gluten medium'), and the other was comprised of autoclaved and moistened barley grain ('grain medium'). The gluten medium was a modification of the Armstrong medium, which contained only glucose and mineral salts, but no protein or free amino acids. To compare the effect of nitrogen source (protein vs. nitrate) on proteinase production, the fungi were also grown in unmodified Armstrong medium ('mineral medium'). Because our aim was to study excreted proteinases, the mycelia were removed from the suspension culture samples by centrifugation and the supernatant was used for the analyses. The grain medium samples were freeze-dried and ground, and soluble protein extracts were prepared from the ground material. The proteinase activities of the growth media and grain extracts were measured at pH 2 using hemoglobin substrate and at pH 5 and 8 using azogelatin. These pH values were chosen because *F. culmorum* had synthesized proteinases that had pH optima of ~2 in maize seedlings (Urbanek & Yirdaw 1978) and proteinases that were maximally active at pH 5 or 8 were produced by

F. graminearum when it was grown in a glucose-casein medium (Griffen *et al.* 1997).

The pH values of the growth media were monitored throughout the cultivation process and the growth of the fungi in the suspension media was monitored by measuring the depletion of glucose. All of the fungi consumed glucose faster in the gluten medium than in the mineral medium. The gluten medium also contained some fatty acids and other compounds (from the added gluten preparation) which may have affected the fungal growth. Very little proteinase activity was produced in the mineral growth medium, but greatly enhanced proteinase levels were generated in the gluten medium. This was in accordance with the findings of Castro *et al.* (1991) and Griffen *et al.* (1997), who reported that proteinases were produced only when protein was present in the growth media. In the gluten medium, *F. culmorum* and *F. graminearum* produced enzymes that were active at pH 5 and 8, but very little activity was detected with analyses carried out at pH 2 (see Figure 1 in publication I). *F. poae* produced mainly acidic (active at pH 2) proteinase(s), between the second and fifth days of growth, but some proteinase(s) that were active at pH 5 and 8 were present in growth medium samples that were removed between days 1.5 and 2.5 and days 6 and 7 of growth.

Proteinase activities were first detected after 1 or 1.5 d in all of the growth media and were maximal when the consumption of glucose (ie. the growth of the fungus) was in its exponential phase (2.0–2.5 d). During that time, the activities of all of the *Fusarium* media were ~0.6 AU/mL when measured at pH 8, with the 2.5-d *F. poae* growth medium containing, in addition, ~1.0 AU/mL of activity at pH 2 (see Figure 1 in I). The alkaline proteinase activities decreased when the glucose was depleted from the media (3 d), but in the *F. culmorum* and *F. graminearum* samples they increased again within 1 d of glucose depletion and reached maximal levels (~1.3 and 2.4 AU/mL, respectively, at pH 8) after 6–7 d. The acidic proteinase in the *F. poae* growth medium also disappeared after 3 d, increased again, and reached its maximum (~1.7 AU/mL, measured at pH 2) at 5 d. After that, the acidic enzyme was suddenly inactivated, possibly due to the drastic change that occurred in the pH of the medium, and the pH 5 and 8 proteinase activities increased (~0.7 AU/mL at pH 8 at 7 d). The production of proteinases may have occured in two or three phases in response to changes in the concentrations of carbohydrates and/or proteins, or to the pH of

the growth medium. These factors are known to affect the synthesis, secretion and/or stabilities of *Fusarium* proteinases (Castro *et al.* 1991, Dunaevskii *et al.* 1995, Rucka *et al.* 1998).

The alkaline proteinases that were produced by all of the tested fusaria were very similar. In all cases, the growth media samples contained enzymes that were active from at least pH 5.0 to 10.5 with maximal activities at pH ~9 (Figure 4a in I), indicating that the majority of the enzymes were 'alkaline' in character. These proteinases also hydrolyzed azogelatin and a synthetic substrate, N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SAAPF*p*NA) (Figure 2 in I). The production of *Fusarium* proteinases that had pH optima between 8 and 10 has been described previously (Castro *et al.* 1991, Griffen *et al.* 1997, McKay 1992, Tomoda *et al.* 1979). The growth media also seemingly contained some neutral proteinases, because each of the pH-dependance curves of the activities had a shoulder at pH ~7 (Figure 4a in I).

Similarly, SAAPF*p*NA-hydrolyzing, alkaline proteinases were detected in the barley grain medium samples (see Figures 3 and 4 in I). This was in accordance with the results of Nightingale *et al.* (1999) and Schwarz *et al.* (2002) who showed that alkaline proteinases were present in FHB-diseased wheat and barley grain, respectively. However, the ratio between the pH 5 and 8 activities was closer to 1 in these samples than in the gluten growth medium samples indicating that, in the grain media, there were at least two different enzymes. In addition, the proteinase activity of the *F. culmorum*-inoculated grain medium sample was slightly higher at pH 6 than at pH 9 (Figure 4b in I).

The acidic proteinase produced by *F. poae* did not hydrolyze azogelatin, so hemoglobin was used as its substrate. Its pH optimum, ~3.5 (Figure 5 in I), was different from those of the *F. culmorum*, *F. oxysporum* and *F. avenaceum* (pH ~2) (Urbanek & Yirdaw 1978) and *F. graminearum* (pH ~5) proteinases (Griffen *et al.* 1997), but similar to that of *F. moniliforme*, which produced an aspartic proteinase whose pH optimum was 3.2 (Kołaczkowska *et al.* 1983). Acidic proteinases occurred in plant tissues that were infected with fusaria or other fungi (Hislop *et al.* 1982, Movahedi & Heale 1990, Urbanek & Yirdaw 1978), but such activities were not detected in the barley grain medium samples that were tested during this study.

3.2 Characterization of the *F. culmorum* alkaline proteinases (II, III)

The alkaline proteinases were purified and characterized, because the most pathogenic *Fusarium* spp. produced these enzymes both in the gluten and grain media. Also, there were indications in the literature that alkaline proteinases were present in *Fusarium*-infected wheats and barleys. Although *F. graminearum* is the fungus that most commonly causes severe FHB epidemics worldwide, the proteinases of *F. culmorum* were studied because it is more common than *F. graminearum* in Finland.

3.2.1 Purification of the proteinases

To produce large amounts of the *F. culmorum* alkaline proteinase(s), the fungus was grown in a gluten-containing medium. The culturing was stopped by removing the mycelia from the broth by centrifugation, after approximately 50% of the glucose had been consumed. At that time, the proteinase concentration of the growth medium was apparently maximized (I) and the fungus was in its active growing phase. The supernatant was concentrated 16-fold by ultrafiltration. The procedure used to purify the two proteinases from this concentrate is presented in Figure 1.

