

Recombinant production and characterization of *Aspergillus niger* prolyl endopeptidase enzyme for gluten-free food production

Belma Şenol¹ 

Özlem Kaplan² 

Rizvan İmamoğlu³ 

İsa Gökçe^{4,*} 

¹Cumhuriyet University, Zara Ahmet Çuhadaroğlu Vocational School, Department of Food Technology, Turkey,

²Istanbul University, Faculty of Science, Department of Molecular Biology and Genetics, Turkey,

³Bartın University, Faculty of Science, Department of Biotechnology, Turkey,

⁴Tokat Gaziosmanpaşa University, Faculty of Engineering and Natural Sciences, Department of Bioengineering, Turkey

*Corresponding Author: isa.gokce@gop.edu.tr

Abstract

Gluten is a protein group found in wheat, barley, rye, and oats, known as cereals. When this vegetable protein is introduced into the body, celiac disease can occur. The use of bacterial and fungal oligopeptidase to ensure the cleavage of gluten into non-toxic fragments are considered a promising alternative for celiac disease. In this study, the *Aspergillus niger* Prolyl EndoPeptidase (AN-PEP) enzyme was cloned into pET22b vector and recombinantly produced in *BL21 (DE3) pLysE* cells. PEP enzyme expressed as inclusion body and was recovered by refolding. And N-terminal His-tagged recombinant protein was purified by nickel affinity chromatography. 280 mg AN-PEP enzyme from 1L bacterial culture was purified at very high yield, and this protein was 90% purity. As a result; It has been determined that the recombinantly produced PEP enzyme can digest gluten. This study shows that recombinantly produced AN-PEP (rAN-PEP) has great potential to use in the production processes of gluten-free foods.

Keywords: Gluten, Celiac disease, *Aspergillus niger*

Introduction

Celiac disease, (celiac sprue, gluten-sensitive enteropathy), is a chronic inflammatory disease that causes destruction of the villi in the small intestinal mucosa and is stimulated by the intake of gliadins in wheat, rye sequins and hordeins in barley (Di Sabatino & Corazza, 2009; Edens et al., 2005). Celiac disease has a very high prevalence in children and adults (seen in rates approaching 5-10% of the population) (Elli et al., 2015; Ortiz et al., 2017). The only current treatment of the disease is a “gluten-free diet” (Comino et al., 2016; Moreno et al., 2017; Rodrigues et al., 2018). The celiac patients need to eliminate gluten from their diet completely. Mammalian digestive enzymes can not easily digest gluten due to protease-resistant domains in gluten (Helmerhorst et al., 2010). Therefore, gluten-digesting enzymes are needed

in food processing to produce gluten-free foods in the food industry. Another approach is the enzymatic hydrolysis of gliadins with oral enzyme supplements in celiac patients (Ciacci et al., 2015; Wei et al., 2020). Prolyl endopeptidases obtained from *Myxococcus xanthus*, *Sphingomonas capsulata* and *Flavobacterium meningosepticum* microorganisms have shown significant potential in pharmacological use (Shan et al., 2004). Aspergillopepsin obtained from the combination of *Aspergillus niger* and *Aspergillus oryzae* is seen to digest gluten in vitro environmental conditions (Ehren et al., 2009). The combination of prolyl endopeptidase from *Sphingomonas capsulata* and barley cysteine endoprotease EP-B2 can also successfully digest gluten fragments in the stomach in clinical trials (Gass et al., 2007; Tye-Din et al., 2010). KumaMax, a synthetic enzyme, has similar results in clinical trials

Cite this article as:

Senol, B., Kapan, O., Imamoglu, R., Gokce, I. (2021). Recombinant production and characterization of *Aspergillus niger* prolyl endopeptidase enzyme for gluten-free food production. Int. J. Agric. Environ. Food Sci., 5(3), 287-293

Doi: <https://doi.org/10.31015/jaefs.2021.3.5>

Orcid: Belma Şenol: 0000-0003-3021-0087, Özlem Kaplan: 0000-0002-3052-4556, Rizvan İmamoğlu: 0000-0002-6306-4760, İsa Gökçe: 0000-0002-5023-9947

Received: 10 February 2021 Accepted: 04 June 2021 Published Online: 09 August 2021

Year: 2021 Volume: 5 Issue: 3 (September) Pages: 287-293

Available online at : <http://www.jaefs.com> - <http://dergipark.gov.tr/jaefs>

Copyright © 2021 International Journal of Agriculture, Environment and Food Sciences (Int. J. Agric. Environ. Food Sci.)

This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC-by 4.0) License



(Krishnareddy et al., 2017). Prolyl endopeptidase, also known as AN-PEP, obtained from *Aspergillus niger*, is an enzyme that digests gluten (Edens et al., 2005; Lopez & Edens, 2005; Stepniak et al., 2006).

E. coli, which is frequently used in recombinant protein production, has advantages such as low cost and rapid production of recombinant proteins (Vallejo & Rinas, 2004). In this study, the AN-PEP enzyme was produced by using recombinant DNA technology, which is one of the modern biotechnological methods. The PEP enzyme gene of *Aspergillus niger* organism was determined using bioinformatics tools, and codon optimization was made. The expression of the PEP gene was performed in *E. coli* BL21 (DE3) pLysE strain. Since the produced PEP protein was found in the form of inclusion bodies in the cell, it was provided to be folded again with the refolding protocol. N-terminally His tagged recombinant protein AN-PEP was purified by nickel affinity chromatography. Then, the activity of the purified AN-PEP enzyme was determined.

