Research Article

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Development of a peptide substrate for detection of sunn pest damage in wheat flour

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Abstract

BACKGROUND: Since the common protease substrates did not give satisfactory results for the determination of Sunn pest protease activity in damaged wheat, different peptide substrates derived from the repeated sequences of high molecular weight glutenin subunits were synthesized.

RESULTS: Hydrolysis of peptides by pest protease was determined by high-performance liquid chromatography. Among three peptides having the same consensus motifs, peptide1 (PGQGQQGYYPTSPQQ) showed the best catalytic efficiency. A novel assay was described for monitoring the enzymatic activity of protease extracted from damaged wheat flour. The selected peptide was labeled with a fluorophore (EDANS) and quencher (Dabcyl) to display fluorescence resonance energy transfer. The proteolytic activity was measured by the change in fluorescence intensity that occurred when the protease cleaved the peptide substrate. Furthermore, the assay developed was modified for rapid and easy detection of bug damage in flour. Flour samples were suspended in water and mixed with fluorescence peptide substrate. After centrifugation, the fluorescence intensities of the supernatants, which are proportional to the protease content of the flour, were determined.

CONCLUSION: The total analysis time for the assay developed is estimated as 15 min. The assay developed permits a significant decrease in time and labor, offering sensitive detection of Sunn pest damage in wheat flour.

Supporting information may be found in the online version of this article.

Keywords: Sunn pest; wheat; peptide substrate; FRET

INTRODUCTION

Sunn pest (*Eurygaster integriceps* Put.) is one of the most important pests of wheat in the Middle East, southern Europe, Central Asia, and North Africa, causing serious yield and quality losses.[1] In Turkey alone, over 300 million USD were spent for sunn pest control and the losses due to the damage were estimated at about 20 million US dollars for the year 2011.[2] Sunn pests attack the leaves, stems, and grains of wheat for feeding, and while doing this they inject their digestive enzymes into the wheat kernel. The insect-damaged wheat contains residual salivary secretion of insects, including proteolytic enzyme(s).[3,4] The protease breaks down the gluten structure, resulting in poor dough and bread properties.[5–9]

During the early stages of kernel development (e.g., milk-ripe stage), much of the kernel content may be sucked out by the insect, resulting in lighter and shriveled kernels that are easily removed in the cleaning section of flour mill. If insect attack occurs during the later stages of kernel development, the kernels retain their normal size and shape and are difficult to remove in the cleaning section of the flour mill.[10] The second type of damage is a major concern for the milling industry, and the level of damage needs to be determined accurately. However, this is not an easy task due the complex characteristics of the damaged kernels.

One of the most widely used methods to detect Sunn pest damage is visual analysis of the kernels. The damaged kernels have characteristic puncture marks surrounded by a pale area, and the puncture marks might be dark colored if infected. The kernels with a sign of pest damage are manually separated and counted. In this method, the damage is quantified by proportioning the number of damaged kernels with the sound ones. However, the number of puncture marks on the kernels was not considered and has a major impact on wheat quality.[2] Although this method is suitable for detecting damage on wheat kernels, it is not applicable on flour samples. The methods based on rheological properties of the dough obtained from flour samples are also used to determine sunn pest damage in the samples.[11] However, requirements for an experienced operator and costly instruments, such as a Farinograph, Extensigraph, and Alveograph, obstruct rapid and easy detection of sunn pest damage in wheat flour.[12] Studies on detecting sunn pest damage have concentrated mostly on the proteolytic effect on wheat proteins. Biochemical analyses, such as gel electrophoresis and high-performance liquid chromatography (HPLC),[8,13] have been used to investigate the protein degradation due to pest proteases. The wheat sedimentation test has been modified to determine sunn pest damage in wheat by adding bromophenol solution and extending the hydration time in the assay.[10,14] A similar autolytic assay, the sodium dodecyl

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sulfate (SDS)-sedimentation test, was developed by Cressey and McStay,[15] for the detection of sunn pest damage in wheat. In this method, the decrease in SDS-sedimentation volume of damaged flour samples is measured after an incubation period in distilled water. Another widely used biochemical method is SDS-protein gel assay, based on measuring gel volumes formed by gluten before and after an incubation with extract obtained from a wheat sample.[16] Reduction in the gel volume gives a measure of proteolytic activity due to sunn pest damage. These biochemical tests have various disadvantages, such as being labor intensive and time-consuming. In order to ensure technological quality, there is an urgent requirement for a sensitive, convenient, and rapid assay for the determination of sunn pest damage in wheat flour samples.

