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Characterisation of proteolytic enzymes of *Eurygaster integriceps Put.* (Sunn bug), a major pest of cereals

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ABSTRACT

Eurygaster integriceps (Sunn pest or Sunn bug) is one of the most significant pests of wheat and is responsible for substantial losses in yield and quality of wheat grain in Europe and Asia. Sunn pest salivary gland-derived proteases and other hydrolases damage grain proteins and starch. Characterisation of protease activities from both Sunn pest salivary glands and Sunn pest-damaged wheat grains revealed a broad range of activities in terms of substrate specificity and diversity of isoelectric point. Neutral and alkaline proteases present in Sunn pest-damaged grains were shown to be capable of hydrolyzing gluten proteins, whilst some proteases were also shown to be active against gelatin. The neutral serine proteases present play the dominant role in degradation of gluten quality. The sensitivity of some proteases to proteinaceous and non-proteinaceous serine proteinase inhibitors was shown, including that of a recombinantly expressed protease. It was found that proteases isolated from Sunn pest salivary glands could be activated by trypsin indicating that they are present as zymogens *in vivo*. Analysis of individual Sunn pest-damaged grains showed great diversity in the proteases present. This work highlights the challenges of developing proteinase inhibitors to manage Sunn pest damage.

Introduction

The Sunn pest, *Eurygaster integriceps* Put. and other wheat bugs of families Scutelleridae, Pentatomidae and Lygaeidae are the most serious insect pests in many countries in the Near East, Southern and Eastern Europe and Pacific region (*e.g.* New Zealand) (Critchley, 1998; Every et al., 2005; Pavlyushin et al., 2015; Dizlek and Ozer, 2016) and climate change is predicted to widen the range of Sunn pest to more Northern countries (Aljaryian et al., 2016). Sunn pests inject a salivary gland secretion containing digestive enzymes into wheat grain endosperm and subsequently ingest the liquefied material, causing a substantial deterioration of wheat quality and yield (Sivri et al., 1999; Vaccino et al., 2006; Özçandir and Özay, 2014; Allameh et al., 2015; Dizlek and Ozer, 2017a). Proteases remaining in the affected grains damage the high molecular weight of glutenin subunits (HMWGS) which are responsible for the most important processing properties of gluten (Shewry et al., 2003). The resulting gluten structure is disrupted and the visco-elastic properties of dough, and hence the breadmaking quality, are impaired (Dizlek and Ozer, 2017b).

The composition and biochemical properties of gluten hydrolysing, Sunn pest-derived, proteases remain incompletely characterized. Since the discovery of the proteolytic nature of the gluten degrading factor in Sunn pest-damaged grains, over 70 years ago, (Kretovich, 1944) there have many attempts to isolate and characterise the proteases of Sunn pest and other wheat-bug pests, for example, Sivri and Koksel (2000) and Every et al. (2005). These authors isolated proteases with molecular masses of approximately 30 kDa. In a previous study Konarev et al. (2011) isolated a 28-kDa trypsin-like protease capable of cleaving HMWGS specifically between the hexa- and nonapeptide repeats, and later cloned and expressed a glutenin hydrolysing protease GHP3 from Sunn pest salivary glands in *Pichia pastoris* (Dolgikh et al., 2014). A Sunn pest salivary gland prolyl endoprotease was also cloned and recombinant proteinase expressed (Yandamuri et al., 2014). Several other trypsin-, chymotrypsin- and elastase-like proteolytic activities have also been detected in the Sunn pest digestive system (Konarev and Fomicheva, 1991; Hosseininaveh et al., 2009; Özgür et al., 2009;

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Saadati and Bandani, 2011; Mehrabadi et al., 2014).

The aim of the present study was to characterise native proteolytic enzymes found in damaged grains and in Sunn pest salivary glands and compare them with recombinant forms in order to provide information for the future design of inhibitors active against Sunn pest proteases.

Materials and methods

Insects and damaged wheat grains

Samples of winter and spring bread wheat grain, damaged by Sunn pest (*E. integriceps*) in 2009–2015 were obtained from various regions of Russia: Rostov, Krasnodar, Stavropol, Saratov, Kurgan and Altai. Additionally, samples of grain damaged by bugs of genus *Eurygaster* Lap. from Central Turkey, (bread wheat cultivars (cv) Bayraktar and Ikizce, and durum wheat cv Ege-88) from South Eastern Turkey were supplied by Dr. H.Koksel and Dr. D.S.Ozay in 2006 (Hacettepe University, Ankara). Grains were stored at room temperature.

Salivary glands were isolated from *E. integriceps* adults collected from the Saratov region in 2006 and Krasnodar region in 2013–2015 which had previously been fed grain prior to dissection. Isolated salivary glands were stored at -80° C.

