Appl Microbiol Biotechnol
DOI 10.1007/s00253-010-2508-4

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

The antifungal protein AFP from *Aspergillus giganteus* prevents secondary growth of different *Fusarium* species on barley

Hassan Barakat · Anja Spielvogel · Mahmoud Hassan · Ahmed El-Desouky · Hamdy El-Mansy · Frank Rath · Vera Meyer · Ulf Stahl

Received: 23 December 2009 / Revised: 9 February 2010 / Accepted: 10 February 2010

Abstract Secondary growth is a common post-harvest problem when pre-infected crops are attacked by filamentous fungi during storage or processing. Several antifungal approaches are thus pursued based on chemical, physical, or bio-control treatments; however, many of these methods are inefficient, affect product quality, or cause severe side effects on the environment. A protein that can potentially overcome these limitations is the antifungal protein AFP, an abundantly secreted peptide of the filamentous fungus *Aspergillus giganteus*. This protein specifically and at low concentrations disturbs the integrity of fungal cell walls and plasma membranes but does not interfere with the viability of other pro- and eukaryotic systems. We thus studied in this work the applicability of AFP to efficiently prevent secondary growth of filamentous fungi on food stuff and chose, as a case study, the malting process where naturally infested raw barley is often to be used as starting material. Malting was performed under lab scale conditions as well as in a pilot plant, and AFP was applied at different steps during the process. AFP appeared to be very efficient against the main fungal contaminants, mainly belonging to the genus *Fusarium*. Fungal growth was completely blocked after the addition of AFP, a result that was not observed for traditional disinfectants such as ozone, hydrogen peroxide, and chlorine dioxide. We furthermore detected reduced levels of the mycotoxin deoxynivalenol after AFP treatment, further supporting the fungicidal activity of the protein. As AFP treatments did not compromise any properties and qualities of the final products malt and wort, we consider the protein as an excellent biological alternative to combat secondary growth of filamentous fungi on food stuff.

Keywords Antifungal protein AFP · Barley · Malting · *Fusarium* · Mycotoxin · Bio-control

Hassan Barakat and Anja Spielvogel equally contributed to this work.

H. Barakat · A. Spielvogel · V. Meyer · U. Stahl
Department of Microbiology and Genetics,
Institute of Biotechnology, Berlin University of Technology,
Gustav-Meyer-Allee 25,
13355 Berlin, Germany

H. Barakat · M. Hassan · A. El-Desouky · H. El-Mansy
Department of Food Science, Faculty of Agriculture,
Benha University,
Moshtohor,
13736 Qaluobia, Egypt

V. Meyer
Department of Molecular Microbiology and Biotechnology,
Institute of Biology, Leiden University,
Sylviusweg 72,
2333 BE Leiden, The Netherlands

F. Rath
Institute of Brewing Science VLB, Department of Raw Material,
Seestrasse 13,
13353 Berlin, Germany

U. Stahl (✉)
TU Berlin, Institut für Biotechnologie,
Fachgebiet Mikrobiologie und Genetik, Gustav-Meyer-Allee 25,
13355 Berlin, Germany
e-mail: ulf.stahl@lb.tu-berlin.de

Introduction

The antifungal protein AFP, a secretory protein of the filamentous fungus *Aspergillus giganteus*, is a small, cysteine-rich protein that bears great potential for future antifungal strategies. The main advantage of AFP is its

Published online: 10 March 2010

specific growth inhibitory effect on filamentous fungi. The growth of several serious human- and plant-pathogenic fungi are efficiently inhibited in growth, whereas the viability of bacteria, yeast, plant, and mammalian cells is not negatively impaired (Szappanos et al. 2006; Theis et al. 2003; Theis et al. 2005). Besides this environmentally friendly mode of action, other properties of the protein make it as a prime candidate for biotechnological application: (1) AFP is remarkably resistant to heat and protease degradation (Lacadena et al. 1995; Theis et al. 2003), (2) it can be produced in a sustainable way by fermentation of *A. giganteus*, and (3) it is active in the micromolar range, i.e., low amounts of the protein are sufficient to combat fungal growth (Lacadena et al. 1995; Theis et al. 2003).

Whereas one of our interests is to unravel the mode of action of the protein (for review see, Meyer 2008), we and other groups also explore different possible applications of AFP and its suitability to substitute for currently used fungicides. For example, when AFP is directly applied to plant leaves or plant roots, the protein efficiently protects the plants against infection by devastating pathogens such as *Magnaporthe grisea* and *Fusarium oxysporum* (Theis et al. 2005; Vila et al. 2001). Other approaches testing transgenic *afp* expressing plants were successful, too—transgenic wheat, rice, and pearl millet were significantly less susceptible towards infections by *Erysiphe graminis*, *Puccinia recondite*, *M. grisea*, and *Sclerospora graminicola*, respectively (Coca et al. 2004; Girgi et al. 2006; Moreno et al. 2005; Oldach et al. 2001). Most importantly, neither the external application of AFP on plants nor transgenic expression of the *afp* gene was detrimental for the plants.