The growth medium concentrate was subjected to size exclusion chromatography (Bio-Gel P30, pH 5) to remove any small contaminants, including the remaining glucose and salts. Most of the larger impurities were removed by separating the proteins by cation exchange chromatography at pH 5. Two major azogelatin-hydrolyzing proteinases eluted concomitantly at this step, but they were separated by carboxymethyl-HPLC at pH 8 (Figure 1 in II). Ion exchange separations at lower pH values did not separate the two enzymes. Both enzyme preparations contained minor protein impurities after this CM-HPLC step. When the enzyme that eluted first was subjected to a second, similar HPLC separation, a small amount of contaminating protein was separated out. This contaminant protein had trypsin-like activity, as it hydrolyzed the trypsin specific substrate N-benzoyl-Val-Gly-Arg *p*-nitroanilide (BVGR*p*NA). However, the second CM-HPLC step was not used routinely to purify the 28.7-

kDa enzyme, because much of its structural protein and proteolytic activity was also lost during the second HPLC separation.

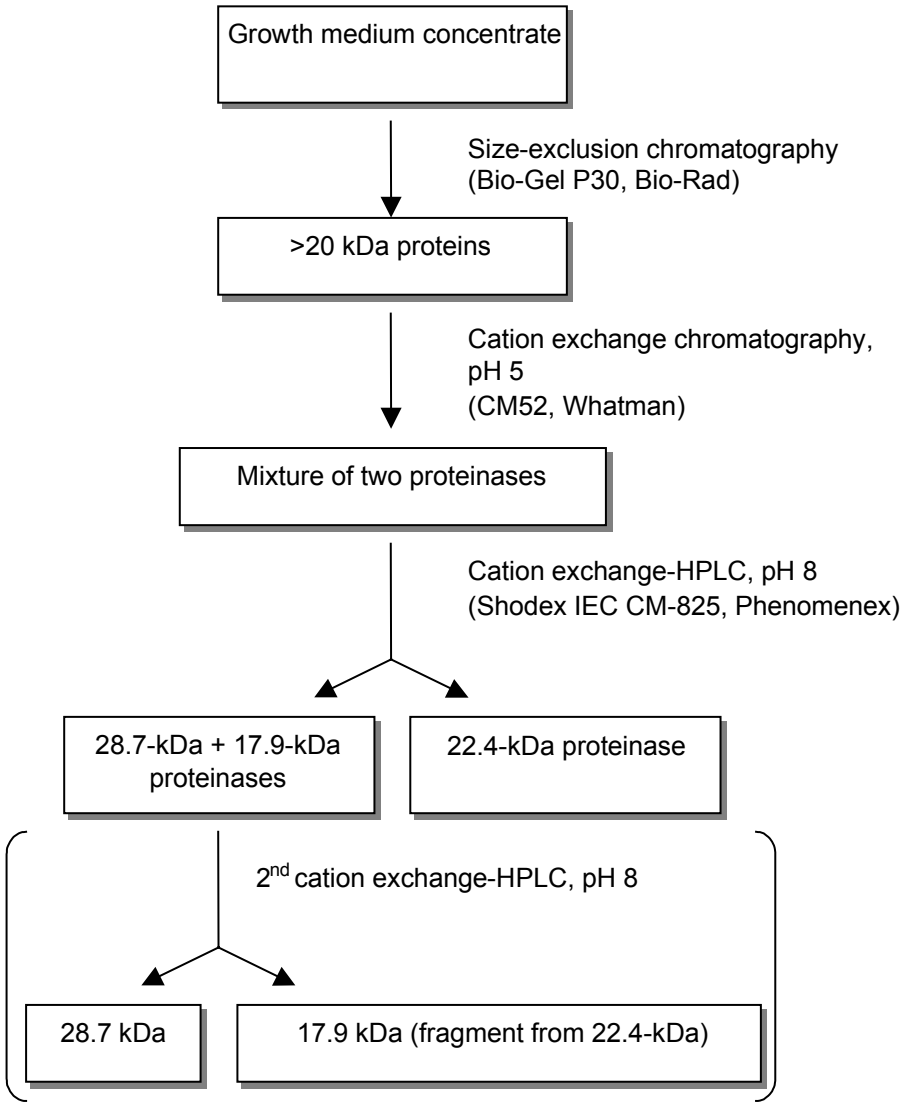


Figure 1. Method used to purify the alkaline serine proteinases of F. culmorum.

The two major proteinases that were separated by the CM-HPLC step had molecular masses of approximately 27 and 25 kDa when analyzed by SDS-PAGE (Figures 2 in II and III). MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry showed that the masses of the enzymes were actually 28 663 ± 50 Da and 22 398 ± 35 Da, respectively. The contaminating trypsin-like enzyme that co-eluted with the 28.7 kDa proteinase, and that was separated by the second CM-HPLC step, had a molecular mass of ~17.9 kDa. The 28.7 and 22.4-kDa proteinases were further characterized.

3.2.2 Determining the mechanistic classes of the purified proteinases

Both alkaline enzymes were inhibited by the serine proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF) and *p*-amidino phenylmethylsulfonyl fluoride (*p*APMSF), while the class specific inhibitors of the cysteine-, metallo- and aspartyl proteinases (EC 3.4.22, 3.4.24 and 3.4.23, respectively) only slightly affected their activities, indicating that they both belonged to the serine proteinase class (Table 3 in II).

The serine proteinases (EC 3.4.21) are divided into six clans and nearly thirty families according to their evolutionary origins, their catalytic amino acids and their three-dimensional structures (Rawlings & Barrett 1994). Subtilisin is a bacterial enzyme that has nearly the same substrate specificity as bovine chymotrypsin. Both subtilisin and chymotrypsin preferentially hydrolyze peptide bonds with large hydrophobic amino acids that occupy the P_1 position (the amino acid on the N-terminal side of the scissile bond of a protein or peptide), but their amino acid sequences are not homologous. The active sites of both of these enzyme families contain a catalytic triad of serine, histidine and aspartic acid, but these amino acids occur in different orders in their primary structures. Trypsin is a bovine enzyme that belongs to the chymotrypsin family, but several microbial proteinases that have similar properties are also categorized into this family.

The 28.7-kDa *Fusarium* enzyme was inhibited by chymostatin (CST), which is a specific inhibitor of chymotrypsin and subtilisin (Table 3 in II). Like the

bacterial and fungal subtilisins, this proteinase was not inhibited by SBBI, which inhibits both bovine trypsin and chymotrypsin. The inhibitor concentrations that were used in this experiment were lower than those utilized for the studies of Marchetti *et al.* (1995), who showed that SBBI inhibited several microbial alkaline proteinases. Thus, this newly purified 28.7-kDa enzyme was denominated a subtilisin-like (SL) proteinase. The 22.4-kDa proteinase was inhibited by the soybean Kunitz inhibitor (STI) and by SBBI, but not by CST, indicating that it was a trypsin-like (TL) enzyme (Table 3 in III). Also like trypsin, it was inhibited only weakly by PMSF, but strongly by *p*APMSF.