Chemicals

Protease substrates *N*-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BApNA) and membranes containing substrates *Z*-Val-Lys-Lys-Arg-TFMCA (Z-VKKR-TFMCA) and D-Val-Leu-Arg-TFMCA (D-VLR-TFMCA) were from Serva Electrophoresis (Germany) and Z-Gly-Pro-pNA (ZGPpNA) from MP Biomedicals (USA). Nitrocellulose membranes (0.45 μm) were from Hoefer Scientific Instruments (USA). Dithiothreitol (DTT), PMSF, CHAPS, pCMB, EDTA-Na_2 were from Sigma-Aldrich (USA).

Heterologous expression of recombinant proteases

Salivary gland proteinase GHP3 (GenBank: ADP06392.1; Konarev et al., 2011) was heterologously expressed in *E. coli* and *Pichia pastoris* cells (Dolgikh et al., 2014). Recombinant proteases were designated as rGHP3e1 (*E. coli*), rGHP3p1 and rGHP3p2 (both *P.pastoris*). rGHP3p1 was expressed as the mature active enzyme and rGHP3p2 as a zymogen, activated by trypsin treatment. Recombinant proline-specific endoprotease (GenBank: EU934738.3) was expressed as described by Yandamuri et al. (2014) and designated in the present work as rPSEP.

Isolation of proteases from damaged grains and salivary glands

Proteases were isolated from single milled damaged grains or from bulked damaged grains with 0.01% *v*/v triton X-100 (1:5 w/v) for 30 min at room temperature. Proteins were concentrated by precipitation with ice cold acetone (1:4) followed by solubilisation of the pellet in 0.01% v/v triton X-100 with 50% *w/v* glycerol in a volume equivalent to the weight of the flour (v/w). Proteases extracted from 10 salivary glands were homogenized with 50 μl of 0.01% v/v triton X-100 and 0.5% w/v CHAPS. For activation studies, protease fractions were treated with immobilized trypsin. *1 mg of dry* Enzygel (Boehringer Mannheim) was incubated with 30 μl salivary gland extract in 0.01 M Tris-HCl buffer pH 8.5 at 37° C for 2 h with shaking.

Gel-filtration chromatography

Gel-filtration was carried out on a 250 × 7 mm column with Sephadex G-100 superfine gel (LKB, Sweden) equilibrated with 0.1% w/v CHAPS in 0.05 M ammonia acetate pH 7.0. The elution rate was 0.075 ml/min. Proteins were monitored at 280 nm.

A protein extract from 600 mg damaged wheat grains from wheat cv. Dzhangal, isolated as described above was dissolved in 250 μl of 0.1% *w/v* CHAPS in 0.05 M ammonia acetate pH 7.0, centrifuged at 10,000 x g for 5 min and the supernatant loaded onto the gel filtration column. Fractions were collected every 10 min, concentrated to a volume of about 50 μl using Centricon 10 units (Amicon, USA) and stored at -25 °C after addition of an equal volume of 70% w/v glycerol.

Detection of gluten degrading proteolytic activity by SDS-sedimentation

The activity of gluten hydrolyzing proteases was estimated using a modified semi-quantitative micro-method of SDS sedimentation. 2–10 μl of extracts from damaged grains or chromatographic fractions were made to a final volume of 30 μl with 10 mM Tris-HCl buffer pH 8.5 with 0.01% *v*/v triton X-100 and added to plastic tubes. A 130 μl suspension of (constantly shaken) bread wheat flour in 10 mM Tris-HCl buffer pH 8.5 with 0.01% v/v triton X-100 (1 g/7 ml) was added to each tube, mixed and incubated at 37° C for 1 h. 130 μl of 4% w/v SDS was added in each tube and the mixtures transferred to narrow (d = 3 mm) plastic tubes and left in a vertical position for 30 min. The height of the column of remaining intact gluten was then measured.

Isoelectric focusing

Isoelectric focusing (IEF) of proteins isolated from salivary glands, grains or size fractionated proteins was carried out on ready for use Phast Gels (GE Healthcare, Sweden) with pH range 3–9, 5–8 and hand cast gels in the pH range 5–11 - using either Multiphor II (LKB, Sweden) or Phast System (GE Healthcare) devices (Konarev and Lovegrove, 2012; Dolgikh et al., 2014). Samples (0.3–4 μl) were loaded close to the anode area of the gel using paper strips. Isoelectric points (pIs) of protein bands were estimated by reference to coloured protein markers, cytochrome *c* (pI 10.6) and horse myoglobin (pI 7.3).

SDS-PAGE

SDS-PAGE was carried out on 12% PAAG. Protein extracts were boiled with 2% w/v SDS containing 1% v/v 2-mercaptoethanol, as previously described (Dolgikh et al., 2014). Gels were stained with Coomassie G-250.