While the approaches tested so far focused on the question on how fungal infections can be prevented in living plants, we questioned whether AFP can also be used to suppress secondary growth of filamentous fungi on harvested plant material. Secondary growth is a common post-harvest problem when (pre-infected) crops become attacked by fungi due to improper storage conditions. In addition, secondary growth is also an inherent and so far unsolved problem of some food processing chains. A very prominent example is the malting process, where barley becomes processed via the three steps steeping, germination, kilning; the first two of which unintentionally adjust excellent environmental conditions for fungal growth. Especially growth of various *Fusarium* species can be observed during the malting process which in turn causes many food safety and quality concerns. Various (secondary) metabolites and enzymes produced by *Fusarium* can affect the quality of the final products malt, wort, and beer. For example, they inhibit the function of process important barley enzymes, cause off flavors, and are thought to be responsible for the gushing phenomenon (Hasan 2001; Laitila et al. 2007; Pekkarinen et al. 2007). Of special importance are heat-stable mycotoxins such as deoxynivalenol, and the trichothecenes HT-2 and T-2, because they survive the kilning process and accumulate in the end-product beer (Bullerman and Bianchini 2007; Hazel and Patel 2004).

The aim of the current study was to test the applicability of AFP to efficiently prevent secondary growth of filamentous fungi on food stuff. As a case study, we chose to apply AFP in the malting process and thus used naturally infested raw barley as starting material. We determined the inhibitory activity of AFP towards fungal species usually detected during malting, applied AFP during steeping and germination, analyzed the levels of fungal infestation, and compared them with levels observed for traditional disinfectants such as ozone, hydrogen peroxide, and chlorine dioxide. Finally, we determined the impact of the AFP treatments on the properties and qualities of the final products malt and wort.

Materials and methods

Fungal strains and materials used A. giganteus IfGB0902, *F. oxysporum* IfGB 4287, and *Altarnaria alternata* IfGB0203 were obtained from the Institut für Gärungsgewerbe (Berlin, Germany). *Fusarium graminearum* DSM 4527, *Fusarium acuminatum*, DSM 62148, *Fusarium tricinctum* DSM 62446, *Fusarium sporotrichioides* DSM 62425, *Fusarium avenaceum* DSM 62161, *Fusarium crookwellense* DSM 8704, *Fusarium poae* DSM 62376, and *Fusarium equiseti* DSM 62202 were obtained from the Versuchs-und Lehranstalt für Brauerei in Berlin (VLB, Germany). Raw barley (*Hordeum vulgare* L., Mary cultivar, season 2007 and Braemar cultivar, season 2008) was obtained from VLB.

Purification of AFP AFP was isolated from *A. giganteus* IfGB0902 culture supernatants according to the method described earlier (Theis et al. 2003), using cellulose phosphate instead of carboxymethyl cellulose as cation exchanger. The concentration of purified AFP was determined by SDS-PAGE (Schägger and von Jagow 1987).

Determination of minimal inhibitory concentration (MIC) The MIC of AFP was determined after (Theis et al. 2003). Added to 200 µl of potato dextrose broth containing AFP at different concentrations ranging from 0 to 50 µg/ml were 10^3 spores of *F. graminearum*, *F. acuminatum*, *F. tricinctum*, *F. sporotrichioides*, *F. crookwellense*, *F. avenaceum*, *F. poae*, and *F. equiseti*, respectively. After 48 h of cultivation at 28°C, fungal growth was evaluated spectrophotometrically at a wavelength of 600 nm. Measurements were carried out in triplicates.

DNA extraction and species-specific polymerase chain reaction (PCR) 10 g steeped, germinated untreated or treated barley were lyophilized and grinded. DNA was extracted twice using 0.25 g ground material as previously described (Fredlund et al. 2008). Species-specific primers (Table 1) were used for PCR-based detection of genomic DNA from *H. vulgare*, *F. graminearum*, *F. sporotrichioides*, and *F. poae*. Standard PCR conditions were used (Sambrook and Russel 2002).

Malt production at laboratory scale 10 g barley kernels, placed on a filter paper in a Petri dish were immersed for 4 h in 4 ml of sterile water, followed by an air-resting period of 20 h (steeping I). Steeping II was performed immediately after (kernels were soaked for 2 h in water, followed by an air-resting period of 22 h). Grains were allowed to germinate for 3-4 days at 15°C. AFP was applied at different concentrations to the samples, either by dissolving it in the steeping water or by spraying it onto kernels. Samples were taken daily to determine the germination vigor (GV, see below), α–amylase activities (see below), as well as fungal contamination using species-specific PCR.

