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Characterization of a Glutenin-Specific Serine Proteinase of Sunn Bug *Eurygaster integricepts* Put.

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Supporting Information

ABSTRACT: Glutenin hydrolyzing proteinases (GHPs) have been purified, by affinity chromatography, from wheat seeds damaged by the Sunn bug *Eurygaster integriceps* (Hemiptera, Scutelleridae). A 28 kDa protein was partially sequenced by mass spectrometry and Edman degradation which showed homology to serine proteases from various insects. Three full length clones were obtained from cDNA isolated from Sunn bug salivary glands using degenerate PCR based on the sequences obtained. The cleavage site of the protease was determined using recombinant and synthetic peptides and shown to be between the consensus hexapeptide and nonapeptide repeat motifs present in the high molecular weight subunits of wheat glutenin (PGQGQQ^GYYPTSLQQ). Homology models were generated for the three proteinases identified in this study using the high resolution X-ray structure of a crayfish (*Pontastacus leptodactylus*) trypsin complexed with a peptide inhibitor as template (PDB accession 2F91). The novel specificity of this protease may find applications in both fundamental and applied studies.

KEYWORDS: Gluten hydrolyzing protease, *Eurygaster intrigriceps*, cleavage site specificity, molecular modeling

INTRODUCTION

Infection of growing wheat and barley crops with insects of the genus *Eurygaster* Lap. (Sunn bug) leads to substantial losses of crop yield and quality in many countries including parts of Eastern and Southern Europe, West and Central Asia, and North Africa.[1−7] More than 10 million Ha of wheat, nearly half of which lies in the Near East, is attacked by the Sunn bug.[4] Losses from Sunn bug infestation result both from seedling loss and failed germination of damaged seed. In Russia alone, the Sunn bug damages from 3.5 to 5 million Ha of wheat per year, with losses estimated at around 300 million US dollars even after chemical treatments (totalling tens of millions of dollars in cost) have been applied.[8] However, the main effect of Sunn bug infestation on crop quality results from the injection into the grain of salivary proteinases. These degrade the wheat gluten proteins, leading to a loss of gluten and dough viscoelasticity and poor processing properties.[9−13] A similar effect occurs by infestation with other true bugs belonging to the genus *Aelia* Fabr. (Pentatomidae) in Europe[14] and by infestation with *Nysius huttoni* White (Lygaeidae) in New Zealand.[15] *Eurygaster* and species with similar effects have already spread to Spain, Italy, and Austria[16−18] and could spread into Western and Northern Europe with changes in climate.

Previously published studies of the digestive enzymes of *Eurygaster maura* and *Nysius huttoni* and other insects that infest wheat grain have shown that they are highly specialized, with narrow specificities for wheat proteins and particularly for the high molecular weight (HMW) subunits of glutenin which are the major determinants of gluten and dough elasticity.[19,15] Although fractions enriched in bug salivary proteinases have been isolated from damaged wheat seeds, the proteinases were not purified sufficiently for sequence analysis.[20−22] Nevertheless, these studies showed that the proteinases of *Eurygaster* and *Nysius* were insensitive to most proteinaceous inhibitors except for potato inhibitor I, which was active against the *Nysius* enzyme.[20] Proteinaceous inhibitors of proteinases and other enzymes have been shown to provide effective defense for plants against many insect and fungal pests in laboratory and glasshouse trials, although they have yet to be exploited in commercially grown crops.[23−26] Furthermore, our current knowledge of the structures and reaction mechanisms of the proteinases of Sunn bug and similar pests is insufficient to allow a logical approach to be taken to the identification or design of specific inhibitors. The aim of this study was therefore to purify and characterize the digestive glutenin-hydrolyzing proteinases (GHPs) from *Eurygaster* spp. and to determine their specificity. This will allow us to evaluate strategies for preventing bug proteinase damage to wheat and to explore their wider applications, for example, in food processing and gluten detoxification for celiac patients.

MATERIALS AND METHODS

Wheat Seed Material. Seeds of various bread and durum wheat varieties damaged by Sunn bug (*E. integriceps*) were harvested from the Krasnodar and Stavropol territories (Krais), the Rostov region (Oblast) of Russia (2002−2006), and from various regions of Turkey (2006).

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The samples contained from 5%−60% damaged seeds which were identified visually and selected for further analysis. Undamaged seeds were also selected and used as controls.

Profile of Proteins from Bug-Damaged and Control Seeds. Samples were ground in a mortar and pestle to a powder, and 20 mg samples were mixed with either six volumes (per weight of sample) of water or six volumes of SDS−PAGE loading buffer and incubated for 1 h, at 37 °C or at room temperature, respectively. A further six volumes of SDS−PAGE loading buffer were then added to all the tubes and extraction continued for another 1 h at room temperature. Samples were then centrifuged at 13,000 g for 5 min, the supernatant heated to 100 °C for 5 min, recentrifuged and separated on 10% (w/v) Tris-borate SDS gels.[27] Gels were fixed in 10% (w/v) TCA and stained with Coomassie R250.

Insect salivary glands. Adults of the overwintering generation of *E. integriceps* feeding on wheat seedlings were collected in June 2004 in the Saratov region of Russia and of the summer populations feeding on developing wheat grains in the Saratov and Samara regions in July 2004 and in the Stavropol territory in July 2006. Salivary glands were hand-dissected from insects using a binocular microscope, submerged in ice-cold physiological buffer containing 2nM $CaCl_2$ and immediately frozen at −80 °C.

Isolation of glutenin-hydrolyzing proteinases (GHPs). GHPs were extracted with water containing 0.01% (w/v) Triton X-100 from damaged milled seeds (1:5 w/v) or homogenized salivary glands (20 organs/100 μL) at 20 °C for 3 min. Equal volumes of 60% (v/v) glycerol were added to the samples for long-term storage at −80 °C.

