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Research Article

In vitro Gluten Degradation Using Recombinant Eurygaster Integriceps Prolyl Endoprotease: Implications for Celiac Disease

Effat Noori[1], Mojgan Bandehpour[2], Mohammad Reza Zali[3], Bahram Kazemi[1,2*]

[1]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[*]*Corresponding author*: Bahram Kazemi, Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tel/ Fax: +98-2188666139. E-mail: kazemi@sbmu.ac.ir

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Background: Celiac disease (CD) is a gluten-sensitive chronic autoimmune enteropathy. A strict life-long gluten-free diet is the only efficient and accepted treatment until now. However, maintaining a truly gluten-free status is both difficult and costly, often resulting in a social burden for the person. Moreover, 2 to 5 percent of patients fail to improve clinically and histologically upon elimination of dietary gluten. Therefore, novel therapeutic approaches, including gluten degrading enzymes, are an unmet need of celiac patients.

Objectives: To evaluate the function of sunn pest prolyl endoprotease for gluten and gliadin hydrolysis in vitro.

Materials and Methods: The spPEP was expressed as a recombinant protein *in E. coli BL21 (DE3),* and its catalytic activity was assessed by SDS-PAGE and RP-HPLC analyses.

Results: Production of a 100-kDa spPEP protein was confirmed by SDS-PAGE and western blot analysis. Also, we demonstrate that spPEP efficiently degrades gluten and α-gliadin (30-40 kDa) in vitro under conditions similar to the GI and is resistant to pepsin and trypsin.

Conclusion: The gathered data demonstrated that spPEP might be a novel candidate for Oral Enzymatic Therapy (OET) in CD and other gluten-related disorders.

Keywords: Autoimmunity, Celiac disease, Gluten, Prolyl endoprotease, Prolamin

1. Background

Celiac disease is a T cell-driven chronic autoimmune enteropathy triggered by partially digested wheat and related cereals gluten proteins. This disorder is characterized by inflammation and villous atrophy of the small intestine, intraepithelial lymphocytosis, and crypt hyperplasia in genetically susceptible individuals (1-4). The prevalence of CD is approximately 1% of the population worldwide, and this rate is increasing, especially in western societies (1, 5, 6).

Gluten comes from wheat and gluten-like molecules found in rye and barley and consists of gliadins and glutenins called prolamins. These prolamins' high proline and glutamine residue content (15% and 35% of all amino acids, respectively) render them resistant to complete enzymatic digestion in the gastrointestinal (GI) tract. (3, 4). These peptides have an affinity for human leukocyte antigen (HLA) DQ2 and DQ8, resulting in both humoral and T-cell mediated adaptive immune responses in the small intestine (2-4).

This multifaceted disorder, if left untreated, is associated with extraintestinal manifestations, including infertility,

rash, arthritis, seizure (7), anemia, osteoporosis, and intestinal lymphoma. This multifactorial disease affects all age groups (8, 9). Currently, a gluten-free diet (GFD) is the only effective treatment option for CD patients. However, it is not always effective or sufficient to relieve symptoms in many CD patients. In addition, adherence to a GFD is very difficult due to the ubiquitous nature of gluten, lack of availability, inadequate food labeling regulations, and social burden (7, 8, 10).

Given the challenges listed above and the high degree of patient dissatisfaction, it is evident that there is an unmet need for supplemental or alternative treatment options for CD (3, 4, 10).

Increasing understanding of the disease mechanism has recently led to the development of alternative non-dietary therapies (4, 10). A major ongoing area of research is the development of enzyme supplementation for the GI tract. The aim of this approach is to digest and detoxify antigenic regions of gluten peptides. Prolyl endoproteases' (PEPs) include attractive nondiet drug candidates, which have been tested for this approach (3, 4, 8, 10). Studies have also demonstrated where endopeptidases, produced by various Eukaryotes and Prokaryotes, degrade gluten peptides in the GI. These gluten detoxifying enzymes can be devised as Oral Enzymatic Therapy (OET). Alternative OET therapies must be safe, available, and effective (3, 4).

The sunn pest, Eurygaster integriceps Puton, belongs to the family Scutelleridae, one of the most important wheat pests (11-13). Darkoh *et al*. first identified the sunn pest prolyl endoprotease (spPEP) as a prolyl endoprotease (11). This enzyme is a serine protease of the S9A family with the ability to digest intact wheat gluten proteins (11, 12).