Malt and wort production in a pilot plant 60 g barley was used as starting material to be processed in small strainer cans (73 × 142 mm), rotating at 2 rpm. Steeping I (5-h water immersion, 19-h air rest, and 14.5°C) was followed by steeping II (2-h water immersion, 22-h air rest, and 14.5°C). Subsequently, steeped kernels were allowed to germinate in growth chambers at 14.5°C and at 90-95% relative humidity. Green malt samples were kilned using the following temperature program: 14.5 ramping to 40°C for 10 h followed by 8 h at 40 ramping to 50°C, followed by 5 h at 80°C, and then de-rooted using cleaning malt machine. Malt extract was gained using 50 g kilned barley malt as starting material, which was milled (Buhler Miag disk Mill, Switzerland) and thereafter mixed with 200 ml water in a mashing bath (Buhler, Switzerland). Mashing was done with continuous agitation at 80-100 rpm and applying a defined temperature program (45°C for 20 min followed by 70°C for 60 min). Subsequently, the mixture was allowed to cool down to 20°C and filtered using Whatman filter (Biometra, Germany). The complete procedure was performed twice.

AFP addition and disinfection AFP was diluted in H_2O and sprayed onto germinated barley kernels to reach a final concentration of 16 μg/g barley dry weight. Ozonated water, chlorine, and H_2O_2 were sprayed onto 800 g germinated barley kernels to reach the following final concentrations: ClO_2 (10 μg/g), ozone (5 μg/g), and H_2O_2 (10% *w*/*w*). The volumes of the solutions were kept as small as possible to ensure complete imbibition by the barley samples. All disinfection treatments were performed after steeping II.

Analytical methods All methods analyzing the quality of malt and wort (GV, malt water content, malt friability, total protein and total soluble nitrogen, free amino nitrogen, β–Glucan content, fermentable sugar content, viscosity, wort color, and gushing potential) strictly followed the protocols of the European Brewery convention (EBC-Analytica 1998). For determination of α-amylase activity, the CERALPHA method was used (Megazyme Int., Ireland). All analytical methods were performed at least in triplicates.

Mycotoxin detection To measure the mycotoxin load of the barley samples, the method described (Lancova et al. 2008) was followed. In brief, 10 g barley or green malt were milled and subjected to ethyl acetate and dichloromethane extraction, respectively. Both extracts were mixed, filtered, and washed twice with ethyl acetate and dichloromethane. The filtrate was evaporated and reconstituted in 200 μl mobile phase (MeOH:H_2O, 30:70 *v*/*v*) and subjected to LC-MS/MS.

Results

PCR-based quantification of *Fusarium* species on barley

In order to qualitatively and quantitatively ascertain fungal growth during the malting process, we established a PCR-

Table 1 Primers used in this study

Primer name	Organism	Sequence (5′-3′)	Reference
Bar R Bar F	*H. vulgare*	GGTACGAACACGCTGGGC CCGGCCTCCACCCGAAGAG	This work
Fg16F Fg16R	*F. graminearum*	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	(Nicholson et al. 1998)
AF330109CF AF330109CR	*F. sporotrichioides*	AAAAGCCCAAATTGCTGATG TGGCATGTTCATTGTCACCT	(Demeke et al. 2005)
FP82F FP82R	*F. poae*	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	(Parry and Nicholson 1996)

based system, which focused on the detection of the three main mycotoxigenic *Fusarium* species that are frequently found on barley—*F. graminearum*, *F. sporotrichoides*, and *F. poae* (Rabie et al. 1997). Specific primers were used for these species as well as for barley to obtain PCR fragment sizes which can be detected by classical and quantitative real-time PCR approaches (Table 1). The specificities of all primer pairs were confirmed by PCR using mixtures of genomic DNAs from all four species as templates (data not shown). We also investigated the sensitivity of the PCR detection method. Sterilized barley kernels were suspended in a spore solution of *F. sporotrichioides* for 15 min. DNA was isolated from barley kernels immediately after this short incubation and after an incubation period of 3 days at 28°C which allowed germination and growth of *F. sporotrichioides* on the barley kernels. For both experimental conditions, fungal DNA could be detected by PCR even in the presence of an excess of barley DNA (1 µg; data not shown). Thus, this approach proved to be valuable to monitor fungal growth on barley samples during the malting process.

Antifungal activities of AFP against barley-specific fungal contaminants

To judge the general potential of AFP to prevent or inhibit secondary growth of fungal species on barley, we determined the MIC of AFP against barley-specific fungal contaminants under in vitro conditions. As depicted in Table 2, *F. graminearum*, *F. acuminatum*, *F. tricinctum*, *F. equiseti*, *F. sporotrichioides*, *F. crookwellens*, *F. avenaceum*, *F. poae*, *F. oxysporum*, *and A. alternata* are highly sensitive towards AFP as their growth was completely inhibited at AFP concentrations between 1 and 15 µg/ml.