Conventional protein substrates, such as azo-casein, azo-albumin, gelatin, cytochrome C, and hemoglobin, have been used for sensitive and rapid detection of total proteolytic activity. Although there have been attempts to use these substrates for the detection of wheat bug (*Nysius huttoni*) proteases, they were not successful due to the high specificity of the enzyme against wheat gluten.[17] A similar study was carried out by Hosseininaveh *et al.*,[18] demonstrating that proteolytic activity in salivary gland and midgut of *E. integriceps* Puton was not detectable by using azo-albumin, azo-casein, and hemoglobin. Although, proteolytic activity in salivary gland and midgut extracts could be measured with fluorescent peptide substrates, which are specific for serine and cysteine proteases, these substrates were not tested for enzyme activity measurement in wheat samples.

The aim of this study was to develop a specific substrate for sunn pest proteases to be used in rapid and easy detection of pest damage in wheat flour. Early studies have shown that proteolytic enzymes of wheat pests are highly specific for the high molecular weight (HMW) subunits of glutenin, which play critical role in functional properties of wheat flour.[17,19] The DNA sequence of HMW glutenin revealed that there are two types of subunits, both having a number of repetitive sequences in the central regions of the polypeptides.[20] These repetitive regions comprise consensus motifs of PGQGQQ and GYYPTS(P/L)QQ. Konarev *et al.*[21] investigated the cleavage site of glutenin hydrolyzing proteases purified from wheat damaged by sunn pest (*E. integriceps*) using synthetic peptides having the repetitive sequences of HMW glutenin subunits (HMW-GSs). The study showed that the purified enzyme was specific for the motif PGQGQQGYYPTSLQQ cleaving between the glutamine and glycine at the sixth and seventh positions of the peptide. Therefore, the synthetic peptides having the sequence of repetitive regions of HMW-GSs have the potential to be used as the substrates of sunn pest proteases. In this study, the reactions between proteases from wheat damaged by sunn pest (*E. integriceps* Put.) and three different synthetic peptides derived from repetitive domains of HMW-GSs were investigated. Furthermore, one of these peptides was used to develop a fluorometric assay for the determination of sunn pest damage.

MATERIALS AND METHODS

Sunn-pest-damaged wheat flour samples

Seeds of bread wheat damaged by sunn pest (*E. integriceps*) were obtained from the Central Research Institute of Field Crops (Ankara, Turkey). Undamaged seeds were also obtained and used as control. The seeds were milled into flour with a laboratory-type roller mill (Sertas Machinery Limited Company, Ankara, Turkey). The flour samples were sieved (Endecotts Limited, London, UK) to obtain flour samples without bran.

Chemicals

Lactic acid (90%), isopropyl alcohol (99–100%), phosphoric acid (85%) and dimethyl sulfoxide were purchased from Merck-Millipore (Billerica, MA, USA). Ethanol was from Riedel-de Haen (Seelze, Germany). Bromophenol blue and Coomassie brilliant blue (G-250) dyes were obtained from Bio-Rad (Hercules, CA, USA). Ethanolamine, Triton X-100, Tris hydrochloride (Tris-HCl) and bovine serum albumin were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Extraction of sunn pest protease from flour samples

E. integriceps protease was extracted from wholemeal flour of severely damaged wheat sample. Flour samples were suspended in pure water and incubated at room temperature for different time periods. Then, samples were centrifuged at 12 000 × *g* for 25 min and supernatants containing protease were obtained. The concentration of flour and the incubation time during extraction were optimized for highest protease activity. In addition to pure water, water containing Triton X-100 and ethanolamine were also used as extraction solutions.

To determine the protease activity in the extracts, 0.8 g undamaged flour sample, 1 mL bromophenol solution (4 mg L^{-1}), and 4 mL extract were mixed in a graduated centrifuge tube and incubated at 37 °C for 2 h in a shaking medium. Flour sample mixed with bromophenol blue solution and enzyme extraction solution was used as control. After incubation, 5 mL sedimentation test solution[22] was added and the tube was shaken for 5 min. Then the centrifuge tubes containing samples were removed from the shaker and placed on a flat surface in upright position. The volume of sediment was read exactly after 5 min. Reduction of sediment volume compared with the control gives a measure of protease activity.