Detection of proteolytic and inhibitory activity

Detection of gliadin hydrolyzing activity

Protease activity was assayed using a gliadin extract made from wheat flour extracted with 70%v/v ethanol, immobilized to the surface of a plastic film in a modified version of Dolgikh et al. (2014). The gluten pellet from flour was homogenized with 100 ml of 70% v/v ethanol and stirred for 30 min using a magnetic stirrer. The extraction in 70% v/v ethanol was repeated three times and the ethanol extracts combined (gliadin extract). 12 ml of 70% v/v ethanol, 12 ml of water and 110 mg of Agarose (Serva) were mixed together and boiled and then cooled to approximately 70°C. 18 ml of gliadin extract was added to the agarose mixture and spread onto the hydrophilic surface of 125 × 250 mm GelBond film, placed on the glass plate installed on a horizontal temperature-controlled plate of the Multiphor II at room temperature. The temperature of the plate was then lowered to 4 °C. After solidification of the gel, the temperature was raised to 37 °C and the gel dried for up to 1.5 h.

Just prior to use, the film was moistened with water for 20 s and then placed onto the surface of the IEF gel on the Multiphor II plate and covered with plastic film to prevent drying. A foam rubber plate 10 × 125 × 250 mm was placed on top of the film, covered with a glass plate and weighted down. The substrate film was incubated with the separating IEF gel at 37 °C for between 25 and 50 mins. The substrate film was then removed and placed in 50 mM Tris-HCl buffer pH 8.5 for 10 min and then transferred to distilled water. After 2–10 min

opalescent bands corresponding to proteinase activity appeared in the gliadin substrate.

Detection of gelatin hydrolyzing activity

A thin layer of gelatin attached to the plastic support of the photographic film ("Foto 65", Russia) was used for detection of gelatin hydrolyzing proteases as described earlier (Konarev and Fomicheva, 1991; Konarev et al., 1999).

Detection of hydrolysis of aminoacyl or peptidyl p-nitroanilides

The method of Ohlsson et al. (1986) modified by Konarev and Fomicheva (1991) was used for detection of proteases by hydrolysis of aminoacyl or peptidyl p-nitroanilides, BApNA and ZGPpNA, in combination with nitrocellulose membrane (NC).

Detection of hydrolysis of fluorogenic peptide substrates

Membranes containing derivatives of trifluoromethylcoumarinyl, *Z*-VKKR-TFMCA and D-VLR-TFMCA were cut in sizes corresponding to Phast separating gels, moistened in 50 mM Tris-HCl buffer pH 8.5, and placed onto the separating gel, covered with plastic film and incubated at 37 °C for 50 min. Membranes were placed on a UV-transilluminator to visualise proteolytic activity.

Inhibitors/activators of Sunn pest proteases

Inhibitors/activators were added to the protease sample prior to IEF at a final concentration 1–2 mM and protease activity patterns for treated and control samples compared.

Gelatin substrate replicas were soaked in 0.1 M Na_2HPO_4 only (control) or with addition of proteinaceous inhibitors (1 mg/ml), aprotinin or soya bean trypsin inhibitor (STI), for 1 h, placed on the IEF gel after separation of proteases and incubated for 2 h at 37° C.

Antibodies

Rabbit polyclonal antibodies specific for the recombinant protease rGHP3e1 were generated from dissolved inclusion bodies derived from heterologous expression of GHP3 in *E. coli* (Dolgikh et al., 2014). The antibodies were used in western blots of SDS-PAGE and IEF gels. Horse raddish peroxidase secondary antibodies and 4-chloro-naphthol were used for western blot development.

Results and discussion

As long ago as 1944 (Kretovich, 1944) the proteolytic nature of the enzymes from Sunn pest-damaged grain have been known, the proteases being studied using standard biochemical approaches (Vilkova and Ekman-Burinskaya, 1977). However, it was not until the 1990s (Every, 1991a,b, 1992, 1993) that methods were developed, to study the proteases of a distantly-related New Zealand pest of wheat *Nysius huttoni*, that permitted the partial purification and early characterisation of some of the proteases from Sunn pest (Sivri, 1998; Sivri and Koksel, 2000). These studies provided the basis for the subsequent purification and cloning of individual isoforms, as well as the expression of proteinases synthesized by the salivary glands of the Sunn pest (Konarev et al., 2011; Dolgikh et al., 2014). The present study uses novel modifications of previous methods, with improved sensitivity, to allow the study of the heterogeneity and variability of Sunn pest proteases isolated from salivary glands and damaged individual wheat grains.

