Gluten-degrading proteases in wheat infected by *Fusarium graminearum* – protease identification and effects on gluten and dough properties.

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Abstract

Recently, we have observed a relationship between poor breadmaking quality and protease activities related to fungal infection. This study aims to identify potential gluten-degrading proteases secreted by fungi and to analyse effects of these proteases on rheological properties of dough and gluten. *Fusarium graminearum* infected grain was used as a model system. Zymography showed that serine-type proteases secreted by *F. graminearum* degrade gluten proteins. Zymography followed by LC-MS/MS analyses predicted one serine carboxypeptidase and seven serine endo-peptidases to be candidate fungal proteases involved in gluten degradation. Effects of fungal proteases on the time-dependent rheological properties of dough and gluten were analysed by small amplitude oscillatory shear rheology and large deformation extensional rheology. Our results indicate that fungal proteases degrade gluten proteins not only in the grain itself, but also during dough preparation and resting. Our study gives new insight into fungal proteases and their potential role in weakening of gluten.

Key words: Wheat, gluten, *Fusarium graminearum*, protease, dough rheology

1 Introduction

Fusarium head blight (FHB) is the fungal disease caused by a range of *Fusarium* spp. and *Microdochium* spp. that can infest heads of cereals. Fungal infection occurs during flowering and is reported to reduce grain yield dramatically worldwide. Besides the severe reduction in grain yield, *Fusarium* spp. produce various mycotoxins, such as deoxynivalenol (DON). Apart from mycotoxins, fungi secrete hydrolytic enzymes targeting the plants' cuticle, cell wall, proteins, lipids, starch and nucleic acids to establish infection and to develop disease [1-7]. Secreted proteases by *Fusarium* spp. were shown to have the ability to degrade gluten proteins in an *in vitro* system [8-10]. Pekkarinen and Jones [11] showed that a trypsin-like proteinase purified from a *Fusarium culmorum* hydrolysed barley storage proteins. However, it is still unclear which fungal proteases in infected wheat can degrade gluten proteins and thus reduce the breadmaking quality of wheat flour.

The breadmaking quality of wheat flour relates to its ability to produce high quality breads (high volume, soft crumb, homogenous pores) during a standard breadmaking processes and is of high commercial importance. This breadmaking performance of wheat flour is largely determined by the quantity and quality of gluten proteins. Gluten proteins are storage proteins synthesised in endosperm cells during grain filling. They comprise two major types, gliadins and glutenins. Gliadins are monomers and consist of α-/β-, γ- and ω-types. Gliadins interact with each other or with glutenin polymers with non-covalent interactions such as hydrogen bonds and hydrophobic interactions [12], and contribute to the viscosity of wheat dough. Glutenins are polymers composed of high molecular weight-glutenin subunits (HMW-GS) and low molecular weight-glutenin subunits (LMW-GS) linked by disulphide bonds. Glutenins, particularly the HMW-GS, are responsible for elasticity of wheat flour dough [13]. The viscoelastic properties of gluten in dough are determined by quality parameters such as the ratio of glutenin to gliadin, the allelic variations of both HMW-GS and LMW-GS and the size of glutenin polymers in wheat flour [14-20]. During dough mixing, gluten proteins form a continuous gluten network providing viscoelastic properties to the wheat dough. Simultaneously, the structure and functionality of gluten undergo significant changes. The molecular structure of the gluten network is not fully understood, but both covalent (disulphide bonds) and non-covalent (hydrogen bonds, hydrophobic interactions and chain

entanglement) intermolecular interactions are thought to be important factors influencing the viscoelastic properties of gluten [21-24].

Fungal infection has been reported to have a detrimental effect on breadmaking quality of wheat flour. Nightingale, et al. [8] visually showed damaged gluten proteins in endosperm cells of FHB grain with scanning electron microscopy. However, they did not find clear differences in crude protein content nor in the composition and size distribution of gluten proteins between FHB and symptom-free grains [8]. Several other studies reported that the amount of glutenin, particularly HMW-GS, was reduced in *Fusarium* infected grains [9, 25-26]. Weaker dough has been reported by measuring the mixing properties of dough made with FHB wheat flour, and fungal proteases were suggested to be involved [9, 25-26]. However, the relationship between fungal proteases and the properties of wheat dough and gluten during the breadmaking process, particularly the period of dough rest when fungal proteases could have deleterious effects, is scarcely reported.

Our earlier study demonstrated that grain samples with extremely weak gluten suffered from infection by *Fusarium* spp. and *Microdochium* spp. [27]. The size of glutenin polymers was severely reduced and gluten-degrading proteases were assumed to be present in these grain samples. We hypothesized that fungi infecting wheat grain secret proteases that degrade gluten proteins both in grain and during dough preparation, and thus causing weak gluten. Grain infected by *F. graminearum* was chosen as a model system in the present study to investigate fungal proteases that target gluten proteins and their effect on gluten properties during breadmaking since the field-inoculation method for *F. graminearum* is well established. Furthermore, the availability of genome and secretome studies for *F. graminearum* aids the identification of candidate proteases of our interest. The presence and activity of proteases in wheat samples harvested from a field inoculated with *F. graminearum* and their ability to degrade gluten proteins were assessed by zymography. Proteome analysis was carried out to identify candidate proteases secreted from *F. graminearum* involved in degrading gluten proteins. Moreover, wheat dough and gluten prepared with and without partial substitution by *F. graminearum* infected samples were studied by extensional and oscillatory shear rheology to examine its effects on the rheological properties of gluten during dough preparation and resting.

2 Material and method

2.1 Wheat samples

Spring wheat cv. Bastian field inoculated with *F. graminearum* was kindly provided by Dr. Morten Lillemo, Norwegian University of Life Sciences. Cultivar Bastian possesses the HMW-GS 5+10 encoded by *Glu-D1* and is classified as the best class for breadmaking in Norway. Briefly, field inoculation with *F. graminearum* was carried out with grain spawn (infected oat kernels) that were prepared with four *F. graminearum* isolates (200726, 200838, 101177 and 101023). The spawn inoculum was dispersed in the field at a density rate of 10 g/m^2 at Zadoks growth stage 32/33. For optimal germination of ascospores, the field was mist irrigated twice for 9 min per hour in the evening after spawn application and four times during anthesis. Details of field experiments with spawn-inoculation are described in Tekle, et al. [28]. Wheat grains were harvested at maturity from four plots (1.5 x 5 m^2, four biological replicates). The average DON content was 24.2 ppm indicating the success of grain infection. The samples are hereafter referred to as *Fg*-infected wheat/grain samples. *Fg*-infected grain samples were milled to wholemeal flour on a Retsch hammer mill with a 0.5 mm sieve. Commercial wholemeal wheat flour (fine ground) containing 30 ppm ascorbic acid (Product name; Regal sammalt mel, fin) from Lantmännen Cerealia (Oslo, Norway) was used as base flour for dough/gluten analyses.

2.2 Analysis of protease activity

In order to assess protease activity, salt-soluble proteins were extracted from *Fg*-infected wholemeal flour or whole grain. One millilitre 100 mM sodium phosphate buffer (pH 7.0) was added to 200 mg flour or approximately 200 mg grain in a 2 ml tube filled with 2.8 mm zirconium oxide beads and proteins were extracted by using a Precellys 24 (Bertin Technology, France). Protein extraction from whole grains were included to improve the extraction of fungal protein. The extraction does not crush the whole grains but loosens the pericarp of *Fg*-infected grain and assists in extraction of fungal proteins, which are located in the outer layers of the grain. The program used for protein extraction was at 6600 rpm for 2 x 15 sec, with a 30 sec pause between shaking. Samples were thereafter centrifuged at 16060 *g* at 4 °C for 20 min and the supernatant was recovered and kept at -80 °C until use. Protein concentration

was measured by a Lowry protein assay (Bio-Rad Laboratories, Inc, USA), using bovine serum albumin as a standard to estimate protein content.