3.2.3 The amino acid sequence homologies between the *Fusarium* and other fungal proteinases

The N-terminus of the SL proteinase was apparently blocked, so its amino acid sequence could not be analyzed directly. To overcome this problem, the purified protein was partially digested with trypsin and the N-terminal amino acid sequences of several of the resulting peptides were ascertained. Four of the peptides had amino acid sequences that were similar to those of portions of the subtilisin-like proteinases of several other fungi (Table 4 in II). These peptides were: 1) GSTSYIYDTSAGSGTYAYIV**D**TGIITSHN, 2) GFNWAANDIISK, 3) SYSNYGTVL and 4) DIFAPGTSVLSS. On average, these peptides were 82% identical with those of a proteinase from *Cephalosporium acremonium*, ~70% identical with enzymes from several *Aspergillus* spp. and *Trichoderma harzianum* and 55–65% the same as those of the *Metarhizium anisopliae*, *Magnaporthe poae* and *Tritirachium album* proteinases (see references in II). Surprisingly, these amino acid sequences were only 52 and 55% identical with those of the corresponding peptides of the subtilisin-like proteinases from the closely related species *F. oxysporum* and *Fusarium* sp. 'S-19-5'. One of the analyzed peptides contained an aspartate residue (bolded in the amino acid sequence of peptide 1) in a position that is conserved in the catalytic triad of all subtilisin-like proteinases (Table 4 in II). The amino acid sequence analyses thus supported the classification of the 28.7-kDa *Fusarium* enzyme to the subtilisin family.

The N-terminus of the *Fusarium* TL proteinase was not blocked, and its amino acid sequence, IVGGTSASAGDFPFIVSISRNGGPW, was 88% identical with

that of the trypsin-like proteinase that occurs in *F. oxysporum*. The N-terminal amino acid sequences of similar enzymes from *Cochliobolus carbonum*, *Stagonosopora nodorum* and *Metarhizium anisopliae* were 64 to 68% identical (see references in III). The N-terminal amino acid sequence of the 17.9-kDa enzyme was identical to that of the TL proteinase. Thus, the 17.9-kDa contaminant was apparently a fragment of the TL proteinase which had at least partially maintained its functionality. Because the 17.9-kDa protein and the SL enzyme eluted concomitantly from the first CM-HPLC step, but were separated during the repeated HPLC (see Figure 1), it appears that the SL enzyme had bound some of the TL molecules, cleaved an ~4.5-kDa fragment from their C-termini, and then retained the remnant of the hydrolyzed TL protein during the first CM-HPLC separation. However, this needs to be confirmed by analyzing the scissile bond amino acids from the TL proteinase.

3.2.4 The temperature and pH dependances of the proteinases

Both of the *F. culmorum* proteinases were maximally active at pH ~9 when azogelatin was used as substrate (Figure 3 in II and Figure 4 in III). The pH optimum curves had shoulders at pH ~7, but it is likely that the buffers were affecting the apparent activities. Although similar results were obtained with the gluten and barley grain media samples (Figure 4 in I), it was unlikely that any neutral proteinase contaminants were present in the purified enzyme preparations.

The enzymes were unstable after purification, especially in alkaline solutions (Table 3), and it appeared that the SL enzyme underwent autolysis. The amino acid sequence analysis of the SL enzyme showed that one of the peptides had resulted from a cleavage on the C-terminal side of a leucine residue (see Table 4 in II). Such cleavages are typical of subtilisin enzymes, but not of the trypsin enzyme that was used to digest the protein to obtain sequencable peptides. Also, the composition of the enzyme solution container (glass or polypropylene) and whether the container was silanized affected the rate of the proteinase inactivation. This implied that the enzyme was also being inactivated due to its adsorbing onto the surfaces of the vessels, as chymotrypsin does (Oshima 1989). The addition of extraneous protein (bovine serum albumin, BSA) to the enzyme solutions stabilized their activities, probably by preventing autohydrolysis, the

surface-catalyzed inactivation, or both. The TL proteinase was also stabilized by the presence of calcium ions, especially under alkaline conditions (Figure 5 in III). Bovine trypsin and chymotrypsin are both stabilized by calcium (Walsh 1970, Wilcox 1970). The stability of the SL proteinase at pH 5 was not affected by calcium ions. Neither Ca^{2+}-ions nor BSA had any direct affect on the proteinase activity assays, under the conditions that were used for this study. The TL was more stable than the SL under slightly alkaline pH conditions, but it was more sensitive to heat inactivation (Table 3). Because both SL and TL were unstable at elevated temperatures, their activities were routinely assayed at 40 ºC.

Table 3. The effects of pH and temperature on the stabilities of the F. culmorum *subtilisin- and trypsin-like proteinases (SL and TL, respectively).*

pH of treatment (at 40 ºC, 90 min)	Remaining activity (%)		Temperature, ºC (at pH 6, 50 min)	Remaining activity (%)	
	SL	TL		SL	TL
4.1	39	50	24	88	90
5.0	35	41	40	55	72
6.0	32	58	50	29	0
6.5	36	58	60	0	0
7.8	28	42			
8.5	0	24			

3.2.5 The substrate specificities of the *Fusarium* proteinases

The abilities of the *Fusarium* enzymes to hydrolyze various synthetic peptides were studied by measuring their activities using N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SAAPF*p*NA), N-succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide (SAAPL*p*NA), N-glutaryl-L-Phe *p*-nitroanilide (GP*p*NA), N-benzoyl-Val-Gly-Arg *p*-nitroanilide (BVGR*p*NA) and Nα-benzoyl-L-Arg *p*-nitroanilide (BApNA). Both proteinases most readily hydrolyzed the tri- and tetrapeptide analogues and only very slowly hydrolyzed GP*p*NA and BA*p*NA. Like the alkaline proteinase of *Aspergillus fumigatus* (Larcher *et al.* 1992), the SL

proteinase hydrolyzed the substrate with Phe at its P_1 position (SAAPF*p*NA) faster than the one that contained Leu (SAAPL*p*NA). The TL proteinase showed a strong preference for BVGR*p*NA. The kinetic constants of the SL and the TL proteinases were determined only for SAAPF*p*NA and BVGR*p*NA, at pH 6 and 9 (Table 4).

Table 4. The kinetic constants of the F. culmorum *proteinases for the synthetic substrates SAAPFpNA and BVGRpNA. The reaction mixtures contained 175 mM Na citrate, pH 6, or Tris-HCl, pH 9, and 4% DMSO.*

Enzyme	Substrate	pH	K_m (mM)	V_{max} (nkat/mg protein)	k_{cat} (s^{-1})
SL	SAAPF*p*NA	6	3.1	1130	33
		9	2.3	2270	65
TL	BVGR*p*NA	6	0.11	2020	45
		9	0.02	3520	79

Some hydrolysis of BVGR*p*NA also occurred in the presence of the SL preparation, and this reaction was inhibited by STI, but not by CST, indicating that the activity was probably due to the contaminating 17.9-kDa trypsin-like enzyme (discussed in section 3.2). Neither STI nor SBBI inhibited the activities of the SL enzyme preparation with the nonspecific proteinase assays (Table 3 in II), indicating that this trypsin-like contaminant was present at a very low concentration and/or it did not catalyze the hydrolysis of azogelatin.