Analytical separation of plant and insect proteins and detection of GHPs. Fractions were separated by isoelectric focusing (IEF) on precoated pH 3−10, 5−7 or 5.5−8.5 gels (Serva or LKB) using a Phast System (Pharmacia) or Multiphor II (LKB).[28] Samples were loaded onto filter paper in a volume of 0.3 to 4 μL and GHP bands detected using a novel glutenin-replica method based on the 'gelatin replicas' procedure.[28,29] 50 g of defatted flour of bread wheat cv. Hereward was extracted by stirring with 200 mL of 0.2% (w/v) NaCl. After 5 min centrifugation at 6000 g the gluten was recovered from the pellet by washing with 0.2% (w/v) NaCl to remove most of the starch. 40 g of wet gluten was then extracted twice by stirring for 20 min with 200 mL 70% (v/v) ethanol to remove gliadins and after centrifugation, twice with 150 mL of 0.15 M acetic acid, for 1 h and 3 h. The acetic acid-insoluble glutenin pellet was used to prepare replicas. 7.5 g of wet glutenin pellet was suspended in 22.5 mL of 0.15 M acetic acid and spread onto the hydrophilic surface of GelBond for agarose plastic film (Serva) (125 × 250 mm) and dried at 50 °C on the horizontal cooling plate of a Multiphor II (LKB) to give a nontransparent layer of glutenin attached to the plastic support. These replicas were applied to IEF separations of protein fractions containing the gluten hydrolyzing proteinases and incubated at 35 °C for 20−50 min. This 'replica' was then incubated in 0.15 M acetic acid for 10 min and washed with water to remove the glutenin solubilized by the proteinases. The replica was then placed in acetic acid and several drops of 5% (w/v) iodine in ethanol were added to stain the traces of starch entrapped in the glutenin layer. The undigested glutenin layer was therefore stained black with transparent bands revealing the positions of GHPs.

Purification of GHPs. Proteins were extracted from 5 g of milled damaged grain with 50 mL of 0.1 M ethanolamine for 3 min at 20 °C [modified from 20], centrifuged at 20,000 g for 10 min at 20 °C and precipitated with four volumes of cold (−20 °C) acetone. The pellet was dissolved in 0.01 M ethanolamine with 0.2 M NaCl and 0.01% Triton X-100, the pH was adjusted to 10.0 and loaded onto a potato chymotrypsin inhibitor I-affinity column (10 mg of potato chymotrypsin inhibitor I (Calbiochem) was coupled to 2 mL of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions). The column was washed with the same buffer and eluted with water/0.01% (v/v) Triton X-100 followed by 0.01 M HCl/0.01% (v/v) Triton X-100. Finally fractions were passed through a gel filtration column (Phenomenex S3000). Fractions with GHP activity were concentrated 10−20 fold by ultrafiltration using Centricon 10 tubes (Amicon). An equal volume of 1% (w/v) CHAPS and 60% (v/v) glycerol was added to the purified GHP samples for long-term storage at −80 °C.

Digestion of recombinant peptides with purified GHP. One mg of recombinant R1X5 peptide [30] (Supporting Information, Figure S3) was dissolved in 100 μL of 0.05 M ammonia bicarbonate containing DTT (2 mg/mL) and incubated at 56 °C for 45 min. 100 μL of iodoacetamide (10 mg/mL) was added and the mixture incubated for 30 min in the dark and then dried under vacuum. The alkylated sample was dissolved in 20 μL of digestion buffer (0.05 M ammonia bicarbonate containing 0.02% (v/v) Triton X-100) and divided into four parts. Purified protease was added to three tubes; 0.3, 1, and 3 μL; to the fourth tube no protease was added to act as a control to identify any contaminating peptides already present in the source material. Enzyme/peptide mixtures were incubated from between 10 min up to 17 h at 38 °C, dried and analyzed by MALDI-Tof MS (for size estimation of hydrolysis products), Q-Tof MS (for *de novo* sequencing) or SDS−PAGE.

Protein Sequencing. Fractions were reduced with 5% (v/v) 2-mercaptoethanol in SDS loading buffer and run on precast NuPage 4%−12% gradient or 16% Tris-Tricine gels (Invitrogen) with MultiMark colored molecular weight standards (Invitrogen). Proteins were either stained with silver[31] or Colloidal G-250 Coomassie Blue (Sigma) or transferred onto a PVDF membrane using semidry blotting and CAPS/methanol buffer and sequenced by Edman degradation (PNAC, Cambridge). For sequencing using mass spectrometry, stained gel slices were excised, destained, and subjected to in-gel digestion with trypsin.[32]

Mass Spectrometry. A MALDI L/R (Micromass, Manchester, UK) was used for MALDI-TOF MS. Data acquisition and processing were performed via the MassLynx 4.0 data system. The mass spectrometer was calibrated in reflectron mode across the mass range 800−3500 Da using 100 fmol of an alcohol dehydrogenase tryptic digest and tuned to a resolution of greater than 10,000 (FHMW). For reflectron mode, 2 mg/mL of α-cyano-4-hydroxycinnamic acid (HCCA) was dissolved in 49.5% (v/v) ethanol, 49.5% (v/v) acetonitrile, and 1% (v/v) of a 0.1% (v/v) TFA solution. Peptide digests were mixed 1:1 with matrix and 1 μL spotted onto the target plate. Glu1-fibrinopeptide B peptide (100 fmol/μL) (Sigma) was used as an external lock mass standard. Mass spectrometric analysis by ESI-MS was as described in ref 33.