2. Objectives

To evaluate the function of sunn pest prolyl endoprotease for gluten and gliadin hydrolysis in vitro under conditions similar to the GI and resistant to pepsin and trypsin.

3. Materials and Methods

3.1. Materials

Protease substrate *N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA)* was obtained from *Sigma-Aldrich Company (Co)*. Restriction endonucleases and other DNA-modifying enzymes were purchased from Takara and Fermentas Cos. Bacterial strains *E. coli* and plasmid Pet-41a were obtained from Novagen Co. The Glutathione Sepharose 4B affinity matrix was purchased from Noavaranzist Co. Buffers and all other chemicals were purchased from Sigma and Merck Cos. Pepsin and Trypsin from bovine pancreas were obtained from Sigma-Aldrich Co. Antibiotics including Ampicillin and Kanamycin were purchased from Sigma Co.

3.2. Cloning of PEP

The pep was selected based on the pep sequence in the GenBank with accession number EU934738, version 3. It was first synthesized into a PUC57 plasmid (GeneCust, Luxemburg). Then, the pep was amplified from the PUC57 plasmid by specific primers. Amplification was carried out in 30 cycles of 5 min at 95 °C, 40 s at 95 °C, 30 s at 58 °C, 2 min at 72 °C, and 10 min at 72 °C. The PCR product and expression plasmid pET-41a (+) were digested with KpnI and EcoRI enzymes (Fermentas). Finally, the PCR product was ligated into pET-41a. The ligation mixture was transformed into *E. coli* Top10 competent cell. The recombinant plasmids were confirmed by PCR, restriction enzyme analysis, and nucleic acid sequencing. Following confirmation, the construct was transformed into *E. coli*, strain BL21 (DE3).

3.3. Expression

One colony of transformed BL21 (DE3) was cultured in LB medium and incubated at 37 °C overnight. The next day it was sub-cultured (0.5mL of culture in 5 mL fresh LB medium) and held at 37 °C with 200 x g agitation until OD. = 0.6. The plasmid promoter was induced with a 1 mM final concentration of IPTG (Isopropyl-Beta-D-thiogalacto-pyranoside) (Merck,) as the inducer, and samples were collected at 0, 2, and 5 hrs.

The cells were harvested by centrifugation (8.000 x g, 4 °C, 10 min), treated with lysis buffer (50 mM Tris base and 2mM EDTA), and subjected to sonication (Hielscher UP200H, 10 × 10 s, amplitude 50 %, 10 min cooling period between each burst). The samples were centrifuged at 12000 x g for 20 min, 4 °C to separate the soluble protein from the inclusion bodies. The pellet was suspended in 7 M urea onice for an hour and fractioned proteins were analyzed using SDS–PAGE and Western blotting.

Laemmli sample buffer was added to 30 µg of protein, heated to 90 °C for 5 min and loaded on to a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS -PAGE), which was run at 100 V, until the dye front migrated just off the gel. The gel was stained with Coomassie Brilliant Blue R250.

3.4. Western Blotting

According to Heidari *et al*. (14), western blotting was performed to confirm the expression of the recombinant PEP. PEP (20 µg) and untransformed cell lysates were separated on a 10 % SDS-PAGE under reducing conditions. Employing the semidry system (Bio-Rad,), protein bands were transferred to a nitrocellulose membrane (Wathman) to perform the western blotting. The membrane was blocked in 4 % skimmed milk in TBS-T (Tris-Buffered Saline, 0.1% Tween 20) overnight at 4 °C. After washing in TBS-T three times, the membrane was incubated for 2 h at room temperature with 1:10000 dilutions of HRP conjugated anti-His-tag monoclonal antibody (Abcam,). After washing in TBS-T, DAB (3, 3'-Diaminobenzidine) substrate (Sigma- Aldrich,) was used for detection.

3.5. Protein Purification

To purify the recombinant protein, *E. coli* harboring spPEP, it was first cultured in 5 mL LB-broth containing Kanamycin at 37 °C overnight. The next day was it was sub-cultured (5mL of culture in 100 mL fresh LB medium) and maintained at 37 °C with shaking (200 rpm) to reach an OD600 of 0.6. Then, the plasmid promoter was induced with a 1 mM final concentration of IPTG, and a sample was collected at 5 hr. The Cell lysates were prepared as mentioned above. The resultant solution was refolded by sequential dialysis (dialysis bag with cutoff of 12 kDa) carried out against PBS) Phosphate-buffered saline (with pH 7. 5 containing 6, 4, 2, and 0 M urea, respectively, and was then applied to a glutathione Sepharose 4B column equilibrated with PBS buffer, pH 7.5. Finally, the column was washed with PBS. GST-fusion protein eluted with 10 mM reduced glutathione in 50 mM Tris pH 8.0, according to Harper *et al*. (15).