However, determination of the in vitro MIC is an artificial approach and does not reflect the conditions present during the malting process. Not only different species can be present on the same kernel surface but they also might interact with each other and could thus react differently on the presence of AFP (Hagen et al. 2007). Moreover, fungal growth can only be inhibited by AFP if the protein is able to penetrate the kernel's surface and if it has a sufficient stability to survive the different steps during malting. We thus performed a lab scale malting and used naturally infested barley as starting material. Applied at three different steps of the malting process was 16 µg AFP/g barley dry weight which we considered critical control points: steeping I, steeping II, and germination (Table 3). Barley germination was monitored over time by visual inspection and kernel samples were taken after different points in time, freeze-dried, and used for DNA extraction. DNA samples were subjected to species-specific PCR approach to determine contamination with *F. graminearum* and *F. poae* (see "Materials and Methods" section). The results of adding AFP during steeping II are exemplarily shown in Fig. 1. Growth of *F. graminearum* increased in untreated samples over time during the malting process. However, contaminations of *F. graminearum* and *F. poae* were markedly reduced when AFP was applied to barley kernels compared to the untreated samples (Fig. 1 and Table 3). The most efficient growth inhibitory effect was observed, when AFP was added during steeping I (about 85%) however, a 50% reduction of fungal growth was still reached when AFP was added at the beginning of the germination period (Table 3).

Table 2 Minimal inhibitory concentrations (MIC) of AFP against different fungi

Organism	MIC µg/ml
F. graminearum	2
F. acuminatum	2
F. tricinctum	1
F. equisiti	5
F. sporotrichioides	1
F. crookwellense	1
F. avenaceum	2
F. poae	15
F. oxysporum	1
A. alternata	2

Antifungal activities of AFP under pilot plant conditions

In order to evaluate the growth inhibitory effect of AFP under industrial settings, we carried out a malting process in a pilot plant, where naturally infested barley kernels were steeped, germinated, and kilned under controlled moisture and temperature conditions: in a two-step steeping process for 48 h, the moisture content of the kernels was raised to approximately 45% at 14.5°C. Subsequently, the kernels germinated in humidified air at 14.5°C for 3-4 days and formed green malt. Finally, kilning at 80°C for 5 h was preceded by a pre-drying period where the temperature was slowly raised from 14.5°C to 50°C during 18 h, to reduce the water content of green malt to 10-12%.

We decided to add AFP after steeping II by spraying an AFP solution over the steeped kernels (16 µg AFP/g barley dry weight; treatment 3, Table 3) and monitored growth of *F. graminearum* and *F. poae* by species-specific PCR over time. As expected, growth of both fungi continuously increased during steeping and germination in untreated samples (Fig. 2). However, the presence of AFP completely blocked further growth of both contaminants after steeping

Table 3 Growth inhibitory effect of AFP (in percent) on two selected test organisms during lab scale malting

	Steeping-I	Steeping-II	Germination	Inhibition of *F. graminearum* (%)	Inhibition of *F. poae* (%)
Control	-	-	-	0	0
Treatment 1	-[a]	-[a]	-[b]	83.3	87.4
Treatment 2	-	-[a]	–	65.8	64.7
Treatment 3	–	–	-[c]	52.8	54.1

– AFP was not added. The experiment was performed in duplicate

[a] AFP was added to steeping water

[b] AFP was added to the wet filter paper

[c] AFP was sprayed on steeped barley

II, demonstrating the excellent potential of the protein to prevent secondary growth of *Fusarium* species. To determine, whether such a strong inhibitory effect of AFP also correlated with a reduced mycotoxin load, we measured the deoxynivalenol (DON) content in malt obtained from AFP-treated or untreated barley kernels. As summarized in Fig. 3, the DON content was significantly reduced in AFP-treated samples, further emphasizing the value of AFP as antifungal agent.

We also compared the inhibitory potential of AFP to common disinfectants such as ozone, hydrogen peroxide, and chlorine dioxide. These disinfection agents are nowadays frequently used in food industries as antimicrobial compounds due to their oxidizing properties (Bengtson et al. 2009). Remarkably, the growth inhibitory potential of AFP is clearly superior to the other compounds tested. Whereas AFP stopped growth of *F. graminearum*, *F. poae*, and *F. sporotrichioides* immediately after addition to the kernels, these fungi continued to grow in the presence of the other compounds before a steady state level was reached (Fig. 4, data not shown).