Proteins in the extract were further precipitated with nine volumes of cold ethanol (−20 °C). Sample was centrifuged at 13 000 × *g* for 30 min and the supernatant was carefully removed. The pellet was dissolved in pure water and protein concentration of partially purified protease solution was determined by the Bradford method.[23]

Proteolysis of synthetic peptides

Three different peptide sequences derived from repetitive regions of HMW-GSs were synthesized by solid-phase peptide synthesis without any modification at the terminals and purified to 95% purity (GenScript Corp., Piscataway, NJ, USA). Peptide1 (PGQGQQ GYYPTSPQQ), peptide2 (PGQGQQGQQGYYPTSPQQ), and peptide3 (PGQGQQGYYPTSLQQ) were dissolved in pure water (8 mg mL^{-1}), and 10 µL of peptides were mixed with 25 µL of protease solution (2.46 mg protein mL^{-1}). The mixtures were diluted to 350 µL with water and incubated at 37 °C for 2 h. Protease solution without peptides and peptide solutions without protease were also diluted and incubated at 37 °C for 2 h. After incubation, solutions were analyzed by HPLC. A complete Agilent 1100 series chromatographic system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an isocratic pump, an autosampler, a thermostat, a degasser, and a diode-array detector was used. The separation was performed on an Agilent Bio SEC-3 Size Exclusion (150 Å, 3 µm, 300 mm length, 4.6 mm ID) analytical column. Elution was carried out at a flow rate of 0.4 mL min^{-1} using pure water. The column temperature was kept at 20 °C, and the elution was monitored at 280 nm.

In order to investigate the effect of pH and temperature on protease–peptide interactions, 10 μL of peptide solution was mixed with 25 μL of protease solution and the mixtures were diluted to 350 μL with 0.05 mol L^{-1} Tris-HCl buffer at different pH values. The reaction temperature was optimized by varying incubation temperatures from 25 to 42 °C.

Fluorometric assay to measure proteolytic activity

The peptide selected as the potential substrate was labeled with a fluorescent donor (EDANS) and quenching moiety (Dabcyl) at the terminals and synthesized with 95% purity (GenScript Corp., Piscataway, NJ, USA). The peptide was dissolved in dimethyl sulfoxide to a final concentration of 30 mg mL^{-1} and then further diluted with pure water. The fluorescence assay was performed using a Cary Eclipse fluorescence spectrophotometer and a quartz microcell (Agilent Technologies, Inc., Santa Clara, CA, USA). The excitation and emission wavelengths were 338 nm and 511 nm respectively, and the temperature was kept constant at 37 °C using a Peltier system. Proteolytic activity measurements were done in a final volume of 100 μL containing 10 μL fluorogenic peptide solution (4 mg mL^{-1}), 50 μL of 0.1 M Tris-HCl buffer (pH 7.0), and 40 μL protease solution. The activity of enzyme was determined by monitoring the fluorescence intensity of substrate every minute. The calculated initial rates were used as a measure of enzyme activity.

Direct determination of sunn pest damage in flour samples

Flour samples containing different amounts of sunn pest protease were prepared by mixing severely damaged flour sample with undamaged flour sample in different ratios. Modified Zeleny sedimentation and Zeleny sedimentation tests were performed for flour samples,[10] and the difference between the sedimentation volumes of the modified Zeleny and Zeleny sedimentation tests (DZ) was used as the measure of sunn pest damage. The samples (0.7 g) having different DZ values were suspended in 4.5 mL of 0.05 mol L^{-1} Tris-HCl buffer. The buffer itself was used as the negative control. Subsequently, 10 μL of the substrate solution (4 mg mL^{-1}) was added to 90 μL of the flour suspension. After 10 min of incubation, samples were centrifuged at 13 000 × *g* for 5 min. Finally, the fluorescence intensities of the supernatants were determined using excitation and emission wavelengths of 338 nm and 511 nm respectively. The excitation and emission slit widths were 10 nm. The fluorescence intensities were plotted against DZ values to obtain the calibration curve.