3.6. Enzyme Activity Assay

Enzyme activity was measured using suc-Ala-Ala-Pro-Phe-pNA as a substrate, according to Cavaletti *et al*. (3). 50 µL of the enzyme with a concentration of 0.2 $mg.mL^{-1}$ were added to 180 µL of 220 µM suc-Ala-Ala-Pro-Phe-pNA in a 0.1 M ethanolamine (MEA (, 0.1M NaCl, and 1 mM DTT buffer at pH 7. Samples were incubated for 120 min at 37 °C. The pNA was detected at 410 nm, reading at intervals of 20 min. One unit was defined as the amount of enzyme that released 1 µmoL of free p-nitroaniline in the reaction mixture per min.

3.7. The SpPEP Stability to Digestive Trypsin and Pepsin Proteases

The enzymatic activity of spPEP was evaluated in the presence of Pepsin and Trypsin, according to Cavaletti *et al*. (3). An aqueous solution of enzymes, alone or in combination, i.e., 0.2 $mg.mL^{-1}$ PEP, 0.1 $mg.mL^{-1}$ Trypsin, 0.1 $mg.mL^{-1}$ Pepsin, was diluted in a buffer of 0.1M MEA, 0.1M NaCl, and 1mM DTT containing suc-Ala-Ala-Pro-Phe-pNA at pH 7 (Trypsin) and pH 4 (Pepsin). Reactions in 200 µLvolume in 96-wells flat bottom micro titer plates were extended for 120 min at 37 °C. Absorbance was monitored at 410 nm, every 20 min. Trypsin and Pepsin without spPEP were processed in the same way. Untransformed BL21 was considered as a control. Each analysis was carried out in duplicate.

3.8. Hydrolysis of Gliadins by SpPEP.

Gliadins were extracted from gluten, according to Gianfrani *et al*. (16). Gliadin (1 mg) were dissolved in 1 mL of buffer (0.1 M MEA, 0.1M NaCl, 1mM DTT) at pH 7.0 and incubated with spPEP (enzyme: substrate, 1:20), at 37 °C for 3 h. Undigested gliadin was considered as the control. The enzymatic hydrolysis was stopped by boiling for 5 minutes, according to Cavaletti *et al*. (3).

The digest gliadin reaction with spPEP was monitored by SDS- PAGE, UV–vis absorption spectroscopy, and RP-HPLC. Digested gliadin samples were dissolved in a Laemli buffer and loaded onto 10% acrylamide gel (Bio-Rad). Electrophoresis was carried out under non-reducing conditions.

RP-HPLC was carried out using a Knauer HPLC system (Berlin, Germany). First, digested and undigested gliadin samples were separated by a C18 column (Perfectsil 100 ODS-3, 5 µm, 250 × 4.6 mm, Germany). Eluent A was 0.1% TFA (v/v) in Milli-Q water and eluent B was 0.1% TFA (v/v) in acetonitrile. Then, the column was equilibrated at 5% B (0.1% TFA (v/v) in acetonitrile). Next, peptides were separated by applying a linear 5–70 % gradient of B over 60 minutes at a 0.5 $mL.min^{-1}$ flow rate. Chromatographic separation was performed at ambient

temperature, and the column effluent was monitored at 280 nm with a UV-Vis detector. The absorption spectrum (UV/visible spectra) of gliadin was determined in the range of 200 to 800 nm with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

3.9. Glutenase Activity

In this assay, developed by Every and Darkoh (11), 200 µL of freshly prepared 5% w/v wheat gluten were dissolved in 0.5 mL of buffer (0.1 M MEA, 0.1M NaCl, 1mM DTT) at pH 7.0. Mixtures were incubated with 50 µL of spPEP at 37 °C for 2 h with vigorous manual shaking every 30 min. Gluten in buffer without enzyme was used as a control. Then, freshly prepared SDS was added to a final concentration of 2%, and incubation continued for 30 min more with a vortex every 5 min. The height of the gluten in the micro tube was measured after centrifugation at 3,000 x g for 10 min.