Protease activities were measured by Zymography. Zymogram gels (12.5 % (w/v) acryl/bis-acrylamide (37.5:1), 275 mM Tris-HCl pH 8.8, 0.05% (w/v) ammonium persulfate and 0.05 % (v/v) tetramethylethylenediamine) were cast with gluten (Amersham Life Science, Cleveland, OH, US) as substrate. The final concentration of gluten substrate in the Zymogram gels was 2 mg/ml with 0.5% (w/v) Sodium dodecyl sulphate (SDS) and 2.5 mM tris(2-carboxyethyl)phosphine hydrochloride. Ten micro gram salt-soluble proteins were separated on zymogram gels at 100 V for 4 h. Zymogram gels were renatured with 2.5 % (w/v) Triton X-100 and washed with 50 mM Tris-HCl (pH 7.6) prior to incubation with 50 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2$ and 0.2 M NaCl at 37 °C overnight. After incubation, gels were stained with 0.1 % (w/v) Coomassie brilliant blue R-250, 50 % (v/v) methanol and 7 % (v/v) acetic acid for one hour, and destained with 20 % (v/v) methanol and 7 % (v/v) acetic acid for two hours. Zymography was also carried out with protease inhibitors; protease inhibitor cocktail P9599 (volume according to manufactures' recommendation), E-64 (10 µM), Pepstatin A (20 µM), Ethylenediaminetetraacetic acid (EDTA, 10 mM), Phenylmethylsulfonyl fluoride (PMSF, 5 mM) and 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, 1 mM) (all inhibitors from Singma-Aldrich, St Louis, MO, USA). Protease inhibitors were added to protein extracts prior to gel electrophoresis as well as in the incubation buffer. The final concentration of each inhibitor is given in brackets above.

2.3 Identification of proteases

To identify proteases in *Fg*-infected grains, 60 µg salt-soluble proteins, extracted as described in *2.2* were reduced with 10 mM dithiothreitol (DTT), alkylated with 55 mM 2-iodoacetamide (IAA) and digested with Trypsin/Lys-C (Promega, USA) on a Microcon-10YM (Merck Millipore, USA) centrifugal filter unit at 37 °C overnight [30] (from here on called Crude-LC-MS/MS). Additionally, 80 µg salt-soluble proteins were also separated on pre-cast NuPAGE™ 12% Bis-Tris Protein Gels (Thermo Fisher Scientific, USA) and stained with Coomassie brilliant blue R-250 as described above (from here on called SDS-PAGE-LC-MS/MS). Each lane of the gel was divided into eight, roughly equal, pieces.

For identification of gluten-degrading proteases, 15 µg salt-soluble proteins were separated on a zymogram gel with gluten, and the gel was stained immediately after electrophoresis, without incubation (from here on called Zymography-LC-MS/MS). The white band was excised and divided into two pieces. Proteins in the gel pieces were reduced (10 mM DTT) and alkylated (55 mM IAA), prior to digestion with Trypsin/Lys-C at 37 °C overnight, and finally peptide extraction was accomplished by sonication. Peptide samples were prepared from four biological replicates for each method. All peptide samples, from gel pieces and digests from protein extracts, were purified and concentrated using a StageTip, C18 material filled in 200 µl pipette tips, according to Rappsilber, et al. [31] and Yu, et al. [32]. Peptides were eluted with 50 µl 70 % acetonitrile (ACN) and dried completely with a speed-vac (Thermo Fisher Scientific, USA). Peptides were resolved with loading buffer (2 % (v/v) ACN and 0.05 % (v/v) Trifluoroacetic acid). All peptides and approximately 2 µg peptides generated from gel pieces and protein extracts, respectively, were loaded onto a trap column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, Thermo Fisher Scientific) and then backflushed with a loading buffer described below onto a 50 cm × 75 µm analytical column (Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. × 50 cm, nanoViper, Thermo Fisher Scientific) for liquid chromatography-mass spectrometry (LC–MS/MS) analysis. Conditions for Ultra-high performance liquid chromatography (LC) were as follows: Loading pump, flow rate 20 µl/min with loading buffer; 2 % (v/v) ACN and 0.05 % (v/v) Formic acid (FA) and Nano/Cap pump, flow rate 0.3 µl/min with gradient of two buffers A (0.1 % (v/v) FA) and B (80 % (v/v) ACN, 0.08 % (v/v) FA). LC-gradient was run for 70 and 90 min for peptides prepared from gel pieces and protein extracts, respectively, from 4 to 45 % buffer B. Peptides from the 12 and 10 most intense peaks obtained from 90 and 70 min elution, respectively, were fragmented and mass-to-charge of these fragmented ions were measured (tandem mass spectrometry, MS/MS) with a Q Exactive™ Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, USA). The Q-Exactive mass spectrometer was set up as follows: a full scan (300–1600 m/z) at R=70,000 was followed by (up to) 10 MS2 scans at R=35,000 using an NCE setting of 28. Singly charged precursors were excluded for MS/MS as were precursors with z > 5. Dynamic exclusion was set at 20 s.

Proteins were identified using the database search program Mascot (http://www.matrixscience.com/) and searched against the *Gibberella zeae* (strain PH-1 / ATCC MYA-4620 / FGSC 9075 / NRRL 31084)

database (ID: UP000070720), *Triticum aestivum* (Wheat) database (ID: UP UP000019116), decoy and contaminants obtained from UniProt (https://www.uniprot.org/). The parameters for Mascot search were as follows: fixed modifications, Carbamidomethylation of Cys; variable modification, oxidation of Met and N-terminal acetylation; missed cleavages, 2; peptide tolerance, 10 ppm; MS/MS tolerance, 0.020 Da. Identified proteins were visualized with Scaffold4 (Proteome Software, USA) with thresholds for protein and peptide being 1.0 % and 0.1% false discovery rate, respectively, and minimum two peptide matches. Proteins identified with over 95 % probability in at least two of four biological replicates were defined as identified proteins.

Prediction of the signal peptide and transmembrane helices were carried out with SignalP v. 4.1 [33] and TMHMM Server v. 2.0 [33], respectively. A sequence containing signal peptide and no trans-membrane helixes outside of the signal peptide was considered as a secreted protein according to earlier secretome studies [1, 3, 6]. The prediction of protein subcellular localization was examined with two programs to have a higher confidence of the localization. One was WoLF PSORT[34], the software used in the secretome study of the *F. graminearum* by Brown, et al. [1] and Lowe, et al. [6]. The other was DeepLoc, a recently developed software by Armenteros, et al. [35], which Savojardo, et al. [36] evaluated to be the best to predict extracellular proteins among similar programs. When the prediction of subcellular localization differed between the two programs, the closest homologs were searched against the annotated SWISS-PROT database with BlastP (https://www.uniprot.org/blast/) and the subcellular localization of the closest homolog was accessed.

2.4 Analyses of dough and gluten

One of the four *Fg*-infected samples from 2.1 was used for analyses of dough and gluten as neither Zymography nor LC-MS/MS analysis showed clear difference between the four biological replicates. All measurements were carried out in duplicates.

2.4.1 Dough preparation

Doughs were prepared by mixing 10 g commercial wholemeal wheat flour with or without substitution by *Fg*-infected flour (0, 10, 20 and 30 %) with 2 % NaCl in a Mixograph (National Manufacturing,

Lincoln, NE., US) for 5 min with fixed water addition (68.2% on flour basis, corresponding to Farinograph water absorption of the commercial wholemeal wheat flour). To study the sole effect of proteases on dough properties, doughs were prepared from 100% commercial wholemeal wheat flour but with different salt soluble flour protein extracts as dough liquid. Aliquots of 5.6 g commercial and *Fg*-infected flour were extracted with 20 ml 2 % NaCl for 30 min at room temperature in a shaking incubator at 200 rpm. The suspension was then centrifuged at 45995 *g* for 20 min at 18 °C and supernatant was recovered and used for dough preparation either directly or after heat treatment at 70°C for 30 min. Assuming complete extraction of proteases, the doughs prepared with extract from *Fg*-infected flour should have the same level of protease as doughs prepared with 20% *Fg*-infected flour (5.6 g flour in 20 mL x 68.2% (water addition) = 20%).