The hydrolytic specificity of the TL proteinase was studied by using it to hydrolyze reduced and pyridylethylated β-purothionin (III). The enzyme hydrolyzed this modified low MW wheat protein most readily at positions where Arg occupied the P_1 position, but minor cleavages also occurred adjacent to Lys residues. A preference for hydrolyzing proteins adjacent to Arg residues is typical of microbial trypsins. When the purothionin substrate was hydrolyzed with the SL proteinase, hydrolyses by the 17.9-kDa trypsin-like contaminant made it impossible to determine the specific SL cleavage sites. The SL did not hydrolyze the purothionin as fast as the contaminant, which could not be totally inhibited or removed from the enzyme preparation.

3.3 The presence of *Fusarium* proteinases in infected barleys (IV)

Two field trials were carried out to study whether the SL and TL proteinases were produced by the fungi that infected developing barley grains. The enzymes were detected by measuring their proteinase activities and by immunoblotting. In the summer of 1998, barley plants were inoculated with *F. culmorum*, *F. graminearum* or *F. poae* in two locations, Hauho and Jokioinen, both in southern Finland. *Fusarium* spore suspensions were sprayed onto the plants when the spikes were emerging from the boot and spike samples were collected every 2 to 3 weeks from inoculation until maturity. The experiment was repeated in Hauho during the summer of 2000 using *F. culmorum*, but in that experiment no samples were collected until 5 weeks after inoculation. Rainy weather during both 1998 and 2000 favored the fungal growth, so that the non-inoculated controls were also infected. However, when the amount of fungus on the various experimental populations was estimated using an agar plating method, the inoculated grain contained more *Fusarium* than the control (Tables 1 and 2 in IV). The *F. culmorum* and *F. graminearum* inoculated Saana barleys also contained higher concentrations of DON and zearalenone than the control grain (Haikara *et al.* 2000).

In 1998, the SL activities were roughly the same in the inoculated and uninoculated (control) kernels that were collected 3 weeks after treatment, but clear differences were observed in the samples that were collected after 5 weeks and later. The SL activities of the *F. poae*-inoculated kernels were nearly the same as those of the control samples, but the grains that were inoculated with *F. culmorum* and *F. graminearum* spores generally contained more than 10 times the SL activity of the control grain (Table 3 in IV). Similar results were obtained from the repeat experiment that was carried out in 2000 with *F. culmorum* (Figure 1 in IV). The TL activities also increased in the *F. culmorum*-inoculated samples, but these activities were much lower than those of the SL enzyme. Trypsin-like proteinase activities have also been detected in loose smut (*Ustilago* spp.) diseased wheat grain (Yamaleev & Ibragimov 1986).

When the year 2000 samples were analyzed by immunoblotting with polyclonal antibodies that had been raised against either purified SL or TL enzyme, both of the proteinases were detected in the *F. culmorum*-inoculated grain samples

(Figure 2 in IV). The proteinases that were present in the grain were therefore structurally the same or very similar to those that were produced by the fungus when it was grown in the gluten medium. Even though the control grain samples were also contaminated with fusaria, neither of the enzymes was detected in them. The control plant infections may have progressed more slowly during both 1998 and 2000, because the control plants were not exposed to as many *F. culmorum* or *F. graminearum* spores as the inoculated plants. Also, the control plants may have been infected at a later date than the inoculated spikes.

Because the proteinases were only detected in samples that were collected more than 3 weeks after the inoculation, it appeared that the proteinases were synthesized during the later phases of the invasion process and were not particularly important for the initial infection. We cannot say, however, that they play no part in the initial infection, because the fungi may have produced such small amounts of enzymes in the beginning of the infection that they could not be detected with the proteinase and immunoblotting assays that were used in these studies. That is, a small amount of fungal enzymes would have been diluted by the large amounts of grain proteins that were present. In addition, the results with the controls should be viewed cautiously, since the majority of the fusaria in the controls could have been species other than *F. graminearum* or *F. culmorum*. The *Fusarium* spp. of the grain samples from this experiment were not identified but, during both 1998 and 2000, *F. avenaceum*, *F. tritinctum* and *F. arthrosporioides* were the most common naturally occurring species in Finland (Yli-Mattila *et al.* 2002). It is not known whether or not these species produce alkaline serine or other proteinases in developing cereal kernels.

Some of the buffer-soluble proteins (albumins and globulins) of the heavily infected field grain samples had been partially degraded, as shown by SDS-PAGE analysis. Certain proteins, with molecular masses of approximately 62, 32, 30 and 25 kDa, had disappeared as the heavily infected grain matured and a 44-kDa protein, which may have been either a fragment of the 62-kDa protein or an *F. culmorum* protein, had appeared (Figure 3 in IV). These changes in the SDS-PAGE protein patterns were most striking in the samples that contained the highest SL and TL activities, implying that the proteins may have been degraded by the *Fusarium* proteinases. However, buffer-extracted barley proteins were not degraded *in vitro* by purified *Fusarium* proteinases. When the purified proteinases were incubated with buffer extracts that were prepared from clean or

lightly infested barley (cv. Mentor) grain, no protein degradation was observed. This may be due to the presence of proteinase inhibitors in the barley grain (IV,V). On the other hand, both the SL and TL proteinases hydrolyzed the C and D class hordeins (barley prolamins, alcohol soluble storage proteins) *in vitro* (Figure 4 in IV, Figure 8 in III). These results indicated that the SL and TL proteinases may be at least partially responsible for the degradation of the grain endosperm matrix proteins that is so obvious in electron microscope photos (Jackowiak *et al.* 2002, Nightingale *et al.* 1999). In addition, the fusaria may produce other proteinases that were not detected in this study.

The mature grain samples were gathered both manually and with a harvester machine. The proteinase activities of the mechanically harvested samples were almost always lower than those of the manually collected ones (Table 3 in IV) and only small amounts of SL and TL proteinases were detected when extracts from the mechanically harvested kernels of the *F. culmorum* -inoculated samples were immunoblotted (Figure 2 in IV). In addition, the buffer-soluble protein SDS-PAGE patterns of the machine-harvested inoculated and control grains were identical (Figure 3 in IV). From these observations it appears that the proteinase activities only reached readily detectable levels in the most shrivelled kernels, which were selected and discarded by the harvester machine. Because of the humid conditions under which the experimental barleys were grown, even the controls were attacked by fungi, so there was no sample of completely fusarium-free control barley whose protein patterns could be compared with those of the *Fusarium*-contaminated grains.