3.10. Gluten Digestion

In vitro gluten digestion assay, according to Yandamuri *et al*. was used to evaluate the gluten digesting abilities of spPEP. In this assay, 2 $mg.mL^{-1}$ of wheat gluten were dissolved in 0.5 mL of buffer PBS plus 1 mM DTT at pH 7.0. Then, mixtures were incubated with 50 µL of spPEP at 37 °C for 90 min with vigorous manual shaking every 30 min. Gluten in buffer without enzyme was used as a control. Next, samples were periodically mixed by vortexing followed by removal of 30 µL at 30, 60, and 90 min for SDS- PAGE analysis of the digestion. The reaction was stopped by putting each sample into heating to 90 °C for 5 min. Finally, the samples were applied to a 10% acrylamide SDS- PAGE mini-gel (Bio-Rad,) under non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R250.

4. Results

4.1. Cloning SpPEP

The spPEP coding gene was successfully cloned in BL21 using the pET-41a expression vector. The recombinant plasmid was confirmed by restriction enzymes (EcoRI and KpnI) and PCR using a plasmid universal primer and a PEP-specific primer. Fragment encoding desired spPEP had 2200 bp appropriate size and digestion pattern (**supplementary data**).

4.2. Expression, Purification, and Western Blot

For protein expression, the bacterial culture media at the logarithmic phase was induced by 1mM IPTG, and recombinant protein was purified using a glutathione Sepharose 4B affinity column as described in the Materials and Methods section. SDS-PAGE and western blotting confirmed spPEP expression and purification. After purification, a single band of 100 kDa parallel with the protein size marker was obtained. Transfer of samples from gels to nitrocellulose membranes was detected with HRP-coupled anti-polyhistidine antibody and DAB. The 100 kDa band was observed in western blotting (**Fig. 1**).

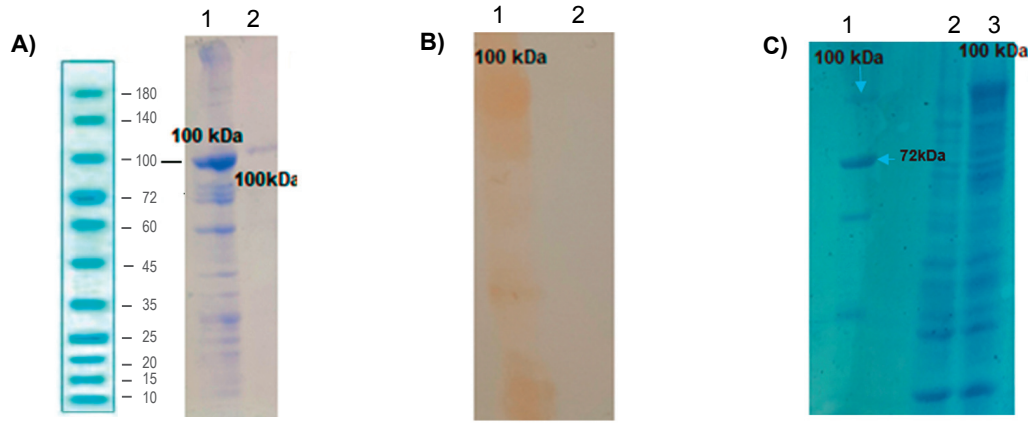

Figure 1. The SDS-PAGE and Western blot analysis of the spPEP. **A**) Lane 1, transformed bacterial lysate; Lane 2, single band of 100 KDa purified recombinant spPEP. **B**) Lane 1, a 100 kDa band observed in western blotting as a result of fusion of the HRP-coupled anti-polyhistidine antibody to the His-tag of spPEP; Lane 2, untransformed BL 21. **C**) Lane 1, molecular weight marker; Lane 2, untransformed BL 21, Lane 3, transformed bacterial lysate after induction with 1 mM IPTG.