Impact of AFP on malt and wort quality

The main goal of a malting process is to provide sufficient sugar extract and free amino acids for the fermentation and specific flavor components for the final product beer. Consequently, high malt quality is not only defined by the absence of any fungal metabolites but also by parameters that have an influence on either the process or the final product. Standard procedures are therefore routinely used to evaluate malt and wort properties such as germination vigor, amylolytic enzyme activities, viscosity, and wort color (EBC-Analytica 1998).

We thus finally assessed any potential impact of AFP on the quality of the products malt and wort. As displayed in Fig. 5, AFP has no negative effect on the germination vigor of barley. Similarly, no negative consequences are provoked by AFP on 14 different malt and wort characteristics (summarized in Table 4), suggesting that the product qualities can be guaranteed when AFP is used as antifungal agent during malting.

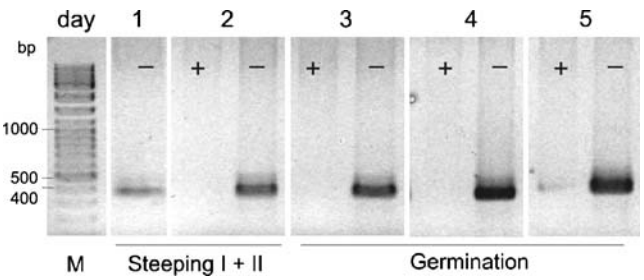

Fig. 1 Effect of AFP on the growth of *F. graminearum* on naturally infected barley during lab scale malting. Each 10 g barley kernels were used as starting material. Steeping I and II were performed as described under "Materials and Methods" section; germination was carried out for 3 days at 15°C. AFP was added at a final concentration of 16 μg/g dry weight barley during steeping II. Growth of *F. graminearum* was determined via species-specific PCR. Similar results were observed for *F. poae* (data not shown). + AFP added; − control without AFP (M) DNA marker

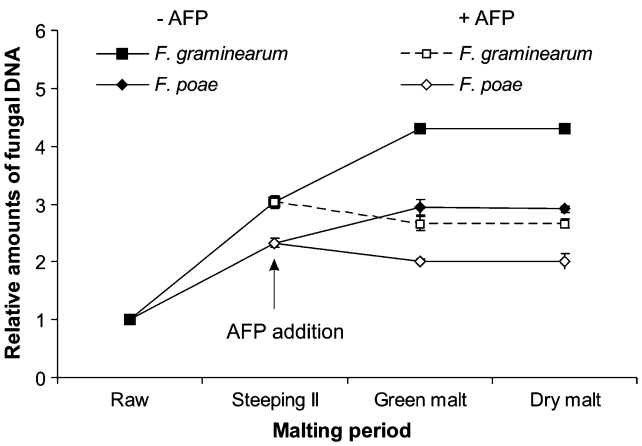

Fig. 2 Secondary fungal growth on naturally infested barley in the presence or absence of AFP. Growth of *F. graminearum* and *F. poae* were determined via species-specific PCR

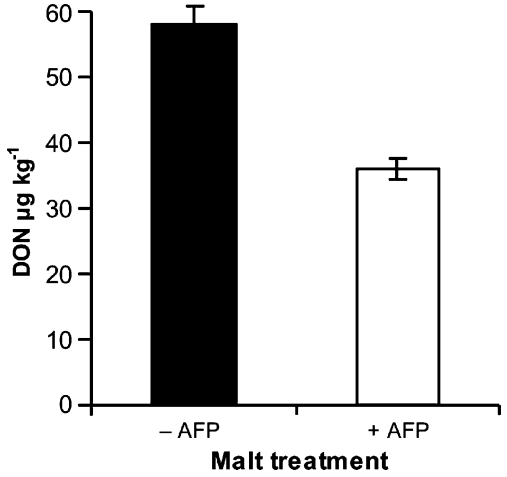

Fig. 3 DON content of the product malt obtained after a pilot plant malting process in the presence or absence of AFP. AFP was added as after steeping II (16 µg AFP/g dry weight). Mean values of a duplicate experiment are given

Discussion

Some food processing chains have to deal with the adversity that they are performed under conditions that strongly support secondary fungal growth, a scenario that is of course not intended. Hence, economically feasible and consumer acceptable solutions have to be found in order to prevent secondary growth. Several approaches are thus nowadays pursued to control and limit fungal contaminations during food processing. For example, chemical (disinfection), physical (sorting), and biological (bio-control) methods are applied to antagonize fungal growth during the malting process. Lactic acid bacteria and yeasts (*Geotrichum candidum*) are added during steeping to decrease the fungal load (Lowe and Arendt 2004). These