3.4 Barley proteins that inhibited the *Fusarium* serine proteinases (V)

When a buffer extract was prepared from finely ground barley (cv. Morex) grain, it strongly inhibited the activities of both the SL and TL proteinases. The inhibition assays were carried out at pH 6, which was the pH of the barley grain medium (see publication I), using SAAPF*p*NA and BVGR*p*NA as substrates. Inhibitor proteins were isolated from the extract by size exclusion and ion exchange chromatographies and were finally purified by reversed phase (RP)-HPLC (Figure 4 in V). The inhibitor purification was monitored by following the abilities of all of the separated fractions to inhibit the SL and TL proteinases,

so that all compounds that inhibited either of the two proteinases could be detected. The purification procedure is diagrammed in Figure 2. The purified inhibitors were identified by their MALDI-TOF mass spectra (Table 1 in V) and from their N-terminal amino acid sequences (Table 2 in V). Several SL proteinase inhibitors were purified and identified as being the chymotrypsin/subtilisin inhibitors (CI) 1A, 1B and 2A and the barley α-amylase/subtilisin inhibitor (BASI). CI-1A and -2A occurred both as intact proteins and as fragments that had varying molecular masses, but CI-1B was always fragmented. The TL proteinase was inhibited by a single purified protein, the Bowman-Birk inhibitor (BBBI). Previous studies by other researchers had shown that each of these proteins could inhibit certain microbial enzymes and that CI-1 can inhibit some alkaline proteinases of *Alternaria tenuissima* (see section 1.4). Our results proved, for the first time, that BASI, the CIs and BBBI could inhibit the proteinases that were produced by the pathogen during its colonization of barley seeds. The major inhibitor of bovine trypsin that occurs in barley endosperm, CMe, was not among the inhibitors identified by this procedure, implying that it does not inhibit the *Fusarium* TL proteinase or, if it does, its inhibition is very weak. However, no purified CMe was available, so its ability to inhibit could not be tested directly. No novel inhibitors were detected, but it is not impossible that other inhibitors, especially weak ones, exist in barley, because they could have been lost during some of the purification steps.

A comparison of the SL and TL enzyme inhibitions that were caused by the various barley inhibitors and by CST and SBBI is shown in Figure 6 of publication V. The inhibitory activities of both intact and fragmented forms of CI-1A and -2A were tested, and the fragmentation did not seem to affect the inhibition of the SL enzyme. Under the assay conditions used, approximately a 1:3 molar ratio of SL proteinase:CI-1A or CST inhibited 50% of its enzyme activity. In accordance with earlier studies (Greagg *et al.* 1994), CI-2A was a more potent inhibitor of SL than CI-1A, because 50% inhibition was reached with an enzyme:inhibitor ratio of only 1:1. When a BASI concentration equal to that of the SL was tested, ~70% inhibition was observed. This value was considerably higher than the ~10% inhibition of *Aspergillus sulphureus* alkaline proteinase that occurred in the presence of a roughly equal amount of BASI (Yoshikawa *et al.* 1976). On the basis of the experimental inhibitor concentration that reduced the TL proteinase activity by 50%, the BBBI was a roughly 7 times more potent inhibitor than SBBI. The fact that BBBI contains

two binding sites for trypsin, whereas only one trypsin molecule can bind to SBBI at a time, probably affects the apparent molar binding efficiency, but other factors must also affect the stability of the enzyme-inhibitor complex.

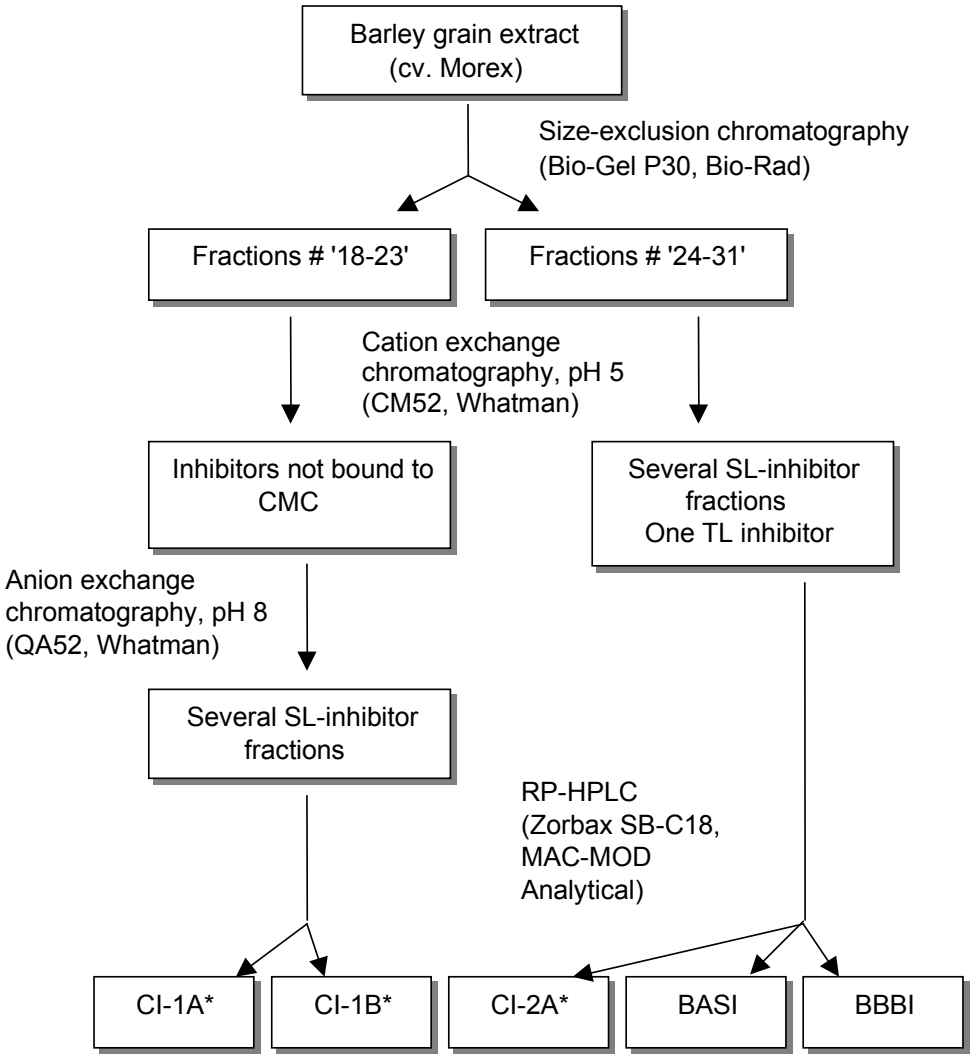


Figure 2. A simplified diagram of the inhibitor purification procedures. The asterisk (*) indicates inhibitors that occurred in multiple size forms.