2.4.2 Small amplitude oscillatory shear tests

Small amplitude oscillatory shear tests can be used to study the viscoelastic properties of dough at rest. Measurements are performed within the linear viscoelastic range of dough and have therefore no influence on the dough structure, which makes them suitable to study the time dependent behaviour of dough. For small amplitude oscillatory shear tests, doughs were rolled out to 4 mm thickness and dough discs of 26 mm diameter were cut out. The dough disc was mounted on a Physica MCR 301 Rheometer (Anton Paar, Stuttgart, Germany) equipped with a parallel plate of 25 mm diameter. A sand blasted lower plate was used to prevent slippage. The temperature of the lower plate was controlled with a Peltier element set at 24 °C corresponding to the dough temperature after mixing. Maximum normal force during sample mounting was set at 5 N with 0.2 N hysteresis. After the gap was adjusted to 2 mm for measurement, a layer of paraffin oil (Merck, Darmstadt, Germany) was applied around the dough to prevent drying. An amplitude sweep was performed at a set frequency of 10 rad/s to determine the linear viscoelastic range of the dough. Measurements within the linear viscoelastic range of dough were performed at an amplitude of 0.01% and a frequency of 10 rad/s. The time dependent changes of storage modulus G` (elastic component) and loss modulus G`` (viscous component) of dough were determined for 45 min starting directly after mounting (no rest period). To quantify the time dependent change of

G` a multiplicative inverse of G` (1/G`) was plotted against measurement time. The slope was calculated by linear regression.

2.4.3 Large deformation rheology

Analysis of large deformation rheology was performed with the SMS/Kieffer Dough and Gluten Extensibility Rig. The method measures the capacity of the gluten network to retain the gas during fermentation by measuring the resistance to extension and extensibility. Gluten was prepared in a Glutomatic 2100 (Perten AB, Huddinge, Sweden) from commercial wholemeal wheat flour with or without substitution by *Fg*-infected flour (0, 20, 30 and 40 %). A 2% (w/v) NaCl solution was used for mixing the dough (1 min) and washing out starch, bran particles and the salt soluble components (9 min). The gluten was centrifuged in a special centrifuge mold in a swing-out rotor (Rotor 5.51) at 3000 *g* at 20 °C for 10 min, and rested in a standard mold at 30°C for 45 min. Three pieces of gluten from each preparation were stretched with the Kieffer rig until they ruptured. The maximum resistance to extension (R_{max}) and distance (Ext) were recorded by a TA.XT plus Texture Analyzer (Stable Micro Systems, Godalming, UK).

To study the effect of fungal proteases on gluten, while omitting the effect of fungal α-amylases on starch in dough, gluten was prepared from incubated dough and measured with the SMS/Kieffer Dough and Gluten Extensibility Rig. Doughs were prepared as described in 2.4.1. and incubated at 30 °C for 0, 45 and 90 min. Thereafter gluten was obtained by washing the doughs with 2 % NaCl in a Glutomatic 2100 and measured with the Kieffer rig as described above.

To investigate the presence of proteases in washed gluten, gluten prepared with and without 20% *Fg*-infected flour as described above was stretched with the Kieffer rig after 45- and 90-min incubation and the results were compared.

2.4.4 Statistical analysis

Minitab 18 was used for statistical analysis. Statistical differences between R_{max}, Ext and slope of 1/G` were assessed for the proportion of *Fg*-infected flour (fixed factor) using regression analysis. Statistical differences between R_{max} and Ext were assessed for incubation time by two-way analysis of variance.

Significance was two-tailed $P<0.05$. Comparison between levels and interaction for two-way analysis of variance was carried out using the Tukey test at a confidence interval of 95%.

3 Results and discussion

We have earlier observed that wheat samples with extremely weak gluten suffered from infection by *Fusarium* spp. and *Microdochium* spp. [27]. The size of glutenin polymers in these samples was severely reduced and gluten-degrading proteases were assumed to be present. Here we confirm the presence of gluten-degrading proteases in wheat samples harvested from a field inoculated with *F. graminearum* as a model system, identify candidate proteases for gluten degradation and investigate their influence on functional dough properties.

3.1 Proteases in wheat grains infected by F. graminearum

Zymography of salt-soluble proteins extracted from *Fg*-infected wheat flour showed a thick smear that indicated strong protease activities (Fig. 1A and Supplementary Fig. SF1). This thick smear was also observed in zymograms without protein renaturing and gel incubation (Supplementary Fig. SF1C). The results revealed that some proteases can digest substrate proteins even under the denaturing condition with SDS. This explains the presence of a thick smear instead of distinct bands in the zymogram. For a comparison, proteins extracted from wholemeal flour of sound grain grown in a greenhouse were analysed with the same method and no bands appeared (data not shown). This result indicates that the level of gluten-degrading proteases in sound grain is lower than the detection level with zymography. Our results clearly showed that proteases detected by zymography were of fungal origin and they could efficiently digest gluten proteins. Although substrate gluten proteins are reduced and their structure differs from native gluten proteins in grain or flour, we assumed that *F. graminearum* secreted proteases also can degrade native gluten proteins. Our results support the study of Eggert, et al. [10] who demonstrated the degradation of gluten proteins during incubation with proteases from *F. graminearum*.

Zymography was also carried out with protease inhibitors to investigate which type of proteases degrade the different substrate proteins (Fig. 1B-H). The intensity of the band in the zymogram was strongly reduced with PMSF and moderately reduced with protease inhibitor cocktail (Fig. 1B and F). Sulfonyl

fluorides of both PMSF and AEBSF react with the hydroxyl of the serine residue, the active site of serine proteases, and inhibit serine proteases [37]. More stable, and less toxic, AEBSF has been used as an alternative for PMSF, however our results showed that AEBSF did not inhibit proteases that degrade gluten proteins. Nevertheless, our results suggest that serine proteases are the main gluten-degrading proteases in *Fg*-infected wheat grains. Pekkarinen and co-authors [11, 38] purified serine proteases from gluten-containing culture medium of *Fusarium culmorum* and one serine protease (trypsin-like enzyme) was able to degrade storage proteins of barley. Hence, some serine proteases secreted by fungi are probably involved in nitrogen acquisition from the host plant.

3.2 Identification of proteases in wheat grain infected by F. graminearum

For identification of fungal proteases, salt-soluble proteins were extracted from whole grain instead of wholemeal flour. It was presumed that the amount of fungal proteins is higher in bran, because fungi grow from the surface of the infected grain. Therefore, protein extraction from whole grain would increase the proportion of fungal proteins, while decreasing the proportion of plant proteins, and thereby increasing the likelihood of identifying fungal protein/proteases by LC-MS/MS analysis. The bran of *Fg*-infected grain was detached from the kernel, partly revealing the starchy endosperm, which remained intact after protein extraction by Precellys 24. This method lowered the protein concentration (data not shown), while protease activities analysed by zymography were higher in protein extracts from whole grain compared to protein extracts from wholemeal flour (Supplementary Fig. SF1A). The results indicate that the protein extracts from *Fg*-infected whole grain increase the proportion of fungal proteins compared to protein extracts from *Fg*-infected wholemeal flour. The salt-soluble proteins extracted from *Fg*-infected whole grain were either digested directly or separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) or zymography prior to peptide generation. The purpose of protein separation by SDS-PAGE was to increase the number of identified proteases by reducing the complexity of the samples. Since our major interest was to identify proteases that degrade gluten proteins, the area of smear from zymograms were excised and peptides were prepared for LC-MS/MS analysis. The proteome database of *F. graminearum* (Gibberella zeae, strain PH-1 / ATCC MYA-4620 /FGSC 9075 /NRRL 31084) consists of 14,160 entries in UniProt, and 267 proteins are listed as peptidases or

proteases (https://www.uniprot.org, Supplementary Table ST1, downloaded Nov, 2018). Mascot searches against *F. graminearum* and *T. aestivum* databases resulted in 172 and 85 identified proteins, respectively, from the Crude-LC-MS/MS, with 12 being fungal proteases (Table 1 and Supplementary Table ST2). Protein analysis by SDS-PAGE-LC-MS/MS improved the number of identified proteins dramatically, and 40 fungal proteases were identified (Table 1 and Supplementary Table ST3). From Zymography-LC-MS/MS, 179 proteins were of fungal origin and 24 of these were proteases (Table 1 and Supplementary Table ST4).