Synthetic Peptides. Two synthetic peptides (NH2-PGQGQQG-YYPTSLQQ-OH and NH2-GYYPTSLQQPGQGQQ-OH) (from Activotec, Cambridge, UK) were dissolved in 50 mM ammonium hydrogen carbonate at 1 mg/mL and 1 μL of purified GHP added. One microliter aliquots were removed at various time points (0, 1, 5, 20, 40, and 60 min, 5 and 24 h) during incubation at 37 °C and 2.5 μL of concentrated acetic acid added, mixed, and evaporated to dryness. The digest was then resuspended in 0.1% (v/v) TFA/30% (v/v) methanol and passed through a SCX zip tip (Millipore) according to the manufacturer's instructions. The elutes were then dried and 1 μL of matrix added, mixed, and spotted onto the MALDI target.

RNA Isolation and cDNA Synthesis. Salivary glands of *E. integriceps* adults of the summer generation collected in the Saratov and Samara regions and Stavropol territory of Russia (organs from 20 bugs from each region) were dissected and quickly frozen. RNA was extracted using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The RNA was further treated with RQ1 RNase-free DNase1 (Promega) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using AMV reverse transcriptase (Promega) on DNase treated salivary gland RNA. Each reaction contained 4 μL of AMV 5× reaction buffer, 0.2 mM dNTP, 30 units AMV reverse transcriptase, 40 units of RNasin ribonuclease inhibitor, 1.5 μg of oligo(dT) primer, 2 μg of RNA template, and nuclease-free water to 40 μL.

Amplification and Cloning of a GHP Gene Fragment. A GHP gene fragment was amplified from *E. integriceps* cDNA by degenerate polymerase chain reaction (PCR) using REDTaq ReadyMix PCR reaction

mix (Sigma). Each reaction contained 12.5 μL of REDTaq ReadyMix (Sigma), 0.2 μM of the forward and reverse primers, 0.5 μL of the RT-PCR template, and nuclease-free water to 25 μL. The primers used were degenNterm and degenInternal (Table S1 of the Supporting Information). The amplification conditions were 95 °C for 2 min followed by 35 cycles of 95 °C for 45 s, 43 °C for 30 s, and 72 °C for 2.5 min, followed by 1 cycle of 72 °C for 5 min. PCR products were purified using the Qiaquick gel extraction kit (Qiagen) and ligated into the pGEM-T Easy plasmid vector using the pGEM-T easy Vector System (Promega).

Sequencing and Sequence Analysis. The cloned inserts of double stranded plasmid (pGEM-T easy, Promega) DNA were sequenced by MWG-Biotech (Eurofins MWG. Raynes Park, London. U.K.) using T7 and SP 6 oligonucleotide primers. Database searches were performed using the Basic Local Alignment Search Tool (BLAST) program through the National Centre for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignments were constructed with the ClustalW program through EMBL-EBI Web site (http://www.ebi.ac.uk/Tools/sequence.html).

Isolation of 5′- and 3′-Fragments Using RACE. For the isolation of 5′- and 3′-GHP gene fragments, reverse transcription and PCR were performed using the SMART RACE cDNA amplification kit (Clontech) and the Advantage 2 PCR enzyme system (Clontech), respectively, on *E. integriceps* cDNA. The gene specific primers used were InternalFor, for 3′ RACE, and InternalRev, for 5′ RACE (Table S1 of the Supporting Information) and the universal primer (Clontech). The amplification conditions were 95 °C for 30 s, 72 °C for 3 min for five cycles, followed by five cycles of 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min, followed by 27 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min.

Isolation of Full-Length GHP cDNAs. For the isolation of full-length GHP cDNAs, reverse transcription was performed, as before. PCR was performed using REDTaq ReadyMix PCR reaction mix (Sigma) on *E. integriceps* cDNA. Each reaction contained 12.5 μL of REDTaq ReadyMix (Sigma), 0.2 μM of the forward and reverse primers, 0.5 μL of the RT-PCR template, and nuclease-free water to 25 μL. The primers used were FL-5′and FL-3′ (Table S1 of the Supporting Information) designed to conserve untranslated 3′ and 5′ regions. The amplification conditions were 95 °C for 2 min, followed by 30 cycles of 96 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.5 min, followed by one cycle of 72 °C for 5 min.

Molecular Modeling of the *Eurygaster* GHPs. Homology models were constructed for the three sequences identified in this study (EiGPH1−3) using the 1.2 Å X-ray structure of a crayfish (*Pontastacus leptodactylus*) trypsin complex with a peptide inhibitor as template (PDB accession 2F91).[34] The synthetic peptides and the potato chymotrypsin inhibitor I were modeled using the backbone atoms of P_6−P_4' (Val_{23}-Pro_{32}) residues of the trypsin inhibitor from the crystal structure as the template. This allowed the positioning of peptide ligand backbones into the canonical conformation observed for the trypsin inhibitor in the template crystal structure. Side chains were replaced by relevant ones, and clashes were avoided by choosing low energy rotamers. Hydrogen atoms were added consistent with pH 7.0, keeping the imidazole ring of the histidine residues in a singly protonated state. Ligands were acetylated at the N-terminus to mimic the backbone of larger peptides and to remove the artificial effect of a charged amine at the N-terminus. Partial charges were assigned using the CHARMM force field, the models were surrounded by a 5 Å layer of water, and the energy of the structures minimized for 2000 cycles of conjugate gradient minimization. The stereochemical quality of the models was assessed with Procheck v.3.5.[35] Protein structures were visualized and manipulated using INSIGHT II (Accelrys Inc., San Diego, CA), and figures were prepared with PyMOL.