Characterisation of proteolytic enzymes present in Sunn pest-damaged grains

Proteins isolated from Sunn pest-damaged grain were size fractioned by gel filtration, Fig. 1 (panel A). Proteolytic activity of fractions was detected using the SDS sedimentation assay. Gluten hydrolysing activity was concentrated in fractions collected at 140, 150 and 160 mins (panel B). A broad range of proteolytic activity was obvious when extracts were incubated with different substrates following IEF (panels C—F). Gliadin hydrolysing proteases (panel C) were present in fractions collected between 140 and 200 mins with a pI of 7.3 and lower and were designated as 'neutral'. As the use of acetic-acid soluble glutenin replicas, gave an identical pattern of protease activity to that observed using the 70% ethanol-soluble (gliadin) substrate, we have used the term "gluten hydrolysing" throughout to describe these enzymes. Proteolytic activity against gelatin was also observed (panel D) with proteases with pIs in the range 7.3–9.0 and were designated as 'alkaline'. Proteolytic activity against the synthetic peptide substrate *Z*-VKKR-TFMCA was detected with pIs in the range 7.5–9.0 (panel E). Finally, proteolytic activity against BApNA was also present between pIs in the range 5.0–6.0 (panel F) and 7.5–9.0 (data not shown). No hydrolytic activity was observed in fractions isolated from undamaged grains (data not shown). The estimated molecular mass of the most active fractions eluting from the gel filtration column was around 30 kDa (140–160 min). The acidic protease activity, capable of hydrolysing BApNa (panel F) eluted slightly early around 140mins, indicating a larger molecular weight.

The proteolytic activity of protein extracts from Sunn pest salivary glands of *E. integriceps* (Fig. 2) showed activity against gluten (panel A) and the synthetic substrate ZGPpNA (panel B). This was in contrast to the recombinant proline endopeptidase (rPSEP) that in conditions of experiment only showed proteolytic activity against ZGPpNA, but not gluten.

Fig. 2 illustrates that Sunn pest salivary gland extracts had proteolytic activity against gluten (panel A, tracks 1–3) with pIs greater than 6.5, but no activity towards gelatin (data not shown). Sunn pest salivary gland proteases were also capable of hydrolyzing the synthetic substrate ZGPpNA (panel B, tracks 1–3) indicating prolyl-specific proteolytic activity, with pIs between pH 5.0 and 6.0. The prolyl-endopeptidase activity revealed in Sunn bug salivary glands was similar to the recombinant rPSEP (panel B, tracks 4 and 5). Under the same conditions no activity towards ZGPpNA was detected in either Sunn pest-damaged or undamaged (control) wheat grains (data not shown).

Activation of Sunn pest salivary gland proteases by trypsin treatment

Proteases isolated from Sunn pest salivary glands initially had weak activity against gluten (Fig. 3A, Track 1) and no activity against gelatin (Fig. 3B, track 1). However, following activation with immobilized trypsin, protease activity was greatly enhanced against gluten (Fig. 3A, track 2) and protease activity against gelatin was also then apparent (Fig. 3B, track 2). The pIs of protease activities against the two substrates were, for gluten below pH 7.3 and for gelatin between pH 7.3 and 10.6. The activation of the protease activity by trypsin coincided with the disappearance of a major band with MW ~30 kDa corresponding to GHP3 proenzyme and the relative strengthening of a band with MW ~28 kDa corresponding to the active enzyme as revealed by western blot (Fig. 3C, track 1, control *vs* tracks 2 and 3, trypsin treated). We therefore speculate that GHP3-related proteases present in Sunn pest salivary glands are in the form of inactive proenzymes and that they are activated upon feeding by a trypsin-like enzyme present *in vivo*. This is supported by data of Dolgikh et al. (2014) who found that trypsin was able to activate the recombinant rGHP3p2 proenzyme. Similar activation seems to take place in case of alkaline proteases. The propeptides of these proteinases, like GHP3, contain an arginine residue at the C-end adjacent to the mature enzyme (Fig. 4). Trypsin-like activating proteinases could thus cleave the polypeptide at arginine releasing the active protease. The GHP3 full size polypetide arginine residue is present in the C-end of the propeptide in position 48 preceding the tetrapeptide sequence "IVGG", typical for the N terminalend of mature trypsin-like serine proteinases (see accession ADP06392, Konarev et al., 2011). Using the Signal-BLAST program (Frank and Sippl, 2008) the signal peptide is 23 amino acid residues long.