To investigate whether the identified proteases are secreted proteases, and thus likely to be involved in degradation of gluten proteins in the wheat grain, subcellular localization of all identified proteases was predicted from their amino acid sequences (Supplementary Table ST5). According to our analyses 20 secretory proteases were predicted (Supplementary Table ST5). Seventeen of them were previously predicted to be extracellular proteases, either in secretome studies, and/or proteomic studies of *F. graminearum* [1, 3-6]. Among our 20 predicted extracellular proteases, 15 were identified by Zymography-LC-MS/MS (Supplementary Table ST5). This indicates that these 15 proteases are capable of degrading gluten proteins. Inhibitor studies with zymography indicate that serine-type proteases (inhibited by PMSF) dominate. In fact, more than half of the secreted proteases identified by Zymography-LC-MS/MS were of the serine-type. Two of these, specifically one carboxypeptidase (FG05797) and one of the uncharacterized proteins (FG11164) seemed to be abundant as they were identified by all three methods. Brown, et al. [2] compared the transcriptome of F. graminearum between symptomless and symptomatic wheat and found some proteases that showed high transcript abundance in symptomatic wheat tissues. When our results were compared to their study, FG05797, FG03467, FG05245, FG06545 and FG03975 were among the proteins whose transcripts were significantly up-regulated in the symptomatic wheat tissues [2]. Proteases with high transcript abundance in symptomatic wheat tissues were presumed to be involved in the development of disease symptoms [2]. Some of them are probably responsible for nitrogen acquisition from host plants. Consequently, the carboxypeptidase (FG05797) is an interesting candidate as it is a serine-type protease. This protease has also previously been reported as a fungal secreted protease in several other studies (Table 2) [1, 3-4, 6]. Carboxypeptidase is an exo-

peptidases that cleaves C-terminal polypeptide bonds [40], hence efficient depolymerisation of intact glutenin polymers by this protease cannot be expected. However, when disulphide bonds of glutenin polymers are reduced, the proteases could cause weaker gluten by removing cysteine residues located at C-terminus in HMW-GS and preventing the formation of disulphide bonds. It has been reported that the disulphide bonds of glutenin polymers are reduced immediately after dough mixing, but is gradually re-established during dough rest [21, 40]. Therefore, it could be hypothesised that the presence of the fungal serine carboxypeptidase in flour efficiently removes cysteine residues located at the C-terminus in HMW-GS during dough mixing and rest, causing a weaker gluten. Moreover, seven uncharacterised proteins that by homology are characterised as serine endo-peptidases were identified and predicted to be extracellular. As endo-peptidases cleave peptide bonds internally, they could efficiently degrade glutenin polymers compared to exo-peptidases. These fungal serine endo-peptidases most likely contribute to gluten digestion both in grain and during dough preparation and resting thereby weakening the gluten. Among seven serine endo-peptidases, one of them (FG11164) is another interesting candidate as this proteases seemed to be abundant.

3.3 The effect of proteases on dough properties during breadmaking process

Infection of wheat with *F. graminearum* has a negative effect on breadmaking quality [9, 25-26]. We have previously shown that the size of glutenin polymers is severely reduced in wheat grain naturally infected by *Fusarium* spp. and *Microdochium* spp. in a field[27]. This reduction in glutenin polymer size (or disulphide bonds in glutenin polymers) found in flour can have a negative effect on gluten properties independent of potential activities of gluten degrading proteases during processing. As enzymes, including proteases, are salt-soluble proteins, the 10 min washing procedure with 2 % NaCl during gluten preparation is presumed to remove proteases from the gluten and minimize their effects during the remaining steps of the gluten large deformation rheology analysis. As proof of their removal neither R_{max} nor Ext differed during incubation of gluten for another 45 min (data not shown). The level of proteases is therefore negligible in washed gluten. Hence the method makes it possible to study the effect of reduced glutenin polymers in the *Fg*-infected flour on the viscoelastic properties of gluten. The highest R_{max} was obtained from gluten made from 100 % commercial flour, and the R_{max} value decreased

with increasing amount of *Fg*-infected flour ($P<0.01$, $R^2_{adj}=0.928$) (Fig. 2A). Simultaneously, the shortest Ext was observed from gluten made from 100 % commercial wholemeal flour, and the value increased with increasing amount of *Fg*-infected flour ($P=0.01$, $R^2_{adj}=0.536$) (Fig. 2B). Gluten lost elasticity and became more extensible when commercial flour was substituted by *Fg*-infected flour, and the changes were more pronounced with increasing amount of *Fg*-infected flour. It cannot be neglected that proteases degrade gluten proteins until they were washed out during gluten preparation, however due to time restriction (less than 10 min) their effect must be limited. The reduction in R_{max} and increase in Ext with increasing amounts of *Fg*-infected flour is therefore likely due to a reduction in glutenin polymer size (reduced disulphide bonds) in *Fg*-infected flour. MacRitchie and Gupta [41] reported a positive correlation between R_{max} and the proportion of unextractable polymeric proteins in total polymeric proteins (%UPP) and a negative correlation between Ext and %UPP. Our results correspond to their findings as increasing amounts of *Fg*-infected flour weaken gluten by decreasing the glutenin polymer size in flour blends.

As proteases from *F. graminearum* will degrade gluten proteins over time as demonstrated by zymography, they could influence the viscoelastic properties of dough by degrading gluten proteins during dough rest and proofing prior to baking. In small strain oscillatory measurements all doughs showed a decrease in G` over time (Fig. 3A). This is in accordance with a previous study reporting a decrease in dough elasticity (increase in tan δ) as a function of resting time [43]. It is already known that the rheological properties of resting wheat flour dough continuously change also during measurement in the linear viscoelastic range due to enzymatic reactions, changes in flour component interactions and relaxation of stresses induced during mixing, shaping and mounting in the rheometer [43]. Substitution of commercial wheat flour by *Fg*-infected flour resulted in an increased rate of decrease of G` (Fig. 3A). Interestingly we found a nearly perfect linear relationship between 1/G` as a function of resting time (R^2 = 0.985-0.996) (Fig. 3B). The slopes of these curves proportionally increased (R^2 in regression = 0,96) with the amount of *Fg*-infected flour (0, 10, 20 and 30 %) substitution (Fig. 4A). When doughs were prepared with different flour extracts the slope of 1/G` was the highest in doughs prepared with salt-soluble protein extracts from *Fg*-infected flour (Fig. 4B). Heat treatment of this extract before

incorporation into the dough resulted in a similar slope than for all other doughs prepared with 2% NaCl solution or extracts from commercial wholemeal flour with or without heat treatment (Fig. 4B). The elastic properties, measured as G`, of doughs made with increasing amounts of *Fg*-infected flour or extract from *Fg*-infected flour decreased more rapidly as a function of incubation time than for other doughs. The fact that the heat-treated extract from *Fg*-infected flour did not show the same effect as its untreated counterpart really points towards a significant role of fungal proteases on gluten protein degradation in dough during resting, which ultimately results in a weakening of the dough structure. Previous research has shown that random depolymerization of single-stranded polysaccharides in dilute solution gives a linear increase in their inverse molecular weight, intrinsic or specific viscosity as a function of their degradation time with the slope corresponding to the rate of chain cleavage [43-45]. This relationship has often been used to elucidate the conformation and structure of polymers in solution e.g. single stranded or double helix etc. [47]. The observed linear relationship between 1/G` and dough resting time (Fig. 3B) in the present study may therefore lend support to the recent proposal [23-24] that individual gluten molecules are linear, not permanently cross-linked, and interact via non-covalent interactions.