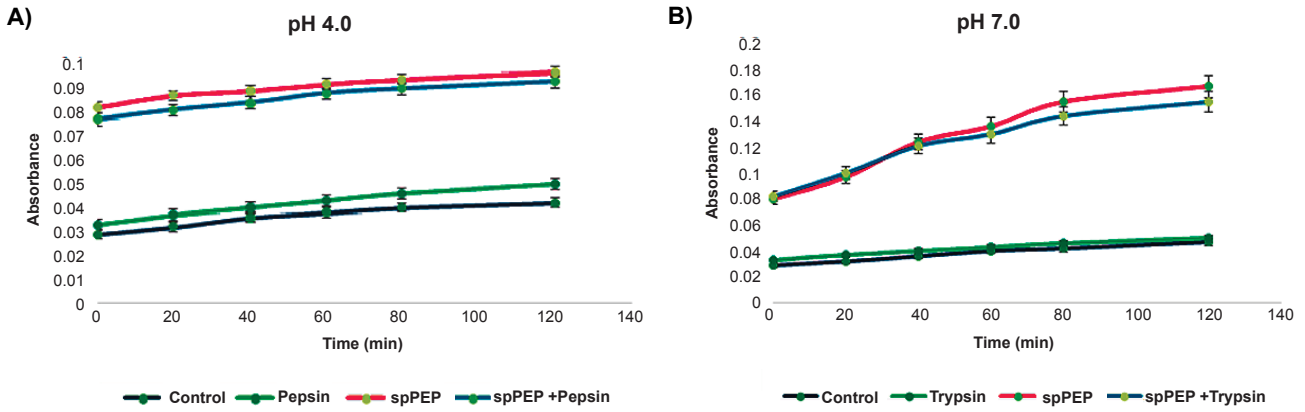

Figure 2. The activity of spPEP was evaluated in the absence and presence of Pepsin (pH 4) and Trypsin (pH 7). A) and **B)**: The reactions were started by the addition of substrate to enzyme samples of spPEP alone (red line), PEP with Trypsin or Pepsin (blue line), Trypsin or Pepsin alone (Green line), and untransformed BL21 lysate (dark blue line). Each reaction condition was tested in duplicate. The final concentrations in the reaction mixture were 0.3 mM substrate and 10 µg spPEP. X-axis: incubation time (minutes), Y-axis: absorbance units read at 410 nm. spPEP= sunn pest prolyl endoprotease

4.3. Enzyme Activity Assay and Stability of SpPEP to Pepsin and Trypsin Proteases

The enzyme activity of spPEP was evaluated with the suc-Ala-Ala-Pro-Phe-pNA substrate. Additionally, the resistance of spPEP to Pepsin and Trypsin digestive proteases was also evaluated. The spPEP activity was assessed with and without Pepsin (pH 4) and Trypsin (pH 7). It should be noted that Pepsin and Trypsin did not hydrolyze the suc-Ala-Ala-Pro-Phe-pNA substrate. Free pNA was released during hydrolysis of the suc-Ala-Ala-Pro-Phe-pNA in 120 min at 37 °C. Notably, spPEP decomposition activity was unaffected by Pepsin and Trypsin. These findings confirm spPEP could be resistant to gastrointestinal tract physiological conditions (**Fig. 2**).

4.4. Hydrolysis of Gliadins by SpPEP

Protein samples were effectively hydrolyzed under conditions mentioned in the Materials and Methods section. The ability of spPEP to hydrolyze whole gliadin proteins was assessed by SDS-PAGE, UV spectra, and HPLC analysis. The main component of gliadins is α-gliadin, with a molecular weight of approximately 35 kDa and consists of several immune stimulant epitopes (17, 18). Because of the complex mixture of whole gliadins, digestion with spPEP (1:50, molar ratio) was extended up to 240 min of incubation. Undigested gliadin was run as a reference. Since peaks assigned to gliadin are reduced after 240 min of digestion, the chromatographic profile was changed. Accordingly, the UV spectra in the range of 200 to 800 nm showed that spPEP could digest gliadin and gel electrophoresis (SDS–PAGE) confirmed this finding (**Fig. 3**).

4.5. Glutenase Activity

The spPEP exhibited glutenase activity. One unit of enzyme activity is defined as a 1-mm/hr change in gel height (19). The difference in gel height between the control and the sample was 3 mm, indicating 3 units of the enzyme. A BL21 sample was used as a control. The decrease in SDS-gluten gel height with spPEP proteinase is shown in **Figure 4**. SDS PAGE was performed after increasing the incubation time for gluten with the spPEP. Results showed both the glutenins and gliadins had been partially digested, illustrating that both are substrates for the spPEP.