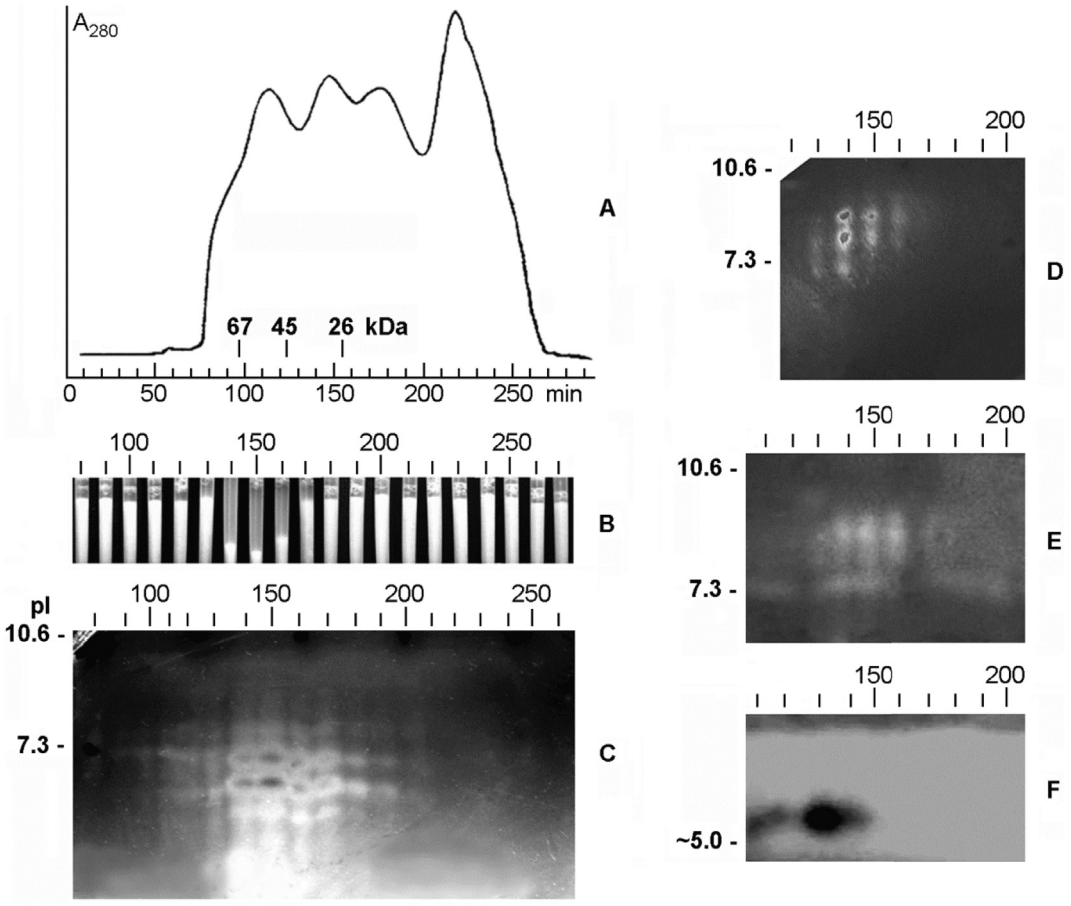

Fig. 1. Characterisation of proteolytic enzymes present in Sunn pest-damaged grains. Panel A. Chromatogram of size fractionated proteins from Sunn bug-damaged grain on Sephadex G-100, positions of molecular weight standards are indicated. Panel B. SDS-sedimentation assay, numbers indicate fractions from size exclusion column. Panels C–F are size-exclusion fractions separated by IEF in the pH range 5–11 and exposed to various substrates. Panel C, 70% v/v alcohol-soluble gluten proteins. Panel D, gelatin; Panel E, *Z*-VKKR-TFMCA; protease activity is observed as white bands against the dark background. Panel F, BApNA, protease activity is observed as black against white (nitrocellulose) background. The tube numbers and tracks marked on the IEF gels relate to fractions from the gel filtration column.

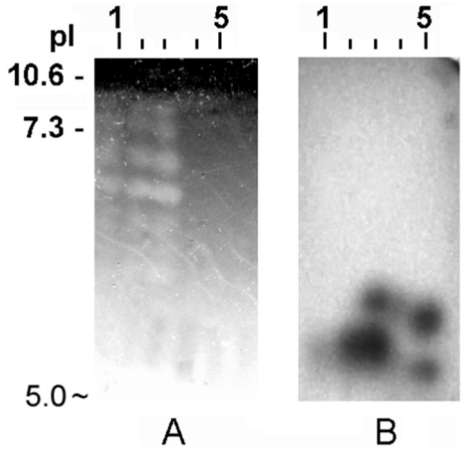

Fig. 2. Parallel detection of neutral protease and prolyl-endopeptidase activities in Sunn pest salivary gland native and recombinant proteins. Extracts from salivary glands and recombinant proteinase rPSEP were separated by IEF in pH range 5–11 and proteases were detected using substrate replicas, gluten proteins soluble in 70% v/v ethanol (panel A) and ZGPpNA (panel B). Panels A and B, tracks 1–3 Sunn pest salivary glands; tracks 4 and 5, recombinant endopeptidase rPSEP.

Therefore, the propeptide is 25 amino acid residues long which corresponds to a molecular weight of approximately 2490. This is consistent with the results shown in Fig. 3, panel (c). The activation of protease zymogens from salivary glands by trypsin *in vitro* has been shown for various insect species (Henrikson and Clever, 1972; Vizioli et al., 2001) but has not previously been suggested for proteases from Sunn pest.