RESULTS

Digestion of Glutenin Proteins of the Durum Wheat Ege-88. The specificity of the GHP for HMW subunits of wheat glutenin is shown in Figure 1, which compares the SDS−PAGE patterns of total seed protein extracts from damaged and undamaged grain of the Turkish durum wheat cultivar Ege-88. This cultivar expresses only two HMW glutenin subunits which are encoded by the *Glu-B1* locus on chromosome 1B and designated subunits 1Bx7 and 1By8 (indicated in Figure 1, track d). Comparison of fractions extracted with SDS sample buffer, from damaged (Figure 1, track a) and undamaged (Figure 1, track b) grains, showed lower intensities of the two HMW subunits in the extract from damaged grain compared to that of undamaged, although the two bands were clearly still present. The same milled grain preparations were therefore incubated in water for 60 min at 37 °C to simulate the conditions during dough mixing and proofing. Total proteins were then extracted with SDS buffer and separated as before (Figure 1, tracks c and d). Comparison of the fractions extracted from damaged (Figure 1, track c) and undamaged (Figure 1, track d) grains showed that more extensive digestion of gluten proteins had occurred in the damaged fraction during incubation, resulting in complete loss of the HMW subunit bands and reduced intensities of the lower molecular mass bands corresponding to other gluten proteins. Thus, although enzymes present in the damaged grain show clear activity against HMW subunits, the same or other enzymes present in the damaged grain are also capable of digesting a wider range of gluten proteins upon prolonged exposure.

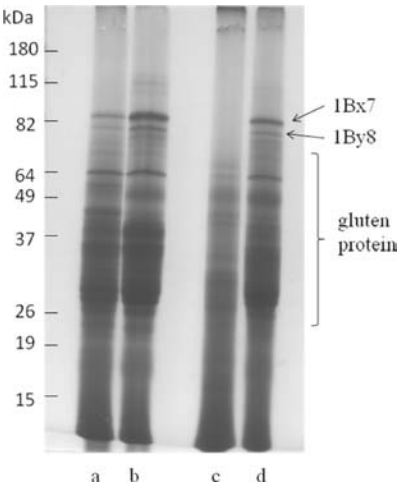

Figure 1. SDS−PAGE of total protein extracted from Eurygaster-damaged and control (undamaged) seeds of the Turkish durum wheat cultivar Ege-88. Molecular weights are indicated on the left. (track a) Damaged seed extracted with SDS sample buffer; (b) control seeds extracted with SDS sample buffer; (c) damaged seed incubated with water prior to the extraction with SDS sample buffer; (d) control seeds incubated with water prior to the extraction with SDS sample buffer. The two high molecular weight subunits (1Bx7 and 1By8) of glutenin present in this durum wheat cultivar are indicated on the right, as are the other gluten protein subunits. Twenty microliters of each extract was loaded onto the gel.

Detection of GHPs in Damaged Seeds and Salivary Glands. Proteases were extracted from either damaged seeds or salivary glands of *E. integriceps* with water, separated by IEF, and identified using a novel glutenin replica method based on the gelatin replica method reported by,[28,29] which identifies protease activity by digestion of a protein-coated film. Figure 2 tracks a−c show glutenin replicas of IEF separations carried out with increasing volumes of extracts from damaged wheat seeds and tracks d−f replicas of increasing volumes of extracts from insect salivary glands. Both

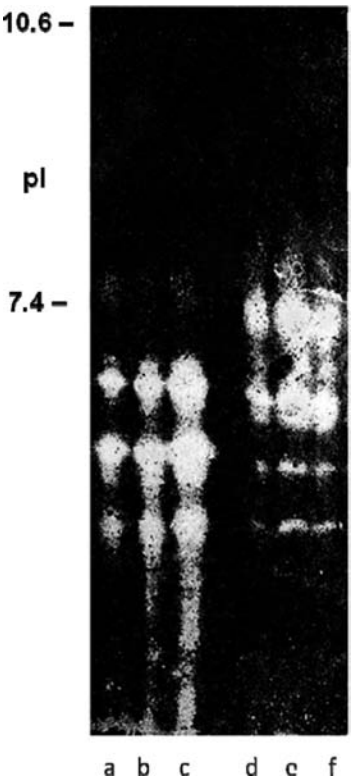

Figure 2. Detection of glutenin hydrolyzing proteinases (GHPs) using the novel glutenin replica method. Detection of glutenin hydrolyzing proteinases (GHPs) in protein fractions extracted from seeds damaged by *E. integriceps* (a−c) and salivary glands of the same pest (d−f) and separated by isoelectric focusing (IEF) in pH range 5.5 to 8.5 using the glutenin replicas method. Increasing volumes, 1, 2, and 4 μL, of protein fractions (a−c and d−f) from both damaged seeds and salivary glands were loaded.

extracts (from damaged seeds and salivary glands) show bands of protease activity with pI between 5.4 and 7.0 (bracket 1, Figure 2), but the extract from salivary glands shows additional activity at approximately pI 7.5 (bracket 2, Figure 2).

Analysis of further samples showed that the production of active GHP by the Sunn bug is seasonal, with substantial activity being detected in the salivary glands of bugs from the summer generation that feed on maturing wheat seed but little activity in salivary glands of the overwintering generation that feeds on green wheat seedlings (Figure S1 of the Supporting Information). Similarly, little activity was detected in gut extracts from either generation (Figure S1 of Supporting Information). This shows that the synthesis of GHPs only occurs in the period when they are required for digestion of seed proteins and only in the salivary glands from where they are injected into the developing grain rather than in the gut where partially hydrolyzed proteins are digested after ingestion.