5. Discussion

Celiac disease is a systemic autoimmune disorder induced by gluten in wheat, rye, and barley. Since gluten is resistant to digest to proteolytic enzymes of the gastrointestinal tract, it can result in intestinal and extra intestinal manifestations in people with Celiac disease (20, 21). Incomplete hydrolysis of gluten is not due to

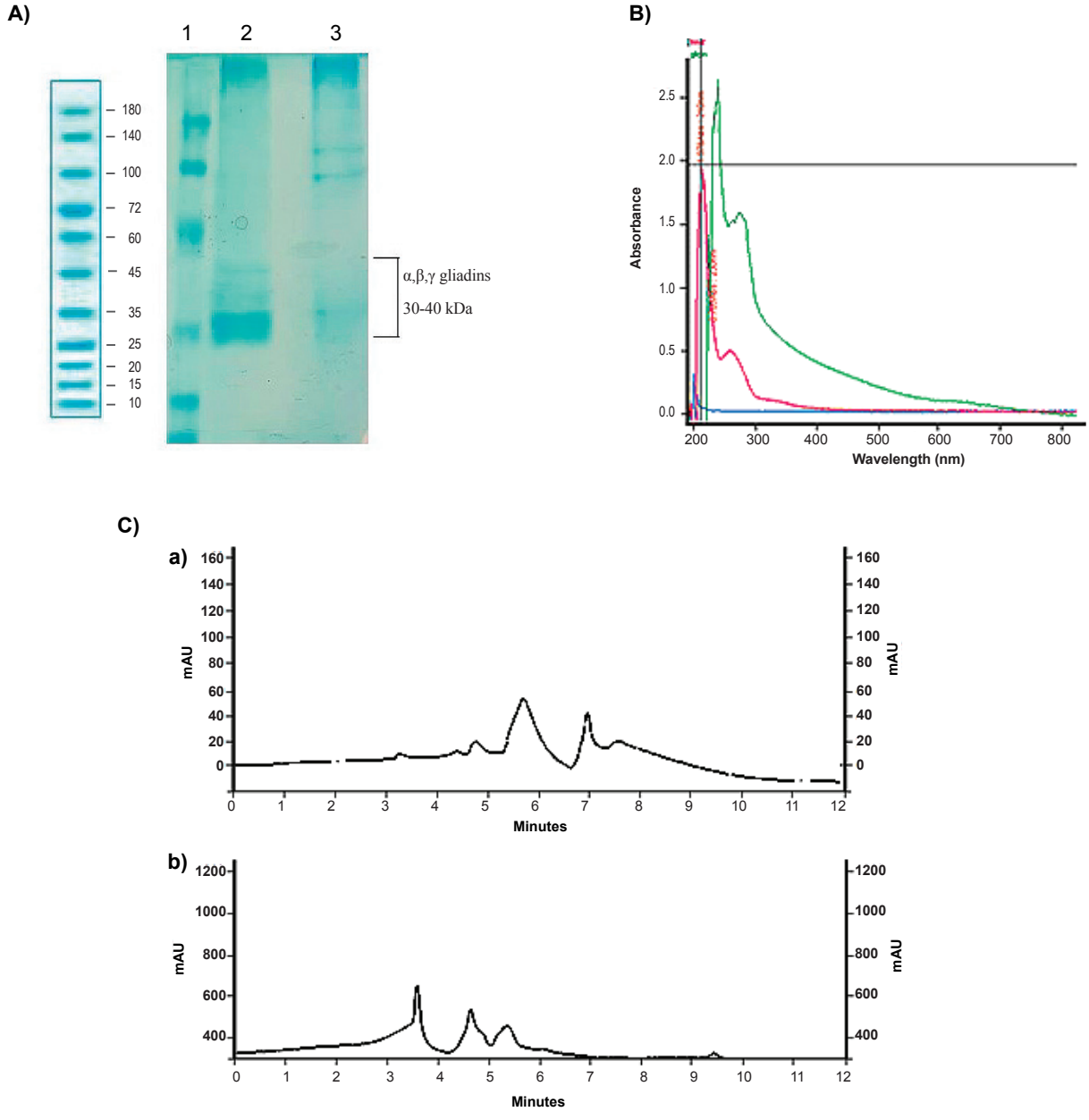

Figure 3. **Analysis of the digestion of gliadin by spPEP. A**) Gliadin digestion by the PEP after 240 min of incubation at 37 °C, pH 7. Gel stained with Coomassie Brilliant Blue R250. Lane 1: Protein Marker, Lane 2: gliadin alone, and Lane 3: gliadin + spPEP. **B**) Enzymatic digestion of gliadin by spPEP. Spectrum generated after incubating gliadin for 240 min at 37 °C with the spPEP. Gliadin alone (green line), gliadin plus spPEP (red line). **C**) RP-HPLC profile of gliadin digestion by spPEP at an incubation time up to 240 min. **a**) Trace of full length Gliadin alone, **b**) gliadin cleavage by spPEP activity.

enzymatic deficiencies in susceptible individuals but rather to gluten's unusual primary sequence (3). Due to its low cost and unique physicochemical characteristics, gluten is found in most processed foods. As a result, it is difficult for celiac patients to follow a lifelong GFD (22, 23).