Interaction of Sunn pest proteases with inhibitors

A number of proteinaceous and low molecular weight inhibitors and activators were used to further characterise the proteases from Sunn pest salivary glands and Sunn pest-damaged grain. The salivary gland proteases activated by trypsin (Fig. 5A, tracks 1 and 2) and proteases from damaged grains (5A, tracks 3 and 4) showed sensitivity to PMSF when gelatin was used as substrate. The same was true for proteases from damaged grains (Fig. 6A and B) and salivary gland proteases activated by trypsin (not shown) with gluten as substrate. Extracts from damaged grains were incubated with (+) and without PMSF and subjected to the SDS gluten sedimentation assay. It was clear that the addition of PMSF reduces the activity of the extracted proteases against gluten (Fig. 6A). IEF of a damaged grain extracts from different wheat cultivars confirmed this effect (Fig. 6B). DTT did not restore their activity. Nor was there any effect of the metalloprotease inhibitor EDTA-Na2, cysteine protease inhibitor pCMB or soya bean trypsin inhibitor upon any of these proteases (data not shown). This was also true for the recombinant protease, rGHP3p2 (panel B, tracks 6 and 7). The recombinant, prolyl-endopeptidase rPSEP (panel B, track 5) did not show

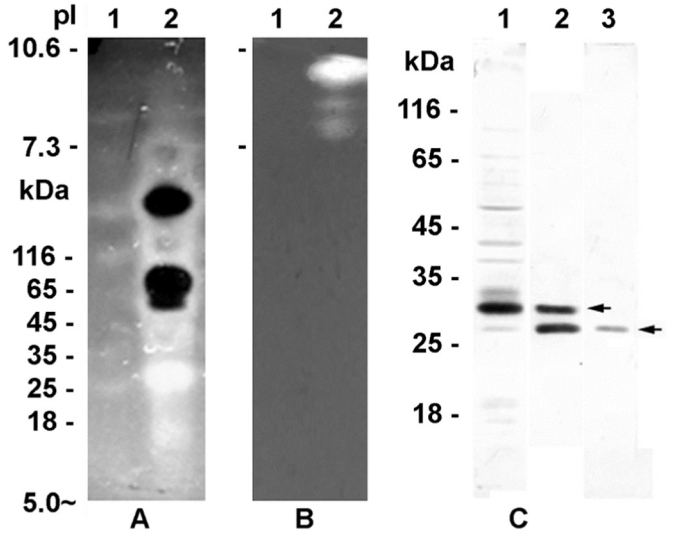

Fig. 3. Activation of Sunn pest salivary gland proteases with immobilized trypsin treatment. Proteins extracted from homogenized salivary glands were separated by IEF (pH range 5–11).
Panel A, gluten substrate. Tack 1, untreated, control; track 2, following trypsin activation.
Panel B gelatin substrate. Track 1, untreated, control; track 2, following trypsin activation.
Panel C, Western blot of salivary gland proteases. Track 1, untreated, control; tracks 2 and 3 following trypsin treatment for 1 and 2 h. Primary antibodies raised against recombinant proteinase, rGHP3e1.

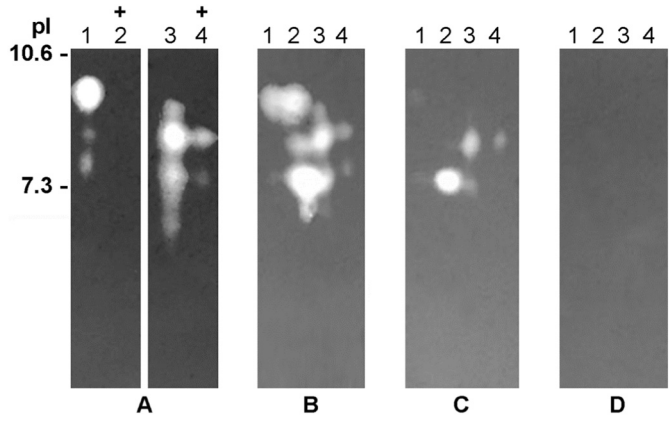

Fig. 5. Effect of inhibitors on alkaline trypsin-activated proteases (gelatinases) from Sunn pest salivary glands and Sunn pest damaged wheat grains. Extracted proteins were separated by IEF and proteolytic activity assayed against gelatin. Panels A, tracks 1 and 2, trypsin-activated proteases from Sunn pest salivary glands; tracks 3 and 4, proteases from Sunn pest-damaged wheat grain. '+' over track indicates extract incubated with 2 mM PMSF.
Panels B, C and D. Tracks 1 and 2, salivary gland extracts treated with trypsin; 3 and 4, extracts from two different bug-damaged grains of cv Dzhangal. Gelatin substrate films were preliminary incubated in pure 0.1 M Na2HPO4 (B, control) and with addition of STI (C) and aprotinin (D) (near 1 mg/ml).