Purification of GHPs. Preliminary studies showed that the *Eurygaster* GHP was inhibited weakly by potato chymotrypsin inhibitor I (not shown), which is consistent with previous studies of the GHP from *Nysius*.[20] This inhibitor was therefore used as an affinity matrix for the purification of GHPs from damaged wheat seed. A protein extract from the damaged seed of the Turkish durum wheat cultivar Ege-88 was loaded onto an affinity column of potato chymotrypsin inhibitor I (PCI-I) linked to Sepharose-4B. After extensive washing with alkaline loading buffer, fractions were eluted with water followed by 0.01 M HCl (Figure 3a). Protease activity in

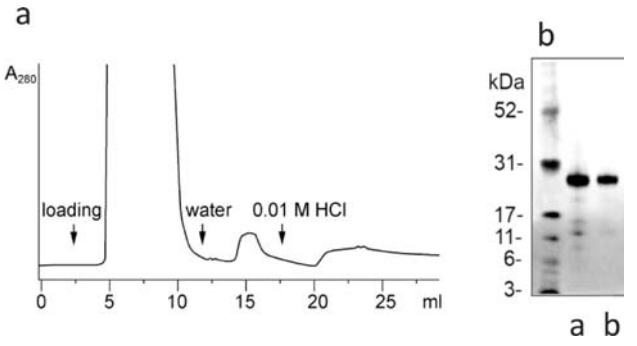

Figure 3. Isolation of GHPs from seeds damaged by *E. integriceps* using affinity chromatography. (a) Elution profile of protein extract isolated from damaged wheat seed, applied to a potato chymotrypsin inhibitor I affinity column. The column was washed with 0.01 M ethanolamine containing 0.2 M NaCl and 0.01% Triton X-100. Proteins were eluted with water and HCl containing 0.01% Triton X-100 and absorbance monitored at 280 nm. (b) Silver-stained SDS−PAGE of fractions eluted from the affinity column from Turkish durum wheat var. EGE-88. Track a, molecular weight markers; b, fraction eluted with water; c, fraction eluted with HCl.

the eluted fractions was monitored using the glutenin replica method, and fractions showing protease activity were then separated by SDS−PAGE (Figure 3b). The fractions eluted with water (Figure 3B, track a) and HCl (Figure 3b, track b) contained major bands of approximately 28 kDa, with some minor bands also being present in the water extract. The latter were removed by gel filtration to give an essentially pure 28 kDa band (data not shown).

Sequence Analysis of GHPs. *N*-Terminal sequencing of the 28 kDa band present in the fractions from affinity chromatography gave the sequence IVGGS/TQALDNEYP, with the presence of both serine and threonine at position 5. These sequences, including the two variants at position 5, were confirmed by analyzing similar fractions from damaged grains of a second Turkish cultivar Bayraktar and a varietal mixture of wheats from Russia (data not shown). ESI-MS sequencing allowed the *N*-terminal sequence to be extended to 17 residues (IVGGS/TQALDNEYPWMVK) and also gave the sequence of an internal tryptic peptide of 12 residues (TI/LNDI/LAI/LI/LPYAQ/K). It should be noted that isoleucine/leucine (I/L) and glutamine/lysine (Q/K) have the same masses and thus cannot be distinguished by mass spectrometry.

Comparison of these amino acid sequences with those in protein sequence databases showed homology to a range of serine proteases from invertebrates, including the tarnished plant bug *Lygus lineolaris* and *Creontiades dilutes*. No related sequences from plant sources were identified, either in protein databases or EST databases (containing over 1 million wheat ESTs, most of which are derived from developing grain), or the recently available shotgun genomic sequence of bread wheat cultivar Chinese Spring (http://www.cerealsdb.uk.net). This supports the origin of the 28 kDa protein being from the Sunn bug and not the wheat grain.

Cloning of *Eurygaster* GHPs. Degenerate primers were designed on the basis of the protein sequences and used to amplify cDNA prepared from *Eurygaster* salivary glands collected from three regions of Russia. Three full length clones were obtained from each of the regions (Ei.GHP1−3, NCBI, GHP1, HM579785; GHP2, HM579786; GHP3, HM579787), showing the highest homology to the trypsin precursor of *Lygus lineolaris* (52.7% similarity; 36.4% identity) and the serine protease of *Creontiades dilutes* (Green Mirid) (order, Hemiptera; family, Miridae), (51% similarity; 35.6% identity) (Figure 4.) Two of the clones encoded proteins with