Numerous alternative strategies, including blocking HLA-DQ2, vaccines, inhibition of transglutaminase 2, and exploration of ancient wheat and gluten degrading enzymes, have been the subject of extensive investigation (17). Among these, Oral Enzymatic Therapy (OET) has been suggested as one of the most effective strategies

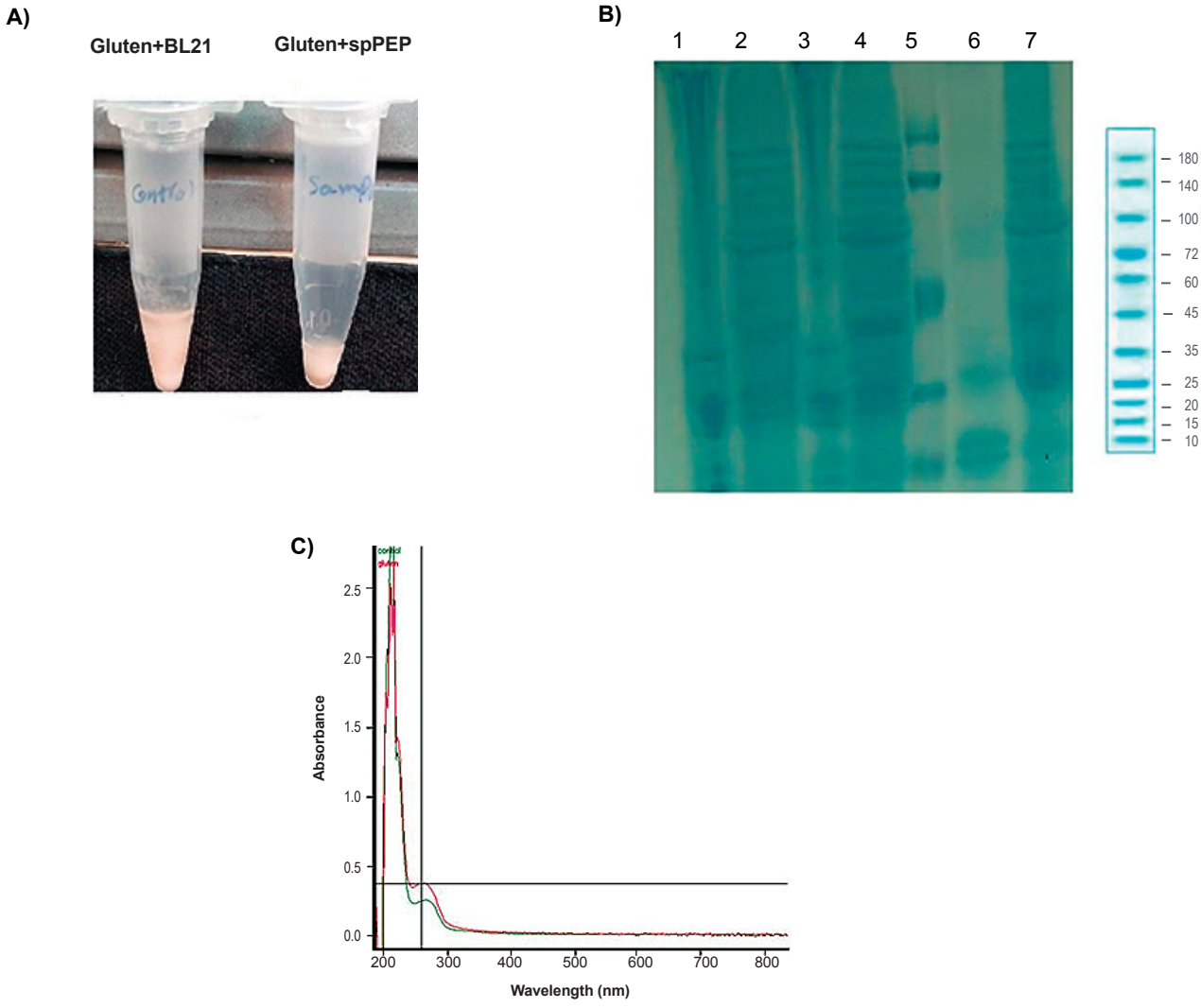

Figure 4. Analysis of the digestion of gluten by spPEP. **A**) Glutenase assay of spPEP in inclusion body lysates, **B**) SDS PAGE analysis of the digestion of gluten by spPEP at 30, 60, and 90 min, lane1: treat, lane2: control at 30 min; lane 3: treat, lane4: control at 60 min; lane5: Marker, lane 6: treat, lane 7: control at 90 min and **C**) Spectrum generated after incubating gluten with the spPEP. Gluten alone (red line), gluten plus spPEP (green line).

to support the gluten-free diet (GFD) (20, 24-26). The use of proteases to digest gluten was proposed in the late 1950s, and a clinical trial conducted in 1976 did not provide a plausible or clear conclusion (2).

Several prolyl endopeptidases (PEP) in concert with the brush border peptidases are currently under investigation for their ability to degrade gluten under physiological conditions (4, 23). For example, endopeptidase AN-PEP derived from the fungus Aspergillus Niger and Latiglutenase (ALV 003) have been extensively evaluated for their efficacy in reducing the immunestimulatory epitopes, but both have failed as a possible treatment for CD in clinical trials (27). Also, properties of Prolyl oligopeptides from Sphingomonas capsulate and Myxococcus Xanthus have been evaluated.

However, these enzymes preferentially digest short peptides, making them unsuitable for treating CD (2).

The use of insect-derived enzymes, including peptidases, lipases, and amylases, have received more attention from various fields in recent years. One of these enzymes is prolyl endoprotease (PEP), derived from Eurygaster integriceps Puton (sunn pest) (28).

Every and Darkoh showed that the spPEP is unique because its natural substrate is whole gluten (11). Wheat gluten, as elastomeric plant proteins, subdivides into two parts, gliadin and glutenin (29). Gliadin, especially α-gliadin monomer, consists of several celiac-active epitopes and is toxic to predisposed people and therefore correlates with the onset and development of CD (17, 30).