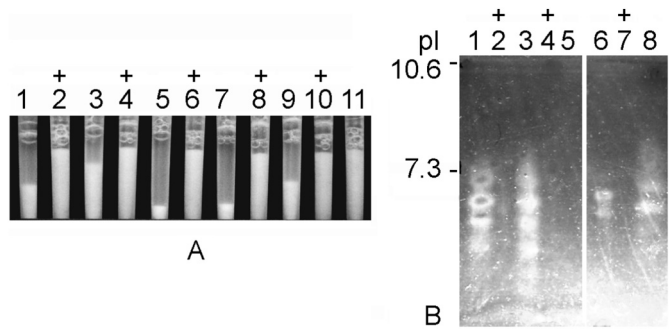

Fig. 6. Interaction of gluten hydrolysing proteases isolated from Sunn pest-damaged seeds and recombinant proteases with PMSF. Panel A, SDS-sedimentation; panel B, grain proteins separated by IEF and proteolytic activity assayed against gluten. '+' indicates addition of PMSF up to 1 mM. Panel A, tubes 1 and 2, Sunn pest-damaged grain extract cv Ege-88; tubes 3 and 4, cv Dzhangal; tubes 5 and 6, cv. Fortune; tubes 7 and 8, Ege-88 sample (for tubes 1–2 and 7–8 extracts obtained from different grains of cv Ege-88 sample); tubes 9 and 10, chromatographic fraction 160 min of damaged proteins of cv. Dzhangal (see Fig. 1); tube 11, control (no protease). Panel B, Sunn pest-damaged grain extract, tracks 1 and 2, cv Fortuna; tracks 3 4 and 8, cv Ege-88; track 5, recombinant prolyl-endopeptidase (rPSEP); tracks, 6 and 7, recombinant protease rGHP3p2.

any activity to gluten proteins under these conditions.

Fig. 5, panels B, C and D illustrate the interaction of isolated alkaline proteases (or gelatinases) with the proteinaceous serine proteinase inhibitors of plant and animal origin, soybean trypsin inhibitor (STI) and aprotinin. Proteases isolated from Sunn pest salivary glands were partially inhibited by STI as were proteases isolated from Sunn pest-damaged grains (panel C). Aprotinin, completely inhibited all activity (panel D). Inhibition of proteases by STI and aprotinin confirms that the alkaline proteases (gelatinases) isolated from Sunn pest salivary glands and Sunn pest-damaged seed are members of the serine proteinase enzyme class. Commercial trypsin was also used as a control and showed the same pattern of inhibition as the experimental extracts (data not shown).

Diversity in protease activity present in Sunn pest-damaged wheat grain

The level of diversity in protease activity present in Sunn pest-damaged wheat grain was examined by characterising protease activity from individual grains collected from different regions of Russia and Turkey (Fig. 7).

The SDS sedimentation method (Panel A) indicated that the level of proteolytic activity varied greatly among single damaged grains. For example, gluten hydrolysing activity was high in tubes 1–4 which corresponded to wheat cvs Fortuna (Tube 1 and 2), cv Grom (Tube 3) and cv Ege-88 (Tube 4). However, proteolytic activity was much lower in damaged grain from wheat cv Ikizce (Tube 6) with gluten sedimentation very similar to the controls (undamaged) grain sample cv Ege-88 (Tube 5) and buffer alone (Tube 21). Grain samples from wheat cv Dzhangal (Tubes 7–9) had variable levels of proteinase activity and grains from wheat cv Altayskaya-70 both low (Tube 10) and high levels of proteolytic activity (Tube 11). Extracts from damaged grains from wheat cv Altayskava-325 had intermediate (Tube 12) and low levels of proteolytic activity (Tube 13). Extracts from damaged grains of wheat cultivars cv Altayskaya zhnitsa (Tubes 14 and 15), cv Stepnava Niva (Tubes 16–18) and cv Umka (Tubes 19 and 20) also had very low levels of gluten hydrolysing activity.