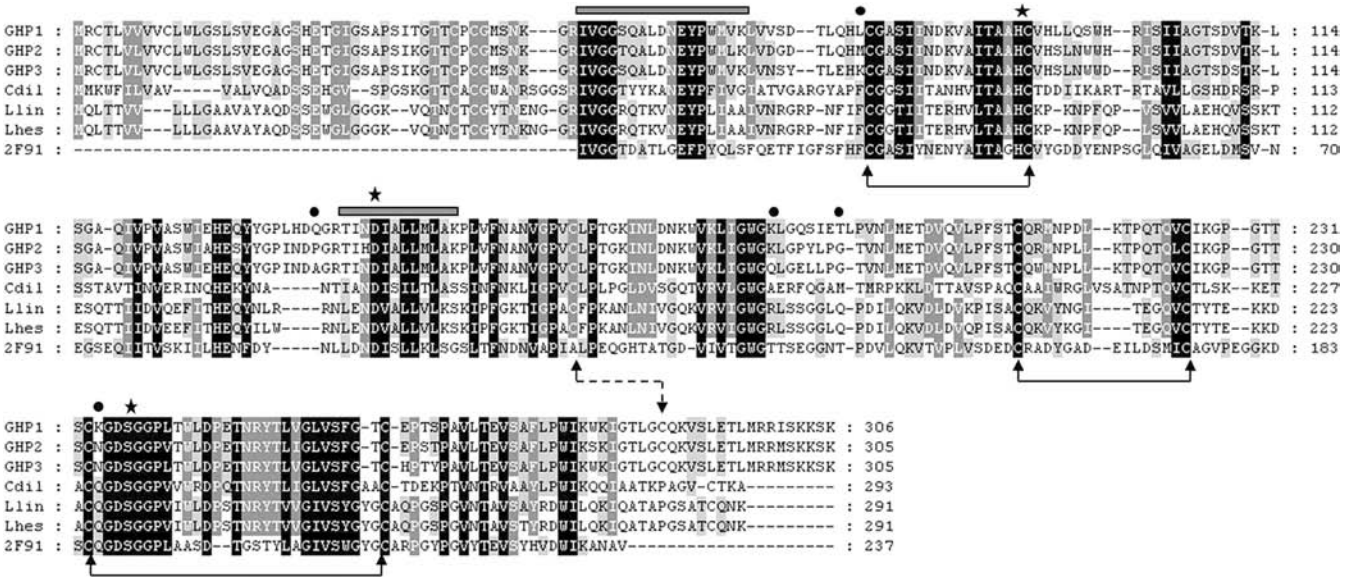

Figure 4. Amino acid sequence alignments. Amino acid sequence Alignment between the three mature GHP (GHP1−3 (NCBI BankIt ID: 1357636) isoforms with that of the crayfish trypsin template (2F91), used for molecular modeling and the three most similar sequences to the GHP's in the NCBI database. Serine protease of *Creontiades dilutes* (Cdil, AAL15154); trypsin precursor of *Lygus lineolaris* (L.lin, AAP12674); and trypsin precursor from *Lygus hesperus* (L.hes, AAK71135). Residues conserved between all sequences are highlighted in black. Partially conserved residues are highlighted with shades of gray. Variant residues that are in contact with the substrate binding cavity are marked with a black dot. The three catalytic residues (His, Asp, Ser) are marked with a star. The disulfide bond pattern is indicated with solid arrows for all the sequences; the dotted arrow indicates the additional disulfide bond present in the three GHPs. Sequences obtained by MS and Edman sequencing initially used for cloning the GHPs are indicated by the gray bar above the sequence.

serine at position 5 and one clone threonine at position 5, confirming the two sequence variants identified by direct sequencing. The essential catalytic triad (histidine, serine, and aspartic acid) characteristic of serine protease enzymes was conserved in the three cloned GHPs (Figure 4, highlighted in bold and with an asterisk above), with the aspartic acid residue being present in the sequence corresponding to the peptide sequenced by ESI-MS.

Determination of GHP Specificity. The HMW subunits of glutenin contain long repetitive domains (between 600 and 900 amino acids), comprising short peptides based on hexapeptide (consensus PGQGQQ) and nonapeptide (consensus GYYPTS**L**-QQ or GYYPTS**P**QQ) motifs.[36] We therefore used two model peptide systems to determine the specificity of the GHP (Figure S2 of the Supporting Information) using synthetic and recombinant peptides.

We initially used a recombinant peptide of 203 amino acid residues, the central part of which comprised 10 tandem repeats of hexapeptide and nonapeptide motifs (PGQGQQ + GHYPASL-QQ).[29] Digestion of this peptide (called R1X5) with the GHP resulted in ladders of fragments when separated by SDS−PAGE (Figure 5). *N*-Terminal sequencing of individual fragments of the ladder obtained by digestion of R1X5 gave the sequence GHY-PASLQQ. This demonstrated that the GHP hydrolyzed the peptide between the hexapeptide and nonapeptide motifs PGQG-QQ^GHYPASLQQ.

We then used two synthetic peptides to confirm the site specificity of the purified GHP. Both were 15 residues comprising the consensus hexapeptide (PGQGQQ) and nonapeptide (GYYPTSLQQ) motifs, but differed in the order of the two motifs (i.e., nonapeptide + hexapeptide or hexapeptide + nonapeptide) (Figure S2 of the Supporting Information). Figure 6 shows the mass spectra of the two synthetic peptides following digestion with the affinity purified GHP. Only the peptide PGQGQQGYYPTSLQQ

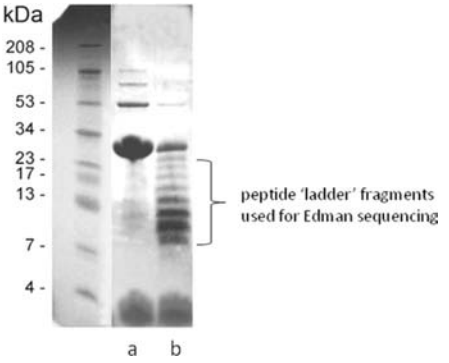

Figure 5. Recombinant peptide digest with purified GHP. SDS−PAGE of products of digestion of recombinant peptide R1X5 incubated with purified GHP. (a) molecular weight marker; (b) peptide R1X5 undigested control; (c) peptide R1X5 plus GHP.

was digested, the peptide GYYPTSLQQPGQGQQ remaining intact. The intact peptide of ~1650 Da, with adducts (probably sodium) from the incubation, give the large peptide peak at ~1673 Da, while the fragment at ~1078 Da indicates the loss of 595 Da, which is equivalent to the hexapeptide sequence PGQGQQ. Thus, the GHP cleaved the peptide between the hexapeptide PG-QGQQ and the nonapeptide GYYPTSLQQ (peak at ~1078 Da) motifs but not between the nonapeptide GYYPTSLQQ and hexapeptide PGQGQQ motifs. This confirms the specificity determined using the recombinant peptide.

Modeling the *Eurygaster* GHPs. On the basis of the specificity demonstrated experimentally, we used molecular modeling to further characterize the substrate specificity of the GHPs, generating homology models for the three GHP isoforms identified in this study.