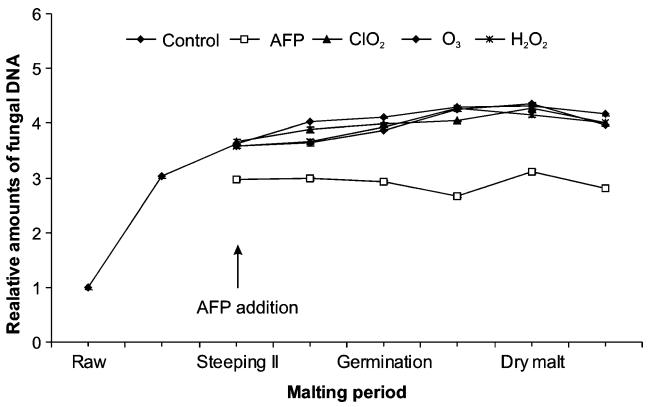

Fig. 4 Antifungal activities of different oxidizing agents and AFP. The compounds were added after steeping II in concentrations commonly used in food industries (10 ppm ClO_2/g dry weight, 5 ppm O_3/g dry weight and 10% (*v*/*w*) H_2O_2). AFP was added after steeping II (16 µg AFP/g dry weight). Growth of *F. graminearum* was determined as described in Fig. 2. Each data point corresponds to one day during the malting procedure, starting with raw barley on day 0

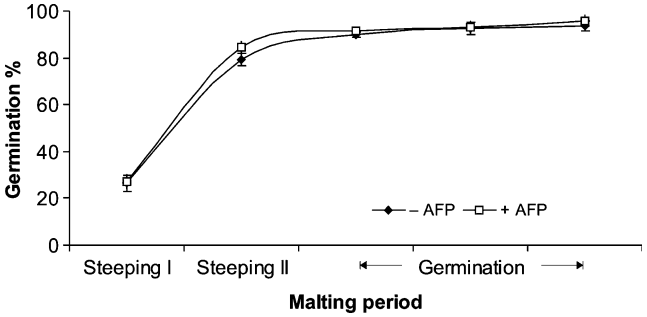

Fig. 5 Kinetics of barley germination in the presence or absence of AFP. AFP was added during the whole process at 16 µg AFP/g dry weight (treatment 1) using 16 µg AFP/g dry weight). One hundred kernels each were counted from a biological triplicate

bio-control strategies are based on the synthesis of antifungal compounds such as lactic acid, acetic acid, hydrogen peroxide, bacteriocins, and other uncharacterized agents (Lowe and Arendt 2004). However, the excretion of these compounds is not uniform. The mode of action of many of these is not understood and the growth inhibiting effects of these cocktails is not predictable. To overcome these limitations, we have studied in this work the potential of the biologically derived protein AFP to prevent fungal growth during the malting process.

Table 4 Malt and wort properties after a pilot plant malting process using barley kernels treated or not treated with AFP

Parameters	Barley treatments	
	Barley	Barley+AFP
Malt properties		
Water content (%)	4.2±0.10	4.1±0.12
Extract (%)[a]	82.3±0.35	81.6±0.29
Crude protein (%)[a]	9.6±0.17	9.6±0.12
Friability (%)	99±0.29	100±0.00
α–amylase CUg^{-1}[a]	259.69±2.34	270.52±3.08
Gushing potential	None	None
Wort properties		
Soluble nitrogen mg/100 g[a]	694±5.77	695±4.04
Kolbach index	45.3±0.40	45.4±0.46
Viscosity (8,6%), mPas	1.49±0.02	1.49±0.03
Fermentable sugar (%)[a]	84.8±0.58	85.8±0.63
pH	5.8±0.02	5.7±0.03
β-Glucan mg/l	74±1.73	59±1.00
FAN, mg/l	171±3.79	174±1.73
Wort color	4.8±0.09	5.1±0.10
Boiled wort color	6.8±0.11	7.0±0.15

The measurements were performed according to standard protocols (EBC-Analytica 1998). Mean values ± standard error of a duplicate experiment are given

[a] Values were calculated based on dry weight

In general, the first aim of the malting process is to increase the water content of the barley kernels during steeping. This water uptake induces the germination process which is characterized by the activation of starch degrading enzymes (e.g., α-amylases) which in turn release fermentable sugars such as maltose and glucose, proteases, and plant cell wall degrading enzymes (Jones and Marinac 2002). The released sugars and amino acids are then used as carbon and nitrogen sources by brewing yeasts or fermenting bacteria during alcoholic or non-alcoholic malt-based beverage production. It has recently been shown that secondary growth of fungi mainly starts after steeping and continues throughout the malting process (Wolf-Hall 2007). Therefore, we decided to add AFP during (treatments 2) and after steeping II (treatment 3). However, due to the fact that no data was available on how efficient and stable AFP is adsorbed to the kernels and whether it is washed out during steeping, the protein was also added during steeping I, steeping II, and germination (treatment 1). All three treatments proved to be very efficient during lab scale conditions and limited fungal growth as exemplarily observed for *F. graminearum*, *F. poae*, and *F. sporotrichioides*. However, supplementation of AFP to the steeping water, where a large amount of water is used, is not feasible under large-scale industrial settings. Thus, we tested the applicability of AFP in a pilot plant malting only for treatment 3. Still, growth of *Fusarium* species was very efficiently prevented, demonstrating the power of the protein and suggesting that spraying of AFP on germinating barley kernels is a straightforward approach to inhibit secondary growth.