Fusarium graminearum secretes various enzymes including α-amylases for the acquisition of nutrients from its host (i.e. wheat grain). However, as wheat dough consists mostly of starch, fungal α-amylases may be partly involved in changing the dough properties observed by the oscillatory measurements. When comparing gluten from dough rested for equal durations, gluten prepared with 20% *Fg*-infected flour showed lower R_{max} and higher Ext ($P<0.01$) than gluten prepared without *Fg*-infected flour (Fig. 5). Maximum resistance to extension decreased and Ext increased with increasing dough incubation time in both gluten samples with and without *Fg*-infected flour ($P<0.01$) (Fig. 5). Wholemeal flour usually contains higher amounts of enzymes compared to white flour. This may explain the relatively large reduction in R_{max} of gluten prepared from rested control dough (100% commercial wholemeal flour) after 45- and 90-min incubation. The maximum resistance to extension tended ($P=0.11$) to decrease more rapidly with increasing dough incubation time in gluten prepared from dough made with *Fg*-infected flour. The result implies that fungal proteases further reduce the resistance to extension of gluten during dough rest. Rheological analyses showed that proteases from *F. graminearum* could have

adverse effects and contribute to weaken gluten during dough mixing and rest. It should be noted that the *Fg*-infected samples used in our study were highly infected as indicated by the high level of DON. Hence the level of proteases might be also high in our samples. The relationship between the levels of proteases and DON in wheat infected by *F. graminearum* deserves further investigation.

Our study demonstrates that serine-type proteases are responsible for degrading gluten proteins in *Fg*-infected grain. Two serine-type proteases, specifically FG05797 and FG11164, seem to be abundant and are thus interesting candidates for further investigation of their function. Rheological analyses revealed that both reduced glutenin polymer size and presence of gluten-degrading proteases in *Fg*-infected wheat flour weaken gluten, most likely by limiting disulphide bond formation and degrading gluten proteins, respectively, during dough mixing and rest. Further research should focus on whether other fungi than *F. graminearum* secrete proteases involved in weakening of gluten. Particular attention should be paid to fungi infecting wheat head which are not yet known to produce mycotoxins, such as *Microdochium* spp. They will not be removed from the food system under the current regulation, and thus the level of fungal proteases can be high and detrimental for the dough properties. Moreover, detailed mechanisms of how those proteases degrade gluten proteins and thus causing weak gluten should be further investigated.

4 Abbreviations Used

FHB, Fusarium head blight; DON, deoxynivalenol; HMW-GS, high molecular weight-glutenin subunits; LMW-GS, low molecular weight-glutenin subunits; *Fg*, *Fusarium graminearum*; SDS, Sodium dodecyl sulfate; EDTA, Ethylenediaminetetraacetic acid; PMSF, Phenylmethylsulfonyl fluoride; AEBSF, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride; DTT, dithiothreitol; IAA, 2-iodoacetamide; CAN, acetonitrile; LC, liquid chromatography; FA, Formic acid; MS/MS tandem mass spectrometry; MS, mass spectrometry; G`, storage modulus; G``, loss modulus G``; 1/G`, a multiplicative inverse of G`; R_{max}, maximum resistance to extension; Ext, distance to extension; SDS-PAGE, SDS–polyacrylamide gel electrophoresis;

5 Acknowledgement

Financial support for this study was provided by a grant from the Agriculture and Food Industry Research Funds and the Norwegian Fund for Research Fees for Agricultural Products (Norwegian Research Council grant number 233993, 294651, 262300). Wheat materials were kindly provided by Dr. Morten Lillemo at Norwegian University of Life Sciences.

Supporting information

Salt-soluble proteins were extracted from *F. graminearum*-infected wholemeal flour (A) or whole grains (A-C) and separated on zymograms copolymerized with gluten that were either incubated at 37 °C overnight prior to staining (A and B) or stained directly after gel electrophoresis (C). WF, Wholemeal flour and WG, whole grain. B and C shows four biological replicates (Supplementary Figure SF1). List of protease and peptidases in the proteome database of *F. graminearum* (Gibberella zeae, strain PH-1 / ATCC MYA-4620 /FGSC 9075 /NRRL 31084) in UniProt (downloaded Nov, 2018) (Supplementary Table ST1). Identified fungal proteins with their identification probability after Mascot search (Supplementary Table ST2-4). Peptides were generated from protein extracted from *F. graminearum*-infected whole grain and analysed by LC-MS/MS (Table ST2). Salt-soluble proteins were extracted from *F. graminearum*-infected whole grain and separated on pre-cast NuPAGE™ 12% Bis-Tris Protein Gels and stained with Coomassie brilliant blue R-250. Each lane of the gel was divided into eight, roughly equal, pieces and proteins were digested in the gel. Peptides were thereafter extracted from the gel pieces and analysed by LC-MS/MS (Table ST3). Salt-soluble proteins were extracted from *F. graminearum*-infected whole grain and separated on zymogram gel copolymerized with gluten and stained with Coomassie brilliant blue R-250 without incubation. White band (smear) was excised from the gel and divided into two pieces and proteins were digested in the gel. Peptides were thereafter extracted from gel pieces and analysed by LC-MS/MS (Table ST4). List of identified fungal proteases and analyses of their subcellular localisation (Supplementary Table ST5). Subcellular localization of all identified proteases in our study was predicted according to their amino acid sequences. A sequence

containing signal peptide and no trans-membrane helixes outside of the signal peptide (analysed by SignalP v. 4.1 and TMHMM Server v. 2.0, respectively) was considered as a secreted protein. The prediction of protein subcellular localization was examined with two programs, WoLF PSORT and DeepLoc. When the prediction of subcellular localization differed between the two programs, the closest homologs were searched against the annotated SWISS-PROT database with BlastP (https://www.uniprot.org/blast/) and the subcellular localization of the closest homolog was assessed.

The supporting information is available free of charge on the ACS Publications website.

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578

Figure captions

Figure 1. Salt-soluble proteins (10µg) extracted from *F. graminearum*-infected flour were mixed with and without protease inhibitors and separated on zymograms. Zymograms were incubated with the respective protease inhibitors at 37 °C overnight prior to staining. (A) without inhibitor, (B) Inhibitor cocktail, (C) E-64; trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, (D) Pepstatin A, (E) EDTA; Ethylenedinitrilotetraacetic acid, (F) PMSF; Phenylmethylsulfonyl fluoride, (G) AEBSF; 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, (H) NEM; N-Ethylmaleimide.

Figure 2. The maximum resistance to extension (R_{max}, A) and extensibility (Ext, B) of gluten. Gluten was prepared from commercial wholemeal flour with and without *F. graminearum*-infected wholemeal flour (0 – 40 %). Error bars show standard deviation of two replicates. * shows significant difference from control (without *F. graminearum*-infected flour) as analysed by Tukey test ($P<0.05$).

Figure 3. Time dependent change in storage modulus (G`, A) and the multiplicative inverse of G` (1/G`, B) during oscillatory measurement of dough prepared from commercial wholemeal flour (solid line), with 10% (dashed line), 20% (dotted line) or 30% (dash and dot) substitution by *F. graminearum*-infected wholemeal flour (lines are averages of two replicates).

Figure 4. The slope of 1/G`. Doughs were prepared with different amounts of *F. graminearum*-infected flour (0-40%) (A) and with 2% NaCl or extracts from commercial wholemeal flour (WF) or *F. graminearum*-infected wholemeal flour (Fg-WF) with/without heat treatment (+70 °C/blank, respectively) (B). Error bars show standard deviation of two replicates. Letters show significant differences as analysed by Tukey test ($P<0.05$).