RESULTS

Extraction of sunn pest protease from flour samples

In order to investigate the interaction between sunn pest protease and synthetic peptides, the protease was extracted from damaged wholemeal flour with water. The effect of flour concentration on the proteolytic activity of the extract was determined and the highest activity was observed at a concentration of 0.35 g mL^{-1} (Fig. S1A). Above this value, the activity decreased slightly as a result of increased viscosity. Extraction time was also optimized in order to enhance the yield. The maximum proteolytic activity was determined when the incubation time was 45 min, although there was no considerable difference above 15 min (Fig. S1B). Water containing different concentrations of Triton X-100 and ethanolamine were also used as extraction solution. However, the proteolytic activities in the extracts were found to be comparable to the one extracted with water (data not shown). Proteases extracted from wholemeal obtained from damaged wheat sample with water were partially purified by ethanol precipitation, and protein concentration in the protease solution was found to be 2.46 mg mL^{-1}.

Proteolysis of synthetic peptides

Three different synthetic peptides, derived from repetitive regions of HMW-GSs, were used to investigate the substrate specificity of sunn pest protease extracted from damaged flour sample. Hydrolysis of peptides by partially purified protease solution was monitored by HPLC. Figure 1A shows the HPLC elution profiles of intact peptide1, enzyme solution and peptide1 incubated with enzyme solution. After incubation with enzyme solution, the absorbance of the peak corresponding to intact peptide1 decreased markedly, while fragments were formed indicating the cleavage of the peptide. Peptide2 and peptide3 showed similar trends when incubated with enzyme solution (Fig. 1(B and C)).

In order to compare the catalytic efficiencies of synthetic peptides, percentage cleavage values were calculated by comparing the peak areas of intact peptide and residual peptide after incubation with the enzyme. After 2 h of incubation, 58.9% of peptide1 was hydrolyzed, while the percentage cleavage values for peptide2 and peptide3 were found to be 21.4% and 4.7% respectively. The effects of pH and temperature on the activity of sunn pest protease were determined. When peptide1 and peptide3 were used as substrates, the maximal activity was observed at 37 °C (Fig. S2). The optimum temperature was 30 °C for hydrolysis of peptide2. The maximum proteolytic activity was observed at pH 7.0 for peptide1 and peptide2, while peptide3 had the highest efficiency at pH 9.0 (Fig. S3). When the percentage cleavage values of synthetic peptides at their optimum conditions were compared (Table 1), peptide1 showed the best catalytic efficiency. Therefore, peptide1 was selected for use as a potential substrate for sunn pest protease.

Fluorometric assay to measure proteolytic activity

Peptide1, comprising 15 amino acids, was labeled with a fluorescence resonance energy transfer (FRET) pair (EDANS–Dabcyl) at the terminals and used as fluorogenic substrate for sunn pest protease. Fluorogenic peptide was mixed with partially purified enzyme solution and the fluorescence changes were monitored for 20 min (Fig. S4). The fluorescence intensity increased linearly as a result of proteolytic activity, and the substrate was quite stable. Protease activity, calculated from the rate of fluorescence increase in 10 min, was proportional to the protein concentration of enzyme solution (Fig. 2). The correlation was linear up to a protein concentration of 1.23 mg mL^{-1}.

In order to assess if the developed fluorogenic peptide can serve as a substrate in the detection of sunn pest damage, labeled peptide1 was mixed with crude extracts obtained from flour samples having different levels of proteolytic activity. The degree of damage in flour samples were expressed as DZ values (the difference between the sedimentation volumes of modified Zeleny and Zeleny sedimentation tests) (Table S1). Flour samples were suspended in pure water (0.35 g mL^{-1}) and incubated for 45 min. After centrifugation at 12 000 × *g* for 25 min, crude extracts containing sunn pest protease were obtained. Fluorescent peptide was incubated with crude extracts and the increases in fluorescence intensities were measured. A severely damaged flour sample having a DZ value of −10 mL showed the highest proteolytic activity. As shown in Fig. 3, the reaction rate decreased with increasing DZ value due to the decrease in protease content. Proteolytic activity