Finally, the effectiveness of AFP was compared to other chemical disinfection methods. Ozone, chlorine dioxide, and hydrogen peroxide treatments are commonly used and recommended for the reduction of surface contamination in the food industry (Demirkol et al. 2008). All three compounds were used in this work at concentrations usually applied in the food industry (Chaidez et al. 2007; Demirkol et al. 2008; Fukuzaki 2006). To our surprise, however, none of these procedures caused a significant reduction of fungal contaminants. Virtually no differences were observed between untreated and disinfectant-treated barley, which supported previous findings (Andrews et al. 1997). In the case of chlorine dioxide, it was proposed that the compound does not penetrate the kernels to reach fungal contamination sites and is thus not efficient enough (Andrews et al. 1997). This would permit the reverse conclusion that the high antifungal efficiency of AFP might also be due to its ability to penetrate into the kernels, a speculation that awaits experimental validation.

We also tested the impact of AFP and the disinfectants on the mycotoxin levels within barley and malt. Mycotoxin formation during the malting process is a severe health risk (Wolf-Hall 2007). Although many water-soluble mycotoxins such as DON are washed out during steeping, secondary growth of mycotoxigenic fungi causes a new formation of mycotoxins (Linko et al. 1998). None of the established disinfectants tested in this study was able to reduce DON levels, even a slight increase was observed (data not shown). A possible explanation for this phenomenon might be that the combination of oxidative stress with low dose fungicides provoked DON synthesis as counteracting response (Magan et al. 2002; Reverberi et al. 2005). In contrast, AFP markedly reduced DON levels, further supporting the fungicidal activity of the protein. However, the impact of AFP on the presence of other mycotoxins has to be tested as well to comprehensively judge any antimycotoxigenic effect of AFP. In the naturally infested barley samples tested in this work, however, the levels of any other mycotoxins tested (zearalenone, nivalenol, T2-toxin, and HT2-toxin) were below the detection limits (data not shown).

Most importantly, the application of AFP during the malting process had no obvious negative impact on the quality of malt and wort. None of the quality parameters put forward by the European Brewing Convention differed in AFP-treated compared to untreated samples. Thus, the antifungal protein AFP can be considered as an excellent biological alternative to combat secondary growth of filamentous fungi on various food stuffs. The advantages of using AFP are obvious—it can be produced in a sustainable way, it is water-soluble and thus easy applicable, it is highly specific against filamentous fungi and it does not interfere with quality parameters of the final product.

Acknowledgments We are grateful to Iris Fechter and Jean-Paul Ouedraogo for excellent technical assistance. This study was partially supported by a grant to HB from the Ministry of High Education, Mission Department, Egypt.

References

Andrews S, Pardoel D, Harun A, Treloar T (1997) Chlorine inactivation of fungal spores on cereal grains. Int J Food Microbiol 35:153–162

Bengtson P, Bastviken D, de Boer W, Oberg G (2009) Possible role of reactive chlorine in microbial antagonism and organic matter chlorination in terrestrial environments. Environ Microbiol 11:1330–1339

Bullerman LB, Bianchini A (2007) Stability of mycotoxins during food processing. Int J Food Microbiol 119:140–146

Chaidez C, Lopez J, Vidales J, Campo NC (2007) Efficacy of chlorinated and ozonated water in reducing *Salmonella typhimurium* attached to tomato surfaces. Int J Environ Health Res 17:311–318

Coca M, Bortolotti C, Rufat M, Penas G, Eritja R, Tharreau D, del Pozo AM, Messeguer J, San Segundo B (2004) Transgenic rice

plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. Plant Mol Biol 54:245–259

Demeke T, Clear RM, Patrick SK, Gaba D (2005) Species-specific PCR-based assays for the detection of *Fusarium* species and a comparison with the whole seed agar plate method and trichothecene analysis. Int J Food Microbiol 103:271–284

Demirkol O, Cagri-Mehmetoglu A, Qiang Z, Ercal N, Adams C (2008) Impact of food disinfection on beneficial biothiol contents in strawberry. J Agric Food Chem 56:10414–10421

EBC-Analytica (1998) Analytica EBC. Section 3 (barley analysis), 4 (malt analysis) and 8 (wort analysis), Hans Carl, Nürnberg-ISBN 3-418-00759-7