Because there is currently no cure for CD, it is important to examine the parts of the gluten protein that contain many immunogenic epitopes (31). In our work, we report the cleavage efficacy of an endoprotease of sunn pest to digest gliadin as a potential alternative treatment option for CD. The spPEP was expressed in a BL21 host. Biological properties of spPEP were maintained, e.g., stability and activity in the presence of Trypsin and Pepsin, efficiency in degradation of the most immunogenic peptide of gluten, as well as of whole gliadin.

Due to the fact that the digestion of proteins is highly active in the duodenum (8, 31, 32), it is practical to evaluate the activity of gliadin degrading enzymes that could be active in this part of the GI tract. The spPEP, which is more effective at the pH of 6 to 7 of the duodenum, must be sufficiently stable to enzymes in this part of the GI tract. Our results showed spPEP gluten-degrading activity is independent of Trypsin and Pepsin proteolysis. Scrutiny of the protein hydrolysis and molecular weights of these peptides was done using SDS-PAGE. Also, RP-HPLC and UV visible analysis demonstrated that spPEP was able to break down whole gliadin and gluten within 2 hours of the spPEP treatment. Analyses performed in our study showed that the candidate spPEP is an efficient glutenase.

Due to the limitations of a GFD for many with CD, the availability of spPEP as an adjuvant approach would be a great benefit to people exposed to inadvertent gluten. Future in vivo studies should provide a supplementary review for the approval of spPEP for CD; however, further analysis is desirable.

6. Conclusion

We have provided proof that spPEP can hydrolyze gliadin into smaller peptides. The present study reveals that spPEP might be suitable for degrading gluten proteins in the GI tract of CD patients or before administration to patients. Therefore, spPEP might be a novel candidate for Oral Enzymatic Therapy (OET) in CD and other gluten-related disorders. However, because different enzymes from various sources do not have the same function and specificity, it is recommended that these enzymes be designed and used as an "enzymatic cocktail".

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Conflicts of interest

The authors declare that there is no conflict of interest.

supplementary data

Confirmation of gene cloning: lane 1 *spPEP* gene PCR product by plasmid universal primers, (3300 bp); Lane 2, specific PCR product of spPEP gene (2200 bp); Lane 3, 1kb DNA ladder marker; and Lane 4, double digestion of recombinant plasmid by *Eco*RI and *KpnI.*

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