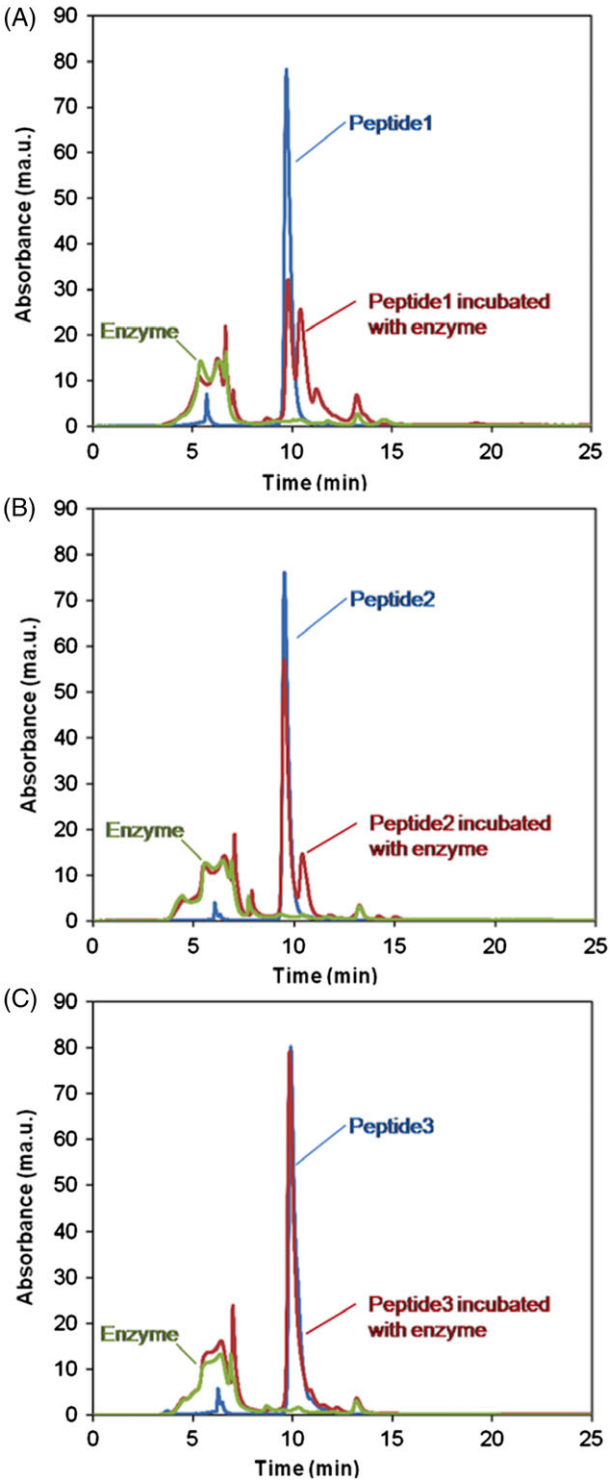

Figure 1. HPLC elution profiles of the intact peptides, peptide1 (A), peptide2 (B), and peptide3 (C), protease solution extracted from damaged wheat flour and the peptides incubated with protease.

Table 1. Optimum pH, temperature, and corresponding percentage cleavage values obtained from the hydrolysis of synthetic peptides by the enzyme solution extracted from damaged wheat flour

Peptide code	Optimum pH	Optimum temperature (°C)	Cleavage (%)
Peptide1	7	37	45.1
Peptide2	7	30	29.9
Peptide3	9	37	12.3

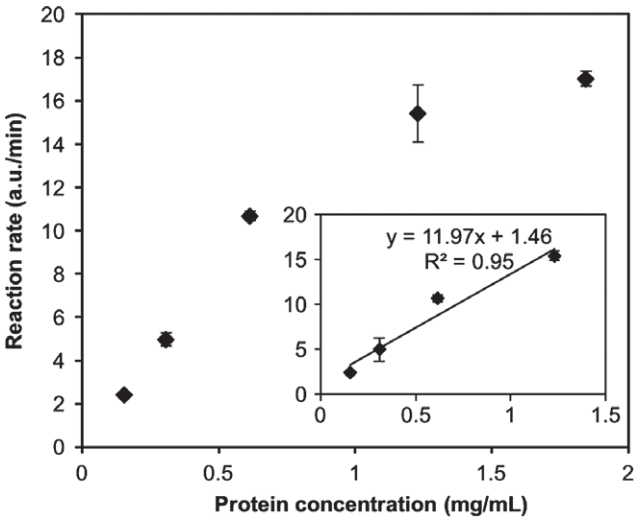

Figure 2. The fluorescence change rate as a function of protein concentration of protease solution extracted from damaged wheat flour.