Fredlund E, Gidlund A, Olsen M, Borjesson T, Spliid NH, Simonsson M (2008) Method evaluation of *Fusarium* DNA extraction from mycelia and wheat for down-stream real-time PCR quantification and correlation to mycotoxin levels. J Microbiol Methods 73:33–40

Fukuzaki S (2006) Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Sci 11:147–157

Girgi M, Breese WA, Lorz H, Oldach KH (2006) Rust and downy mildew resistance in pearl millet (*Pennisetum glaucum*) mediated by heterologous expression of the *afp* gene from *Aspergillus giganteus*. Transgenic Res 15:313–324

Hagen S, Marx F, Ram AF, Meyer V (2007) The antifungal protein AFP from *Aspergillus giganteus* inhibits chitin synthesis in sensitive fungi. Appl Environ Microbiol 73:2128–2134

Hasan HA (2001) Phytotoxicity of pathogenic fungi and their mycotoxins to cereal seedling viability. Acta Microbiol Immunol Hung 48:27–37

Hazel CM, Patel S (2004) Influence of processing on trichothecene levels. Toxicol Lett 153:51–59

Jones BL, Marinac L (2002) The effect of mashing on malt endoproteolytic activities. J Agric Food Chem 50:858–864

Lacadena J, Martinez del Pozo A, Gasset M, Patino B, Campos-Olivas R, Vazquez C, Martinez-Ruiz A, Mancheno JM, Onaderra M, Gavilanes JG (1995) Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. Arch Biochem Biophys 324:273–281

Laitila A, Sarlin T, Kotaviita E, Huttunen T, Home S, Wilhelmson A (2007) Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors. J Ind Microbiol Biotechnol 34:701–713

Lancova K, Hajslova J, Poustka J, Krplova A, Zachariasova M, Dostalek P, Sachambula L (2008) Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25:732–744

Linko M, Haikara A, Ritala A, Penttilä M (1998) Recent advances in the malting and brewing industry. J Biotechnol 65:85–98

Lowe DP, Arendt EK (2004) The use and effects of lactic acid bacteria in malting and brewing with their relationships to antifungal activity, mycotoxins and gushing: a review. J Inst Brew 110:163–180

Magan N, Hope R, Colleate A, Baxter ES (2002) Relationship between growth and mycotoxin production by *Fusarium* species, biocides and environment. Eur J Plant Pathol 108:685–690

Meyer V (2008) A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. Appl Microbiol Biotechnol 78:17–28

Moreno AB, Penas G, Rufat M, Bravo JM, Estopa M, Messeguer J, San Segundo B (2005) Pathogen-induced production of the antifungal AFP protein from *Aspergillus giganteus* confers resistance to the blast fungus *Magnaporthe grisea* in transgenic rice. Mol Plant Microb Interact 18:960–972

Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW, Joyce D (1998) Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiol Mol Plant Pathol 53:17–37

Oldach KH, Becker D, Lorz H (2001) Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. Mol Plant Microbe Interact 14:832–838

Parry DW, Nicholson P (1996) Development of a PCR assay to detect *Fusarium poae* in wheat. Plant Pathology 45:383–391

Pekkarinen AI, Longstaff C, Jones BL (2007) Kinetics of the inhibition of *Fusarium* serine proteinases by barley (*Hordeum vulgare* L.) inhibitors. J Agric Food Chem 55:2736–2742

Rabie CJ, Lubben A, Marais GJ, Jansen van Vuuren H (1997) Enumeration of fungi in barley. Int J Food Microbiol 35:117–127

Reverberi M, Fabbri AA, Zjalic S, Ricelli A, Punelli F, Fanelli C (2005) Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production. Appl Microbiol Biotechnol 69:207–215

Sambrook J, Russel DW (2002) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, NY

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379

Szappanos H, Szigeti GP, Pal B, Rusznak Z, Szucs G, Rajnavolgyi E, Balla J, Balla G, Nagy E, Leiter E, Pocsi I, Hagen S, Meyer V, Csernoch L (2006) The antifungal protein AFP secreted by *Aspergillus giganteus* does not cause detrimental effects on certain mammalian cells. Peptides 27:1717–1725

Theis T, Wedde M, Meyer V, Stahl U (2003) The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization. Antimicrob Agents Chemother 47:588–593

Theis T, Marx F, Salvenmoser W, Stahl U, Meyer V (2005) New insights into the target site and mode of action of the antifungal protein of *Aspergillus giganteus*. Res Microbiol 156:47–56

Vila L, Lacadena V, Fontanet P, Martinez del Pozo A, San Segundo B (2001) A protein from the mold *Aspergillus giganteus* is a potent inhibitor of fungal plant pathogens. Mol Plant Microbe Interact 14:1327–1331

Wolf-Hall CE (2007) Mold and mycotoxin problems encountered during malting and brewing. Int J Food Microbiol 119:89–94