Even though some differences were observed in the inhibition efficiencies of CI-1A, CI-2A and BASI, more complicated analysis methods must be applied in order to make truly meaningful comparisons of the inhibitory abilities of these proteins. The CI-1 and -2 proteins are slow, tight-binding inhibitors of bovine chymotrypsin and of bacterial subtilisin (Longstaff *et al.* 1990, Greagg *et al.* 1994). To determine whether the purified proteins inhibited the *F. culmorum* proteinases via the slow, tight-binding mechanism, their inhibitory activities were measured with an 'extended-time assay' (unpublished results). In these experiments, the reactions were started by adding the enzyme to the substrate solution that contained the inhibitor (E added to S+I) or by adding the preincubated enzyme-inhibitor mixture to the substrate solution (E+I added to S). Then the hydrolysis of the substrate was monitored at 405 nm wavelength for 16 h. The enzyme concentration was kept low enough in these reactions that less than 3% of the substrate was hydrolyzed during the entire assay, so that the effect of substrate depletion on the enzyme activities was negligible. As shown in Figure 3A and 3B, the addition of purified CI-2A and BBBI proteins to hydrolyses catalyzed by the SL and TL enzymes, respectively, resulted in curved hydrolysis time courses. This is consistent with their having caused slow, tight-binding inhibition. There was some evidence for slow-binding inhibition with CI-1A, but the results with this inhibitor were ambiguous. The inhibition mode of CI-1B was not tested. Because the enzyme-inhibitor complexes equilibrated slowly, the Michaelis-Menten kinetic analysis method was not appropriate for determining the inhibition constants of this system. On the other hand, the BASI did equilibrate rapidly with the SL enzyme (Figure 3C); that is it bound faster than, but not as tightly as, CI-2A. Because the inhibition modes of CI-2A and BASI are different, it is difficult to determine which of them is the more potent inhibitor. CI-2A can inhibit more strongly than BASI at low concentrations but, because its binding to the enzyme is slow, the enzyme has time to hydrolyze some substrate molecules before significant inhibition occurs. However, once the CI-2A is bound to the enzyme, it dissociates slowly and thus does not allow the substrate to occupy the active site. On the contrary, BASI can bind to the enzyme quickly, but because it also dissociates rapidly, the substrate has more of a chance to occupy the active site of the enzyme.

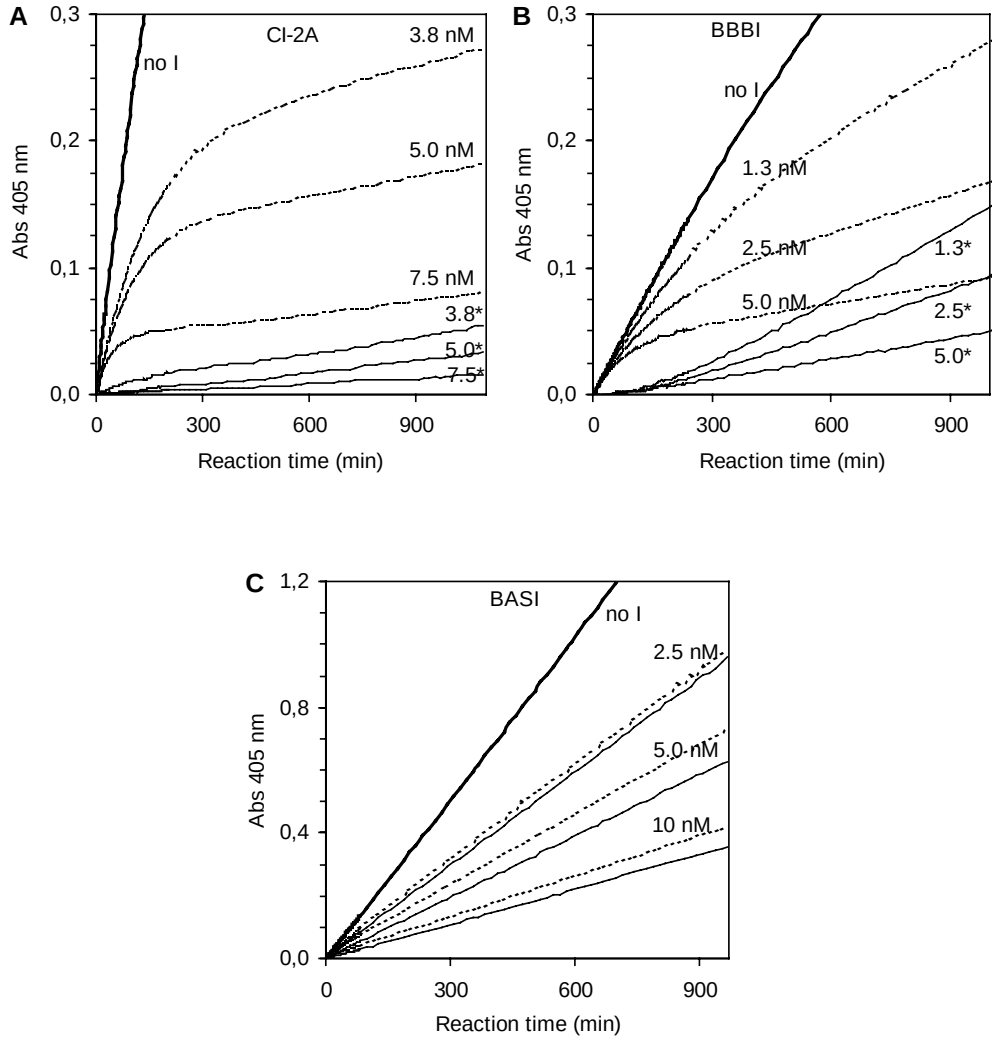


Figure 3. Hydrolysis of synthetic substrates (S) by F. culmorum *SL or TL proteinase enzymes (E) in the presence of various barley inhibitors (I). The analyses were at pH 6 and 28 ºC. (—), E was incubated for 30 min at 28 ºC with I before the reactions were started; (---), E was added to a premixed S+I solution. The inhibitor concentrations (nM) used are indicated next to each curve. For clarity, an asterisk (*) next to an inhibitor concentration indicates that the reaction was carried out with preincubated E+I. A) S = 7 mM SAAPFpNA, E = 0.15 nM SL proteinase and I = CI-2A; B) S = 1 mM BVGRpNA, E = 0.03 nM TL proteinase and I = BBBI, and; C) S = 7 mM SAAPFpNA, E = 0.15 nM SL proteinase and I = BASI.*

The interactions between the proteinases and their inhibitors in the grain depend, first of all, upon their proximities in the grain tissues. Secondly, providing that the inhibitors are in close contact with the enzymes, the inhibition depends on their association and dissociation constants and their concentrations, as compared with those of the substrates (grain proteins that the enzymes can hydrolyze). In order to inhibit the degradation of potential substrates in the grain, the inhibitors need to bind to the enzyme more readily than the substrates. In this work, the inhibition of hordein hydrolyses by SL and TL were tested only with CST and STI, respectively (III and IV). Approximately a 300-fold molar excess of STI, relative to the TL enzyme, was required to stop the hydrolysis of the hordeins under the assay conditions that were used. However, it is expected that BBBI would inhibit the *Fusarium* TL enzyme more strongly than STI, because less than a 10-fold excess of BBBI to TL proteinase was needed to inhibit nearly 100% of the enzyme's BVGR*p*NA-hydrolyzing activity (Figure 6B in V). In the presence of the same substrate (BVGR*p*NA), only ~80% inhibition was observed in the presence of a 330-fold excess of STI (Table 3 in III). Similarly, a 300-fold excess of CST over SL was used to inhibit the hydrolysis of C- and D-hordeins, but both CI-2A and BASI were stronger inhibitors than CST (Figure 6A in V).