Figure 5. The maximum resistance to extension (R_{max}, A) and extensibility (Ext, B) of gluten. Gluten was prepared from dough made of commercial wholemeal flour without (solid bar) and with *F. graminearum*-infected wholemeal flour (20 % inclusion level, open bar). Dough was incubated at 30 °C for 0, 45 or 90 min prior to washing with Glutomatic 2100. Error bars show standard deviation of two replicates. Letters show significant differences as analysed by Tukey test ($P<0.05$).

Table 1. Number of protein hits after Mascot search and identified fungal proteases

Protein analysis method	Number of protein hits			Number of identified fungal protease
	F. graminearum	*T. aestivum*	Total	
Crude-LC-MS/MS	172	85	257	12
SDS-PAGE-LC-MS/MS	614	433	1047	40
Zymography-LC-MS/MS	179	540	719	24

Table 2. List of predicted extracellular fungal proteases identified from *F. graminearum*-infected wheat grain

Protein name	Entry[a]	Gene name	Gene ontology (Molecular function)	Method[b]			Previously reported proteases				
				Crude	SDS-PAGE	Zym	Brown et al., 2012	Yang et al., 2012	Ji et al., 2013	Paper et al., 2007	Lowe et al., 2015
Carboxypeptidase											
	I1RP45	FG05797	serine-type carboxypeptidase activity [GO:0004185]	x	x	x	x	x	x		x
Extracellular metalloproteinase (Fungalysin)											
	I1RI44	FG03467	metalloendopeptidase activity [GO:0004222]; zinc ion binding [GO:0008270]	x	x	x	x				x
Peptide hydrolase											
	I1RR22	FG06527	aminopeptidase activity [GO:0004177]; metal ion binding [GO:0046872]	x	x					x	
	I1RMQ1	FG05245	metal ion binding [GO:0046872]; peptidase activity [GO:0008233]	x	x		x				x
	I1RR36	FG06545	metal ion binding [GO:0046872]; peptidase activity [GO:0008233]		x						
	A0A0E0RRY2	FG01818	metal ion binding [GO:0046872]; peptidase activity [GO:0008233]	x	x	x		x			x
Uncharacterized protein											
	I1RJF8	FG03975	aspartic-type endopeptidase activity [GO:0004190]		x	x	x	x			x
	A0A098DS79	FG09141	glutathione hydrolase activity [GO:0036374]	x	x	x					
	I1S379	FG11249	metallocarboxypeptidase activity [GO:0004181]; zinc ion binding [GO:0008270]		x	x	x			x	x
	I1RER9	FG02169	metallocarboxypeptidase activity [GO:0004181]; zinc ion binding [GO:0008270]		x	x					
	I1RM69	FG05052	metallopeptidase activity [GO:0008237]		x						x
	I1S1J0	FG10595	serine-type endopeptidase activity [GO:0004252]			x	x				x
	I1RHQ4	FG03315	serine-type endopeptidase activity [GO:0004252]		x	x	x			x	x
	I1RR60	FG06572	serine-type endopeptidase activity [GO:0004252]		x	x	x				x
	I1RB96	FG00806	serine-type endopeptidase activity [GO:0004252]		x	x	x	x			x
	I1S3S6	FG11472	serine-type endopeptidase activity [GO:0004252]		x	x	x				x
	I1RGU8	FG02976	serine-type endopeptidase activity [GO:0004252]		x	x	x				x
	I1S303	FG11164	serine-type endopeptidase activity [GO:0004252]	x	x	x				x	x
	I1S2N6	FG11036	serine-type peptidase activity [GO:0008236]	x	x		x				x
	I1S2I7	FG10982	serine-type peptidase activity [GO:0008236]		x	x	x				x

Subcellular localization of identified proteases was predicted according to their sequences by analysing with SignalP, TMHMM, DeepLoc and Wolf PSORT Identified proteases possessing a signal peptide, no transmembrane α-helix and predicted to be extracellular were listed in the table. [a]UniProtKB/Swiss-Prot entry name, [b]Method for protein analysis. Crude, Crude-LC-MS/MS; SDS-PAGE, SDS-PAGE-LC-MS/MS; Zym, Zymography-LC-MS/MS

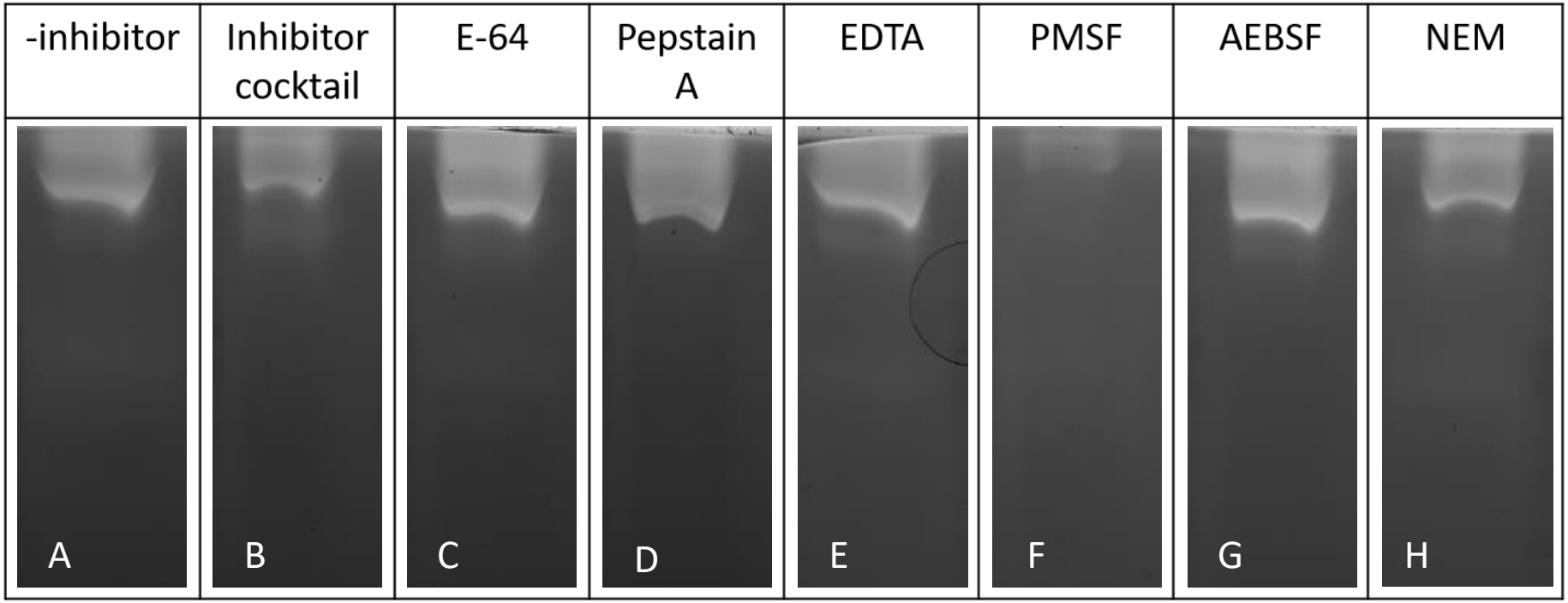

-inhibitor
Inhibitor cocktail
E-64
Pepstain A
EDTA
PMSF
AEBSF
NEM
A
B
C
D
E
F
G
H

Figure 1.