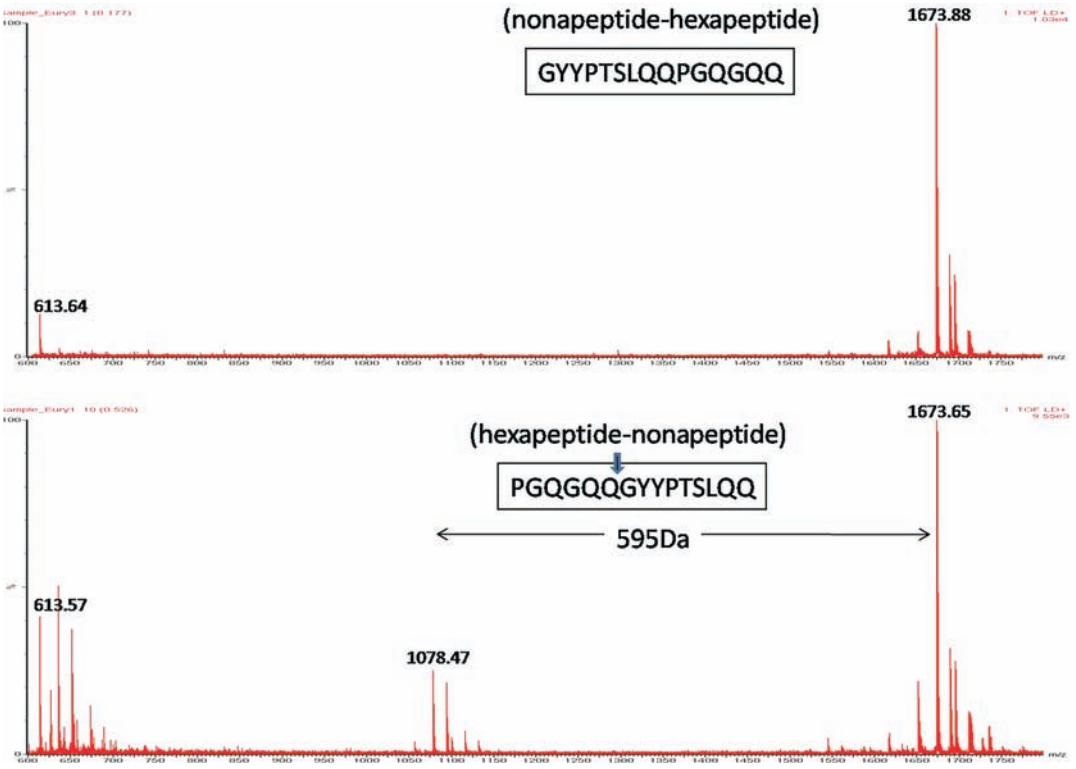

Figure 6. MALDI-TOF MS of synthetic peptide digest with purified GHP. MALDI traces of synthetic peptides incubated with purified GHP for 20 min at 37 °C. Peptide/enzyme mix was then cleaned by passage through a SCX zip tip prior to mass spectrometric analysis. Top panel, peptide GYYPTSLQQPGQGQQ. Lower panel, peptide GQGQQGYYPTSLQQ. The arrow indicates the site of cleavage of this peptide, on the basis of the mass of the major peak at 1078 Da.

The portion of the three *Eurygaster* GHP sequences that was modeled (the mature protein missing 22 amino acids at the *C*-terminus) showed approximately 34% identity and 48% similarity to the crayfish sequence used as the template (as this showed the highest similarity in the Protein Structure (PDB) database) (*A. leptodactylus* trypsin, PDB code 2F91) with 7% of the residues being in gaps located in loops between the main secondary structure elements (Figure 4). The three catalytic residues (His_{43}, Asp_{96}, and $Ser_{188/189}$) were located in conserved core regions which also contain six cysteine residues involved in the formation of three disulfides bonds with a pattern identical to that observed in the crystal structure template. An additional pair of cysteine residues (Cys_{116} and $Cys_{240/241}$) present in the GHP proteins are involved in the formation of another disulfide bond with the C-terminal end of the protein on the opposite side from the active site. This part of the protein could not be modeled in this study because it did not have a counterpart in the template structure. After energy minimization, comparison of the backbone of the GHP models with that of the crystal structure template reveals a seven residue loop extension corresponding to Gly_{85}−Gly_{91} located just before the catalytic aspartate (as illustrated for GHP3 in Figure7a and b and Figure S3a of the Supporting Information). The only major difference between the three GHPs modeled is an extended loop formed by residues Glu_{142} to Pro_{145} in GHP1 compared to GHP2 and 3 (Figure S3a and b of the Supporting Information).

Modeling Substrate Binding. Modeling the peptide PGQGQQGYYP (called R6) (which was present in the synthetic peptide used to determine the enzyme specificity (Figure S3 of the Supporting Information) fitted very well in the binding pocket of all three models with main chain atoms of the ligand involved in two intrachain H-bonds between the carbonyl oxygens of P5 Gly and P2 Gln and the amide hydrogens of P3 Gly and P1′ Gly, respectively (for example, GPH3 + R6, Figure S4a and b of the Supporting Information), and are likely to contribute to stabilization of the substrate in an optimal low energy conformation favorable for catalytic activity.

To corroborate the digestion profile of the R1X5 peptide, we modeled *in silico* the peptide PGQGQQGHYP (i.e., with histidine at the P2′ position) (Figure S2 of the Supporting Information). After docking and energy minimization, the P2′ histidine fitted very well into the S2′ pocket of all models, which is consistent with the observation that the peptide PQGQGQQGHYPASLQQ is cleaved between the PQGQGQQ and the GHYPASLQQ to liberate the GHYPASLQQ peptide (identified experimentally by *N*-terminal sequencing of the peptide ladder produced by digestion of R1X5) (Figure 5). Further details of the molecular modeling including calculation of interaction energies and modeling of cleavage site specificity are given in the Supporting Information.

DISCUSSION

The Sunn bug is an economically important pest in the Middle East, Central and West Asia, North Africa, and parts of Europe. It is particularly damaging when infesting developing grain as it injects enzymes into the grain that specifically digest gluten proteins, leading to the loss of processing quality. Analyses of wheat samples damaged by *E. integriceps* (and probably also the related species *E. maura* L.) originating from different sites in Russia and Turkey indicate that they contain multiple forms of GHP, which differ in their pIs but are almost identical on SDS−PAGE and by *N*-terminal sequencing.