3.5 Studies on an unidentified *F. culmorum* proteinase

One of the gluten-containing growth medium batches contained abundant amounts of a third *F. culmorum* proteinase that differed from SL and TL. This enzyme was partially purified by anion exchange chromatography at pH 5 (unpublished results) and it hydrolyzed SAAPF*p*NA and azogelatin more readily at pH 6 than at pH 9. Even though it was able to degrade the same synthetic substrate as the SL enzyme, this 'neutral' enzyme was not similar to either of the alkaline proteinases, because it was not inhibited by CST, STI or SBBI. Its molecular mass could not be determined, because it migrated very slowly on 10% SDS-PAGE when run under reducing conditions, implying that its size was larger than 250 kDa, and MALDI mass spectrometry is not suitable for determining the masses of proteins larger than 200 kDa. Interestingly, neither this *F. culmorum* enzyme nor the acidic proteinase of *F. poae* was inhibited by crude barley grain extracts (unpublished results). We were not able to determine whether it was synthesized in the *Fusarium*-inoculated developing kernels, but

this enzyme may be the same one that was observed in the barley grain medium samples in which *F. culmorum* was grown (see Figures 3b and 4b in I). This large enzyme did not cross-react with antibodies that were raised against either the SL or TL proteinase.

4. General discussion

The purpose of this dissertation was to investigate the interactions that occur between *Fusarium* fungi and cereal spikes and, especially, how the *Fusarium* proteinases and grain inhibitors each affect the development of the disease FHB. To attack cereals, the fungi seem to utilize a web of various factors that support each other; any one of which, if missing, might not interfere unduly with the ability of the fungus to attack the host cells. Fungal toxins apparently play a central role in the FHB-infection process (see section 1.2.2), but it is likely that various hydrolytic enzymes and hormone-like compounds also influence the invasion rate. In addition, it is not inconceivable that some host responses, eg. possibly programmed cell death, may also enhance the fungal colonization.

The presence of fungal serine proteinases in infected plant tissues has been shown in several studies, but none of them seems to be an essential pathogenesis factor. However, as discussed in section 1.2.3.3, the role of a single type of proteinase is difficult to establish, because other similar hydrolases could easily replace a non-functional or missing enzyme. The proteinases probably do help the fungus to grow and invade faster by degrading the host proteins to provide nutrition. It is extremely difficult to determine the importance of the 'nutritional' role of proteinases, because host cells contain other nitrogen sources besides proteins that the fungus can absorb and utilize. These sources could be free amino acids and small peptides that are newly synthesized by the plant or that are released from degraded proteins by the plant's endogenous proteinases (Simpson 2001). In addition, the fungi can synthesize amino acids from plant carbohydrates and inorganic nitrogen (eg. NO_3^-, NH_4^+) via their normal metabolic mechanisms. These sources could be sufficient to support some fungal growth, but the fungus might presumably grow faster in the presence of protein and proteinases, because then its free amino acid pool should be greater. This was supported by our observation that the fungi consumed glucose faster, ie. they were presumably growing faster, in the gluten medium than in the mineral medium (I). Proteinases may play other roles during the fungal invasion, such as suppressing the host's defenses by inactivating PR-proteins or modifying the fungal cell wall morphology, eg. during branching.

Because the SL and TL proteinases are present in *Fusarium*-infected barley grain, they probably could facilitate fungal invasions. In this work, the enzymes

were detected only in heavily infected grain, implying that they were produced during the latter stage of the development of the disease. On the other hand, if the enzymes were synthesized during the initial fungal attack or during the early stages of invasion, they probably would occur in localized areas around the penetrating hyphae. This could be studied by immunomicroscopy, which allows one to detect local accumulations of enzymes, even if the enzymes are not detectable in grain extracts by immunoblotting or by carrying out activity assays. In addition, to completely define the functions of the fungal proteinases, the possible presence of other types of *Fusarium* proteinases in the host plants needs to be investigated.

In considering the importance of the proteinases in general, one also needs to take into consideration the fact that serine proteinase inhibitors are very common in plants. In some plant tissues, eg. in tomato leaves, the expression of certain proteinase inhibitors is induced by wounding or by pathogen attacks. The induction of cereal grain inhibitors by pathogens has not been established, and most of the inhibitors are synthesized in the embryo, the aleurone layer and/or the starchy endosperm tissues between 2 and 4 weeks after anthesis. These proteins comprise one part of the storage reserve that is used for seedling growth but, conveniently for the plant, they also inhibit pathogen proteinases, as shown in this work. Although the interactions of the SL and TL with these inhibitors have only been established *in vitro*, it seems likely that the proteinase inhibitors can bind to the SL and TL proteinases in the grain. However, if the syntheses of the inhibitors truly start no earlier than 2 weeks after the anthesis, they would not be able to help defending the wheat or barley florets from infections that occur at anthesis. In addition, it is not known whether any of these inhibitors are produced in the glumes, lemma, palea or pericarp, which are the first tissues to be colonized by the fungi. Thus, they may play no defensive role during the early stages of infection, but they may hinder the fungal growth in the aleurone, starchy endosperm and embryo tissues when the hyphae reach them. In addition, the importance of these inhibitors in plant defense depends on how important the roles are that the SL and TL proteinases play during the infection. BBBI and CMe seem to affect the permeability of fungal plasmalemmas (Terras *et al.* 1993), but it is not known whether any of the other inhibitors have antifungal properties other than those that are related to the inhibition of proteinase activities.

Presuming that the proteinase inhibitors have important roles in impeding fungal invasions, it should be possible to use them to increase the resistance of barley and/or other cereals to FHB. However, considering the infection pathway, i.e. the fungus first colonizes the glume, lemma and palea, it would probably be useful to target the expression of BBBI, together with either BASI or CI-2A, to these tissues. Because the inhibitors already are abundant in the endosperm and/or embryo tissues, it does not seem likely that enhancing their expression in those tissues would significantly increase the plant's resistance to the fungus. The structures that cover the developing seed contain little protein, but the inhibitors located there might retard the maceration of the seed's cell walls and/or increase the efficiency of the plant's defense system by preventing the inactivation of the defense proteins. In addition, if the proteinases are involved in the constructing of fungal cell walls, the inhibitors might hinder the fungal growth directly (i.e. not just from affecting its nutrition).