Fig. 7B and C illustrate the activity and substrate specificity of the proteases present in the extracts from the same individual grains towards gluten and gelatin respectively. Specificity towards synthetic peptides *Z*-VKKR-TFMCA and D-VLR-TFMCA was also observed (Fig. S1

Fig. 4. The primary structure of N-terminal part of full length Sunn pest protease GHP3 polypeptide (GenBank: ADP06392.1). The blue arrow indicates the cleavage site between the signal peptide and the propeptide. The red arrow the cleavage site between the propeptide and mature protease. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

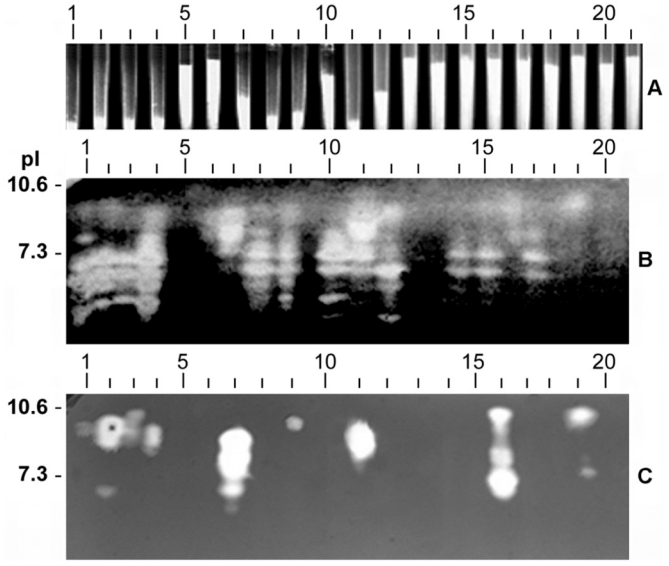

Fig. 7. Proteolytic activity from individual Sunn pest-damaged grains. Proteolytic activity assayed by SDS-sedimentation method (panel A) and IEF combined with substrate replicas (panels B, gluten, and panel C, gelatin). Tubes (A)/tracks (B and C) 1 and 2, cv Fortuna; 3, cv Grom; 4 and 5 cv Ege-88 (5, undamaged grain control); 6, cv Ikizce; 7–9, cv Dzhangal; 10 and 11, cv Altayskaya 70; 12 and 13, cv Altayskaya-325; 14 and 15, cv Alayskaya zhnitsa; 16–18, cv Stepnaya Niva; 19 and 20, cv Umka; tube 21, control (gluten alone).

in Supplement). It was clear that extracts with the highest levels of proteolytic activity based on the SDS sedimentation assay also displayed the higher number of proteases, after separation by IEF and assayed using the substrate replicas, with the highest level of activity towards gluten the proteases present displayed a broad range of pI (acid through to alkaline). This is illustrated in Fig. 7A and B by tracks 1–4 and 7–12. Some extracts had proteolytic activity against both gluten and gelatin, for example from an individual grain of wheat cv Dzhangal, alkaline protease activity (Fig. 7B and C, track 7) but no activity against the synthetic substrates (Fig. S1A and S1B in Supplement, tracks 6 and 7). Extracts from the damaged grains of wheat cv Stepnaya Niva, showed low activity in the SDS sedimentation assay (tube 16), a narrow range of activity against gluten (Fig. 7B, track 16) and high activity against gelatin with proteases with pI in both the neutral and alkaline range (Fig. 7C, track 16). Therefore, it is clear that the protease extracts from damaged grains had a wide pI range, variable levels of specificity and showed variability in composition, for example, cv Dzhangal, tracks 7–9. The diversity present in proteolytic activity within wheat cultivars and presumably Sunn pest may therefore in some cases cause problems when looking for wheat cultivars resistant to Sunn pest or finding a single protease inhibitor to combat protease damage in the grain. It should be noted that Sunn pest was, by far, the most prevalent insect pest of wheat grain in all areas it was collected in.

Conclusions

Size fractionation and IEF, in conjunction with substrate replica detection methods, have allowed the characterisation of several proteolytic enzyme activities from Sunn pest salivary glands and Sunn pest-damaged wheat grains. Analysis has shown a range of neutral and alkaline serine proteases present in bug-damaged grains. Proteases with neutral pI ranges from Sunn pest-damaged grains were shown to be active against gluten and proteases with mostly alkaline pI range were active both against gluten and gelatin. Neutral serine proteases appear to play a major role in the deterioration of gluten quality in wheat flour. A high degree of diversity in protease composition was detected when proteases were isolated from individual grains from different wheat cultivars and from different geographical areas. Proteases isolated from Sunn pest salivary glands could be activated by incubation in trypsin, we therefore hypothesize that an inactive zymogen of the major gluten-hydrolysing protease is synthesized in Sunn pest salivary glands and may be activated by a trypsin-like protease *in vivo* once feeding commences. Recently the presence of a GHP3-like enzyme in bug-damaged grain was confirmed using scFv-fragment antibodies (Dolgikh et al., 2017). Therefore, it appears that neutral (including GHP3), alkaline and some other forms of proteolytic enzymes maintain their activity after harvest and through storage of Sunn pest-damaged grains. The natural diversity in proteolytic activity observed means that effective control of Sunn pest damage with the use of protease inhibitors may be difficult. This is of particular importance as Sunn pest salivary gland, neutral proteases are almost entirely insensitive to known proteinaceous inhibitors and it is these that are primarily responsible for the gluten quality deterioration.

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Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2019.02.001.

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