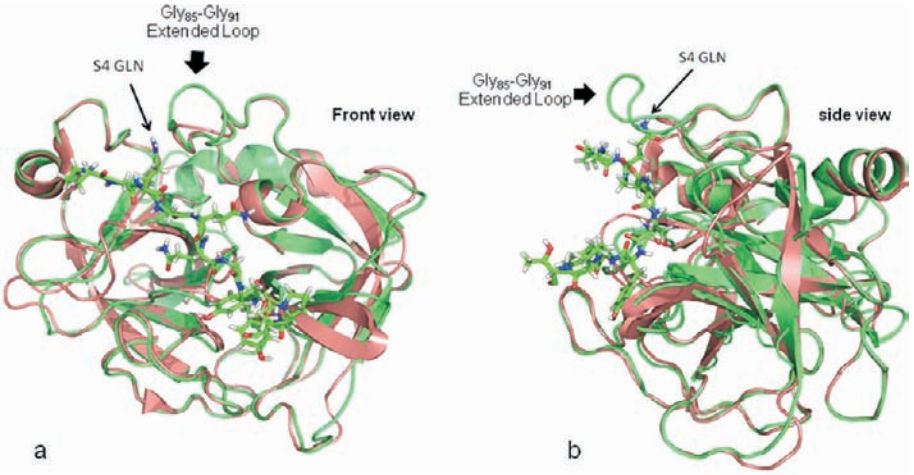

Figure 7. (a and b) Molecular modeling of the three GHP sequences. (a) Front view and (b) side view, superimposed GPH3 (green ribbon) and the crayfish trypsin template structure 2F91.pdb (pink ribbon) showing the Gly_{85}-Gly_{91} extended loop present in the GHP models (arrow). The docked R6 peptide (PGQGQQGYYP) is shown as a stick model in atomic colors (C atoms in green). The P4 glutamine residue is also indicated.

We have used affinity chromatography and novel detection methods to purify a major GHP from seeds of wheat infested with the Sunn bug *E. integricepts*. The partial amino acid sequence of the purified protease was used as a basis to isolate full length cDNA clones from Sunn bug salivary glands. The proteinase has some limited sequence identity with serine proteinases of various invertebrates and vertebrates and is highly specific for the HMW subunits of wheat glutenin, cleaving within the repeated peptide sequences which comprise around 80% of the HMW subunits. It was, however, largely insensitive to proteinaceous inhibitors of serine proteinases, which is similar to Sunn bug salivary and gut α-amylases, which were also mostly insensitive to amylase inhibitors.[37]

The *Eurygaster* GHP was specific for the peptide bond *between* the glutamine and glycine residues in adjacent hexapeptide and nonapeptide motifs (PGQGQQ↓GYYPTSLQQ) but did not cleave between the same two residues *within* the hexapeptide motif (PGQGQQ). In contrast, the GHP from the New Zealand wheat bug *N. huttoni* has been reported to cleave the glutamine/glycine bond *within* the hexapeptide (PGQ↓GQQGYYPTSLQQ).[15]

The presence of inhibitors of α-amylase and proteinases in plant and in particular in plant storage organs such as seeds has been suggested as a strategy to confer resistance to insect and other invertebrate pests, and its effectiveness has been demonstrated in model plant systems and crops grown under controlled conditions.[38−41] However, it has not yet proved to be applicable in commercial crop production systems. The new data presented here might permit the identification or design of specific GHP inhibitors. Wheat cultivars vary in their susceptibility to gluten damage by *Eurygaster* enzymes[8,18,42] indicating that it may be possible to select for GHP-resistant glutenin proteins based on an understanding of enzyme specificity and mechanisms of action. The novel and highly specific activity demonstrated for the GHP described here could also be exploited in food processing, using a recombinant enzyme to fine tune the structure and properties of the gluten network for specific food applications.

The high specificity of the enzyme may also be exploited in enzyme therapy for celiac disease. Celiac disease is estimated to occur in at least 1% of the general population in Western Europe and North America and results from an autoimmune response which is triggered by the binding of gluten peptides to T cells of the immune system in some individuals with the human leucocyte antigens (HLAs) DQ2 and DQ8. The HLA-DQ2 antigen is present in about 95% of celiac patients,[43] and detailed studies by a number of workers have led to the identification of the major antigens which trigger the autoimmune response. The identification of prolyl and glutaminyl endoproteinases that specifically cleave within these epitopes has led to clinical trials of enzyme therapy, in which the endoproteinases are ingested together with gluten-containing foods.[44,45] Less is known about the HLA-DQ8-associated form of celiac disease, which affects about 6% of celiac patients without HLA-DQ2 and 10% of patients with HLA-DQ2.[43] However, van de Wal et al.[46] defined the minimal glutenin epitope for the HLA-DQ8 form of celiac disease as containing the sequence QQGYYPTS, which includes the cleavage site for the *Eurygaster* GHP (QQ↓GYY). It would therefore be interesting to determine the effectiveness of the recombinant form of the *Eurygaster* proteinase in eliminating this epitope from wheat proteins present in food systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Detailed account of molecular modeling; sequences of oligonucleotide primers used for PCR and cloning of GHPs from *E. integriceps* salivary glands; detection of glutenin hydrolyzing proteinases (GHPs) in salivary glands and whole guts from overwintering and summer generations of *E. integriceps* separated by isoelectric focusing (IEF); recombinant, synthetic, and *in silico* peptides used for the determination of GHPs site specificity; molecular modeling of GHPs; Connolly surface representation of GHP3 and of GHP3 mapping the 30 variant amino acids on the surface of the protein; interaction energy profiles between GHPs 1, 2, and 3 models and the ligand peptides used in the docking experiments; Connolly surface representation of GHP3 mapping the 30 variant amino acids on the surface of the protein; interaction energy profiles between GHPs 1, 2, and 3 models and the ligand peptides used in the docking experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

GHP,gluten hydrolyzing proteinase; SCX,strong cation exchange.

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