Because it is possible that the fungal toxins can interfere with the translation of induced defense proteins, expression of the inhibitor genes under a pathogen-inducible promoter might not be effective. Hence, it would be better if the inhibitors were synthesized in the floret and/or during the very early stages of kernel development by using a promoter that is especially active in that tissue and/or during that time. However, the inhibitors may play some physiological roles that are not yet known and their synthesis in the "wrong" tissues or developmental stage may have adverse effects on the grain development. A transient expression of the inhibitors during the early infection stages would have the benefit of protecting the caryopsis when it is most vulnerable to FHB, but it would prevent an excessive accumulation of the inhibitors. Production of the inhibitors in the husks of wheat and barley would also have the benefits of not affecting the digestability of the grain by humans and animals or the germination of the seed. Even if the inhibitor gene expression can be successfully targeted to the husk tissues, the fungus might be able to adjust to their presence by producing different proteinases that they do not inhibit.

5. Conclusions and future research

This work showed that the *Fusarium* spp. (*F. culmorum* and *F. graminearum*) that are most pathogenic in causing FHB in wheat and barley produced alkaline proteinases when grown in cereal protein media and in FHB-diseased barley kernels. Concurrently with this study, other researchers have found similar results with *F. graminearum*, but until now the *Fusarium* enzymes that were produced in the cereal grain had not been characterized. This work also showed that barley grain contains proteins that inhibit the activities of the SL and TL fungal proteinases and delineated which proteins were the strongest inhibitors. The roles that the proteinases and their inhibitors may play during the progression of FHB have been discussed in this dissertation, but more research is needed to prove or disprove their proposed roles. Researchers have previously proposed that various proteinase inhibitors may play a role in defending grains from fungal attack. However, it was important to show, as is done in this thesis, that the proteinases whose activities these inhibitors block actually are produced and occur in the diseased grain. Even though the exact roles of the proteinases that formed during the infection were not defined during this study, the different proteins, both attacking enzymes and defending inhibitors, have now been defined so that their roles can be studied in more detail in the future. In order to obtain better information about the *Fusarium* proteinase-barley inhibitor interactions, antibodies were raised against the purified CI-2A, BASI and BBBI inhibitors and against SL and TL during this study. These can be used to conduct immunomicroscopic studies on the synthesis of the enzymes during infection and to localize the inhibitors and enzymes. These studies have already been started, in conjunction with Dr. Salla Marttila, who is using the antibodies to study the developing and maturing tissues of barley spikelets that are infected with *F. culmorum* (Marttila *et al.* 2002).

The specific antibodies can also be used to develop quantitative (ELISA) assays for the enzymes and inhibitors. The activity assays used to measure the activity/inhibition characteristics of the purified enzymes and inhibitors are not very reliable for quantifying their amounts in extracts that contain both, because they can interact to form complexes that will not show either inhibitory or enzymatic activities. Using ELISA assays will allow, for example, the determination of whether the increased levels of fungal proteinases that occur in barley spikes after infection lead to an increase in the amounts of inhibitors that

are present in the grain. ELISA tests thus might help to clarify what actually happens during the fungal invasion.

Studies using proteinase-deficient *F. graminearum* and *F. culmorum* mutants that cannot express either the SL or TL genes could also be used to study the roles of these proteinases during FHB pathogenesis, but such mutant strains have not been developed yet.

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Pekkarinen, A.I. and Jones, B.L. Trypsin-like proteinase produced by *Fusarium culmorum* grown on grain proteins. J. Agric. Food Chem. 2002. Vol. 50, pp. 3849–3855.

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Pekkarinen, A.I. and Jones, B.L. Purification and identification of barley (*Hordeum vulgare* L.) proteins that inhibit the alkaline serine proteinases of *Fusarium culmorum*. J. Agric. Food Chem. Submitted.

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The serine proteinases of *Fusarium* grown on cereal proteins and in barley grain and their inhibition by barley proteins

Abstract

Fusarium head blight (FHB, scab) of wheat and barley is one of the most devastating diseases of cereals. Severe FHB epidemics have occurred all over the world, resulting in major yield and quality losses that cause problems to producers and to various industries that use grain as raw material. Scabby grain processes poorly and the toxins that are produced by the fungi cause potential health risks to humans and animals. The *Fusarium* fungi colonize cereal spikes and utilize the grain components for their own nutrition and reproduction. One of the interesting aspects of the infection mechanism is the question of how important is the hydrolysis of the host plant proteins by the invading fungus. Previous studies have indicated that protein degradation occurs in infected grains, implying that the fungi produce proteinases during the colonization of the kernel tissues. In addition, it has been proposed in the literature that host plants may use various proteinase inhibitors to defend themselves against pathogens. The purpose of this dissertation was to pinpoint and characterize the proteinases that are synthesized by *Fusarium* species to degrade grain proteins during infection and to identify and thoroughly examine any proteins in barley that can inhibit those enzymes.

In this study, it was shown that species that cause FHB, *F. culmorum*, *F. graminearum* and *F. poae,* produced alkaline proteinases when grown in cereal protein media. Two proteinases were purified from a *F. culmorum* culture filtrate by using size-exclusion and ion exchange chromatographies. Both of the enzymes were maximally active at pH ~9 and 40–45 °C, but they were unstable under those conditions. The mechanistic classes of the enzymes were determined by measuring the effects of class-specific proteinase inhibitors on their activities and this indicated that they were subtilisin- and trypsin-like proteinases. In addition, portions of their amino acid sequences were homologous to those of other fungal proteinases that have been categorized into these classes. Both of the proteinases hydrolyzed C- and D hordeins (barley storage proteins) *in vitro*. The presence of these enzymes in field grown, FHB-infected barley was demonstrated by activity assays using N-succinyl-Ala-Ala-Pro-Phe *p*NA and N-benzoyl-Val-Gly-Arg *p*NA as substrates and by an immunoblotting method. These proteinases were inhibited by several barley proteins, which were then purified and identified. The subtilisin-like proteinase was inhibited by the barley α-amylase/subtilisin inhibitor (BASI) and by the chymotrypsin/subtilisin inhibitors 1A, 1B and 2A (CI-1A, -1B, -2A). The trypsin-like enzyme was only inhibited by the barley Bowman-Birk inhibitor (BBBI). The roles that these proteinases and their inhibitors may play during the *Fusarium*-infection are discussed.

Keywords

cereals, barley, *Fusarium*, fungi, plant pathogens, proteinases, proteinase inhibitors, proteolytic enzymes

Activity unit

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