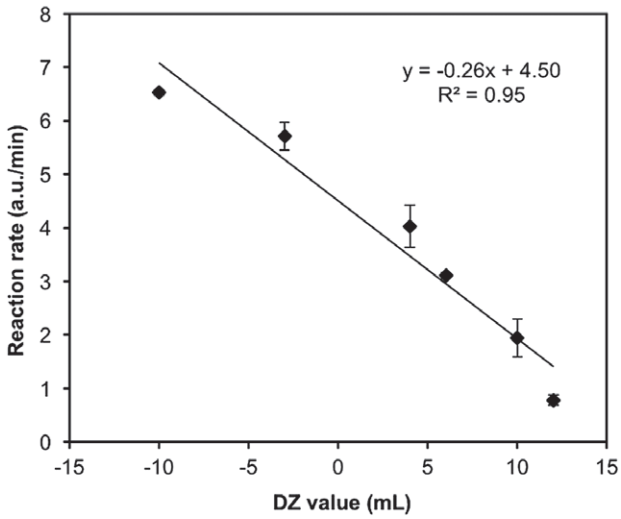

Figure 3. The fluorescence change rate as a function of DZ value (difference between the sedimentation volumes obtained from modified Zeleny and Zeleny sedimentation tests).

was linear in the range of −10 to +12 mL DZ value. The sensitivity of the assay developed was determined as 0.26 a.u. min^{-1} mL^{-1} DZ value. These results show that the substrate developed is sensitive to sunn pest protease activity. The total analysis time was 80 min, including the extraction of protease from the flour sample and fluorescence measurement.

Direct determination of sunn pest damage in flour samples

High sensitivity, short analysis time, and ease of application are crucial for the analysis of sunn pest damage. Therefore, we modified the procedure, including the extraction of protease from flour, by eliminating the extraction step and providing direct measurement of the proteolytic activity in flour samples. Accordingly,

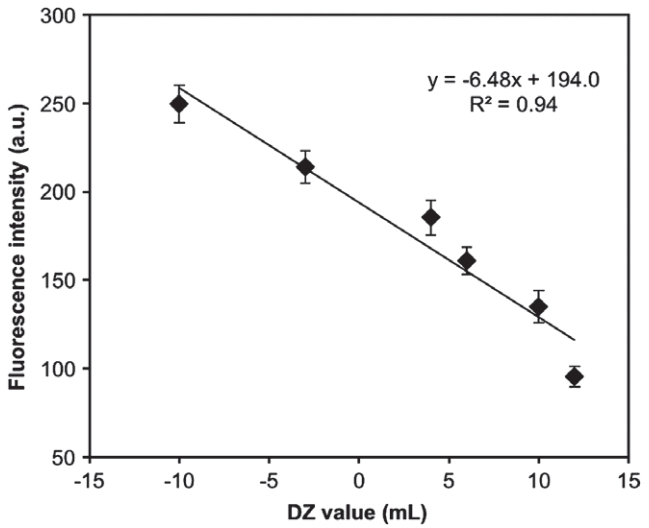

Figure 4. Calibration curve depicting the fluorescence intensity as a function of DZ value (difference between the sedimentation volumes obtained from modified Zeleny and Zeleny sedimentation tests).

flour samples were suspended in Tris-HCl buffer and mixed with substrate solution. After 10 min of incubation, samples were centrifuged at 13 000 × *g* for 5 min to remove any turbidity that may interfere with the fluorescence measurement and the intensities were recorded. It was found that cleavage of labeled peptide by flour samples containing sunn pest protease gave increases in fluorescence that were linear and correlated with the damage levels of the samples (Fig. 4). The calibration curve was found to be linear in the range −10 to +12 mL DZ value, and the sensitivity was calculated as 6.48 a.u. min^{-1} mL^{-1} DZ value, which is much higher than that obtained from the previously developed procedure.