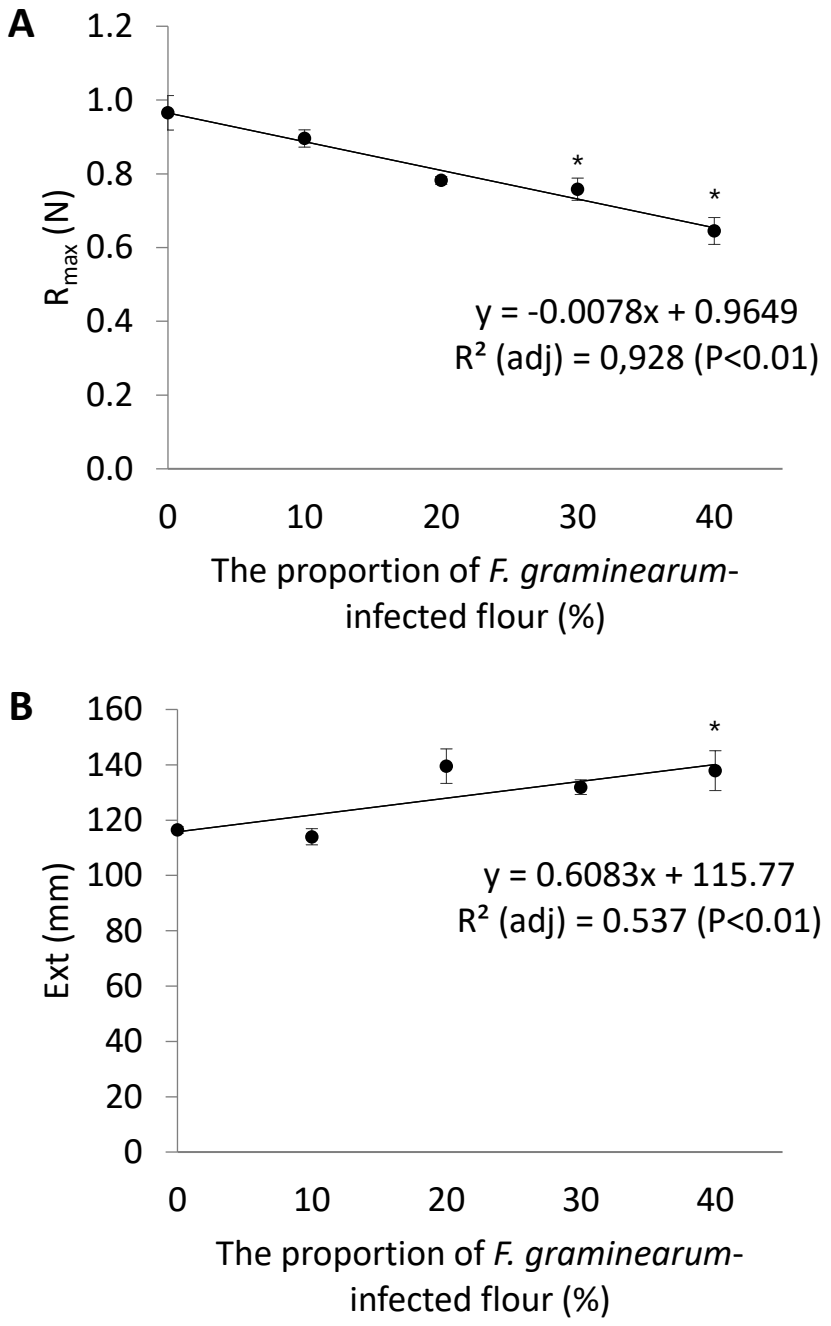

A
1.2
1.0
0.8
0.6
0.4
0.2
0.0
R_{max} (N)
*
*
y = -0.0078x + 0.9649
R² (adj) = 0,928 (P<0.01)
0
10
20
30
40
The proportion of F. graminearum-infected flour (%)
B
160
140
120
100
80
60
40
20
0
Ext (mm)
*
y = 0.6083x + 115.77
R² (adj) = 0.537 (P<0.01)
0
10
20
30
40
The proportion of F. graminearum-infected flour (%)

Figure 2.

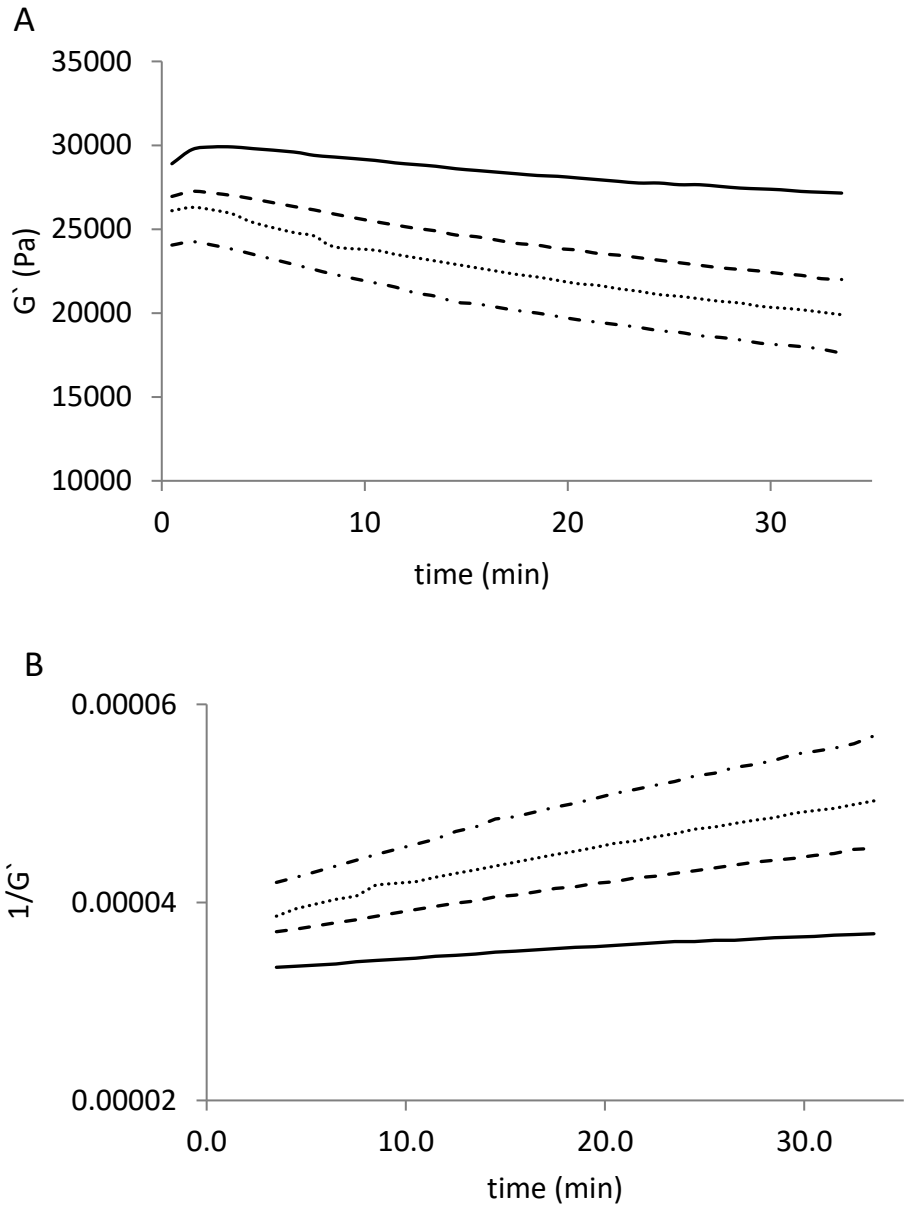

A
35000
30000
25000
20000
15000
10000
G` (Pa)
0
10
20
30
time (min)
B
0.00006
0.00004
0.00002
1/G`
0.0
10.0
20.0
30.0
time (min)

Figure 3.