DISCUSSION

Detection of the level of sunn pest damage in wheat is crucial as it is one of the most important determinants of technological quality in the areas affected by the sunn pest. While the assays based on the proteolytic activity on wheat gluten are useful, they are very time-consuming and labor intensive, and therefore inappropriate for on-site detection. Thus, peptides or proteins that sunn pest protease acts on can serve as substrates more amenable to bioassay test kits and biosensors. Early attempts regarding the proteins and synthetic substrates used in protease assays have failed due to the substrate specificity of sunn pest protease.[17] In this study, we conceptualized that repetitive regions of HMW-GSs on which sunn pest protease acts might be used as the specific substrates for bug protease. Hence, three different sequences having the same motifs of PGQGQQ and GYYPTS(P/L)QQ were chosen as potential substrates and the efficiency of synthesized peptides was determined using enzyme solution extracted from sunn-pest-damaged wheat flour. Among the peptides, peptide1 (PGQGQQGYYPTSPQQ) showed the best catalytic efficiency. The addition of GQQ motif (peptide2) reduced the catalytic efficiency of protease, and replacement of proline with leucine (peptide3) caused a drastic decrease in proteolytic activity. Although one may think that sunn pest protease is highly specific for the sequence of peptide1, optimum pH and temperature values also varied with the sequence used. Thus, it seems likely that the protease solution extracted from damaged flour contains multiple proteolytic enzymes having different catalytic properties.[24]

After comparing the peptide substrates of sunn pest protease, we selected the sequence of peptide1 to devise a fluorescent substrate. The N and C termini were labeled with fluorescence donor and acceptor to display FRET, which was lost after cleavage. Thus, the activity of the protease was monitored by changes in fluorescence intensity. The fluorescence intensity was increased linearly upon addition of protease solution extracted from sunn-pest-damaged sample. To verify the suitability of the substrate for the detection of pest protease, we tested the substrate with different concentrations of protease solution and the rate of fluorescence increase was proportional to the concentration. For further validation of the substrate, we tested flour samples prepared by mixing severely damaged flour sample with undamaged sample in different ratios. The changes in fluorescence intensity were monitored after mixing the developed peptide with the crude extracts of flour samples. The proteolytic activity values obtained from the rate of fluorescence increase were proportional to those obtained from a modified Zeleny sedimentation test, one of the most commonly used assays in the detection of bug damage. The reduction of the sedimentation values obtained from modified Zeleny and Zeleny sedimentation tests (DZ values) is accepted as a measure of bug damage in wheat samples. However, for the flour samples with high gluten quality, positive DZ values can be obtained despite the presence of sunn pest protease.[25] By using the substrate developed, the proteolytic activity was observed even for those samples having positive DZ values, as sunn pest proteases could be directly detected regardless of the gluten content and quality of wheat flour. These results revealed that the method developed is promising for use in sensitive and selective detection of sunn pest protease in flour samples. In addition, the method developed could be easily modified in further studies to detect sunn pest damage in kernels by optimizing the extraction of protease from wheat.

We further modified the assay developed to achieve more rapid and easy detection of bug damage in flour samples. In the modified version, the extraction step was eliminated and fluorogenic peptide substrate was directly mixed with suspended flour sample. After 10 min of incubation, the mixture was centrifuged and the fluorescence intensities of supernatants were measured. When the sensitivity and assay time were considered, the performance of the modified method was much better than that of the procedure including the extraction step.

In conclusion, a specific sunn pest protease substrate was developed for measuring pest damage in wheat flour. Furthermore, the peptide substrate developed was the utilized in a FRET-based assay allowing a quick and easy detection of pest damage. By using the method developed, sunn pest damage in flour samples can be determined in minutes without the requirement of a specialist. In addition, the peptide substrate can also be used in colorimetric assays and test strips. Although the assay developed is promising, a full validation should be performed, including investigation of the effect of wheat type, to reveal the accuracy, specificity, and reproducibility of the assay. The results indicate that the peptide substrate used in this study can be exploited for developing novel methods for the detection of different types of pest damage in wheat.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Critchley BR, Literature review of sunn pest *Eurygaster integriceps* Put. (Hemiptera, Scutelleridae). *Crop Prot* **17**:271–287 (1998).
2 Dizlek H and Islamoglu M, Effects of sunn pest (*Eurygaster maura* L. Heteroptera; Scutelleridae) sucking number on physical and physicochemical characteristics of wheat varieties. *J Appl Bot Food Qual* **88**:10–15 (2015).
3 Paulian F and Popov C, Sunn pest or cereal bug, in *Wheat*, ed. by Hafliger E. Ciba-Geigy, Basel, pp. 69–74 (1980).
4 Parker BL, Amir-Maafi M, Skinner M, Kim JS and El Bouhssini M, Distribution of sunn pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae), in overwintering sites. *J Asia-Pac Entomol* **14**:83–88 (2011).
5 Every D, Farrell JA, Stufkens MW and Wallace AR, Wheat cultivar susceptibility to grain damage by the New Zealand wheat bug, *Nysius huttoni*, and cultivar susceptibility to the effects of bug proteinase on baking quality. *J Cereal Sci* **27**:37–46 (1998).
6 Lorenz K and Meredith P, Insect-damaged wheat: history of the problem, effects on baking quality, remedies. *Lebens-Wiss Technol* **21**:183–187 (1988).
7 Sivri D, Köksel H and Bushuk W, Effects of wheat bug (*Eurygaster maura*) proteolytic enzymes on electrophoretic properties of gluten proteins. *New Zeal J Crop Hortic* **26**:117–125 (1998).
8 Sivri D, Sapirstein HD, Koksel H and Bushuk W, Effects of wheat bug (*Eurygaster maura*) protease on glutenin proteins. *Cereal Chem* **76**:816–820 (1999).
9 Köksel H, Sivri D, PKW N and Steffe JF, Effects of transglutaminase enzyme on fundamental rheological properties of sound and bug-damaged wheat flour doughs. *Cereal Chem* **78**:26–30 (2001).
10 Köksel H, Atli A, Dag A and Sivri D, Commercial milling of suni bug (*Eurygaster* spp.) damaged wheat. *Nahrung* **46**:25–27 (2002).
11 Karababa E and Ozan AN, Effect of wheat bug (*Eurygaster integriceps*) damage on quality of a wheat variety grown in Turkey. *J Sci Food Agric* **77**:399–403 (1998).
12 Dizlek H and Özer MS, Effects of of sunn pest (*Eurygaster integriceps*) damage ratios on rheological characteristics of wheat flour. *Qual Assur Saf Crop* **9**:47–54 (2017).
13 Aja S, Pérez G and Rosell CM, Wheat damage by *Aelia* spp. and *Erygaster* spp.: effects on gluten and water-soluble compounds released by gluten hydrolysis. *J Cereal Sci* **39**:187–193 (2004).
14 Greenaway WT, Neustadt MH and Zeleny L, Communication to the editor: a test for stink bug damage in wheat. *Cereal Chem* **42**:577–579 (1965).
15 Cressey PJ and CL MS, Wheat-bug damage in New-Zealand wheats: development of a simple SDS-sedimentation test for bug damage. *J Sci Food Agric* **38**:357–366 (1987).
16 Every D, General proteinase assay by formation of SDS-protein gels of proteolyzed substrate proteins. *Anal Biochem* **197**:208–212 (1991).
17 Every D, Sutton KH, Shewry PR, Tatham AS and Coolbear T, Specificity of action of an insect proteinase purified from wheat grain infested by the New Zealand wheat bug, *Nysius huttoni*. *J Cereal Sci* **42**:185–191 (2005).
18 Hosseininaveh V, Bandani A and Hosseininaveh F, Digestive proteolytic activity in the sunn pest, *Eurygaster integriceps*. *J Insect Sci* **9**:1–11 (2009).
19 Sivri D, Sapirstein HD, Bushuk W and Koksel H, Wheat intercultivar differences in susceptibility of glutenin protein to effects of bug (*Eurygaster integriceps*) protease. *Cereal Chem* **79**:41–44 (2002).
20 Lásztity R, The chemistry of wheat, in *Cereal Chemistry*, 1st edn, ed. by Lásztity R. Akadémiai Kiadó, Budapest, pp. 85–92 (1999).
21 Konarev AV, Frédéric B, Marsh J, Vilkova NA, Nefedova LI, Sivri D *et al.*, Characterization of a glutenin-specific serine proteinase of sunn bug *Eurygaster integricepts* Put. *J Agric Food Chem* **59**:2462–2470 (2011).
22 AACC, Methods 56-60.01 and 56-61.02, in *Approved Methods of the American Association of Cereal Chemists*, 11th edn. American Association of Cereal Chemists, St. Paul, MN (2009).
23 Bradford MM, A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).
24 Ozgur E, Yucel M and Oktem HA, Identification and characterization of hydrolytic enzymes from the midgut of sunn pest of wheat (*Eurygaster integriceps*). *Int J Pest Manage* **55**:359–364 (2009).
25 Dizlek H and Özer MS, Effects of sunn pest (*Eurygaster integriceps*) damage ratio on physical, chemical, and technological characteristics of wheat. *Qual Assur Saf Crop* **8**:145–156 (2016).