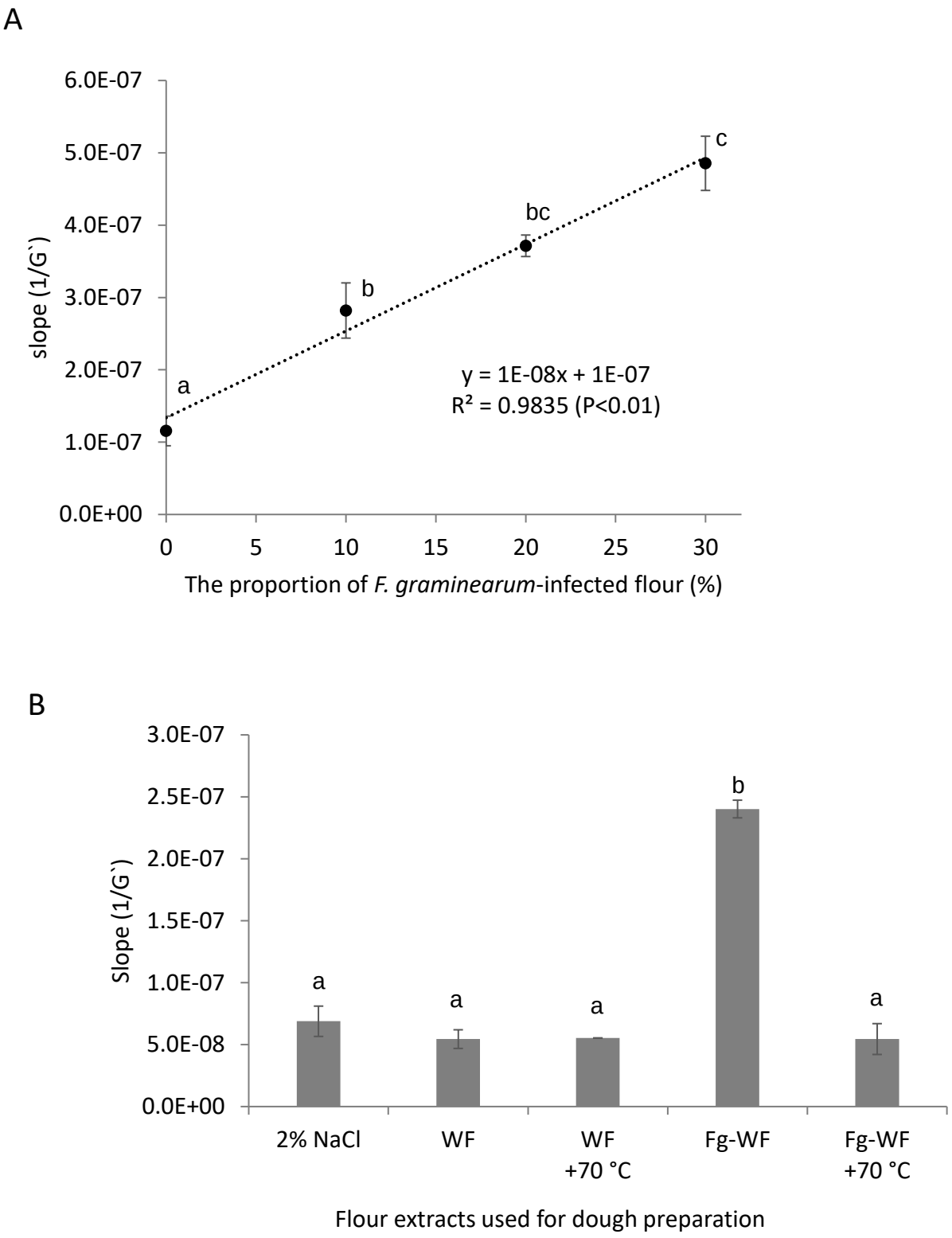
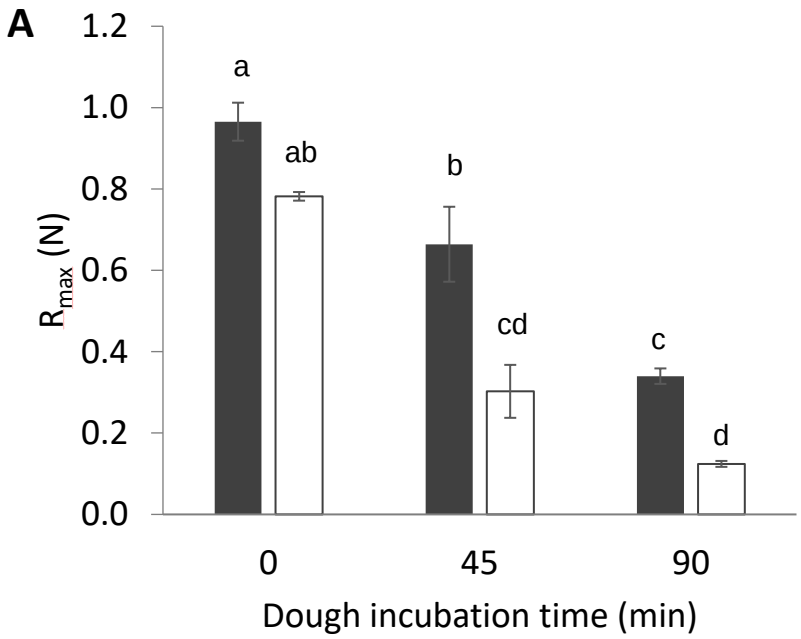

A
6.0E-07
5.0E-07
4.0E-07
3.0E-07
2.0E-07
1.0E-07
0.0E+00
slope (1/G`)
a
b
bc
c
y = 1E-08x + 1E-07
R² = 0.9835 (P<0.01)
0
5
10
15
20
25
30
The proportion of F. graminearum-infected flour (%)
B
3.0E-07
2.5E-07
2.0E-07
1.5E-07
1.0E-07
5.0E-08
0.0E+00
Slope (1/G`)
a
a
a
b
a
2% NaCl
WF
WF +70 °C
Fg-WF
Fg-WF +70 °C
Flour extracts used for dough preparation

Figure 4.


A
1.2
1.0
0.8
0.6
0.4
0.2
0.0
R_{max} (N)
a
ab
b
cd
c
d
0
45
90
Dough incubation time (min)

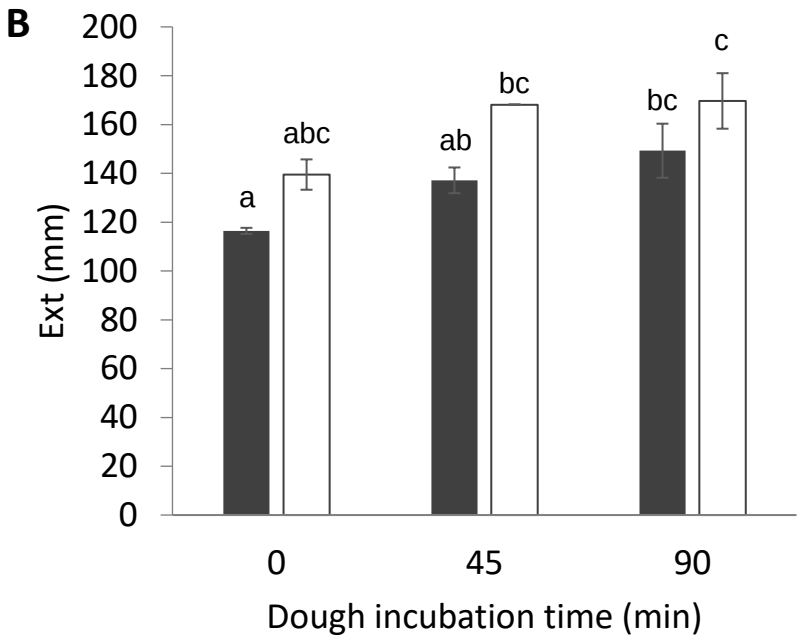

B
200
180
160
140
120
100
80
60
40
20
0
Ext (mm)
a
abc
ab
bc
bc
c
0
45
90
Dough incubation time (min)

Figure 5