Materials and Methods

Construction of AN-PEP expression plasmid

The DNA sequence of the PEP enzyme was taken from NCBI (National Center for Biotechnology Information) (Accession number: AX458699.1). The codon optimization was performed for the *E. coli* K12 strain in the gene sequence from NCBI using bioinformatics tools via using jCat codon optimization program (<http://www.jcat.de/>). The codons found in the nucleotide sequence of the PEP gene were replaced by codons expressed in *E. coli* K12 strain with higher frequency. Additionally, N terminal 6 His tag nucleotide sequence has been added to allow easy purification of the produced protein. The pET22b vector was chosen as the cloning vector. The gene sequence regulated by bioinformatics tools was synthesized by Biomatik company.

Expression of AN-PEP enzyme in *E. coli* BL21(DE3) pLysE cells

The expression of AN-PEP protein was carried out similar to the protocol detailed in our previous study (Kuduğ et al. 2019; Kaplan et al. 2021). *E. coli* BL21 (DE3) pLysE strain was used in protein expression studies. *E. coli* BL21 (DE3) pLysE cells containing the pET22b-ANPEP plasmid were transferred to 50 ml LB media and incubated in a shaker incubator at 37°C at 240 rpm. The cells were induced with IPTG when OD₆₀₀: 0.7. Then, samples were taken from the cells incubated for 4 hours to be analyzed in SDS PAGE.

The recovering from inclusion body of rAN-PEP Enzyme

Two protocols were used to recover the rAN-PEP enzyme from the inclusion body.

Protocol 1

The cells suspended with Tris-HCl buffer (50 mM Tris, 0.1 mM DTT, pH: 8.0) were lysed by sonicator. The lysed cells were centrifuged at 16,000xg for 15 minutes, and the pellet was washed twice with resuspension buffer (50 mM Tris-HCl (pH: 8.0), 2.5% triton X-100, 20% sucrose) (5ml/g). The pellet was incubated for 5 hours with 50 mM Tris-HCl buffer (pH: 8.0) containing 8 M urea after centrifugation at 16,000xg for 15 min. A cold refolding buffer (100 mM Tris-HCl, 10 mM DTT, 20% glycerol (pH: 8.0)) was added dropwise to the

supernatant. This supernatant was stirred at 4°C for 16 hours. After the completion of the mixing process, centrifugation was carried out for 15 minutes at 16,000xg. The supernatant was taken and dialyzed in 20 mM Tris HCl (pH: 8.0) containing 100 mM NaCl. The purification of the PEP enzyme from the supernatant obtained was done with a column containing Ni-NTA agarose (Melissis et al., 2010).

Protocol 2

The cells suspended with 20 mM Tris-HCl, 0.5 M NaCl (pH: 8) were lysed by sonication and centrifuged at 16,000xg for 20 minutes. The pellet was dissolved by adding 0.5 M NaCl, 2% Triton-X, 2 M urea 20 mM Tris-HCl pH: 8 solution on the pellet and this process was repeated twice. The solution was centrifuged at 16,000xg for 20 minutes. The pellet was mixed with 6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol pH: 8 buffer at room temperature for 30 and centrifuged at 16,000xg for 20 minutes. Refolding and purification processes of the obtained supernatant in the column containing Ni-NTA resin. Refolding was achieved by generating urea gradient (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, and 6M-0M Urea Buffer) from the column. Urea gradient was provided by passing through the column at a rate of 0.1-1 ml/min, with 5 ml of buffer containing 6M, 5M, 4M, 3M, 2M, 1M, and 0M urea, respectively. Protein was finally eluted from the column with 20 ml of 20 mM Tris-HCl, 0.5 M NaCl, 300 mM imidazole, 1 mM 2-mercaptoethanol pH: 8.0 buffer (Palmer & Wingfield, 2012).

AN-PEP Enzyme Activity Assays

It was determined using the methodology reported by Edens et al. (Edens et al., 2005). The substrate (Z-Gly-Pro-pNA, benzyloxycarbonyl-glycine-proline-p-nitroanilide) was first dissolved in 40% 1,4 dioxane at 60°C to prepare 250 µM solution. PEP activity was determined by spectrophotometric monitoring of the release of pNa from the Z-Gly-Pro-pNA substrate at a wavelength of 410 nm (Edens et al., 2005). To determine the pH value which the enzyme showed optimum activity, McIlvaine buffer, between pH 2.2-8.0, and Tris-HCl buffer at pH 9-10 were used. To determine the effect of temperature on enzyme activity, activity measurements were made at temperatures between 25°C-70°C. Based on the highest absorbance value, % relative enzyme activity was determined. To determine the thermal stability of the enzyme, the enzyme was incubated for 30, 60, and 90 minutes at 30°C, 40°C, 50°C, 60°C temperatures. Then, the stability of the enzyme was determined by determining the activity at optimum pH and temperature conditions. To determine the effect of substrate concentration on enzyme activity, enzyme activity at optimum conditions was defined against the amount of substrate that varies. Measurement results were interpreted using the Michaelis-Menten kinetic model and Lineweaver-Burk models. For the enzyme-substrate specificity, the kinetic models used were evaluated by calculating K_m and V_{max} values.

The experimental steps of this study are illustrated as a summary in Figure 1.

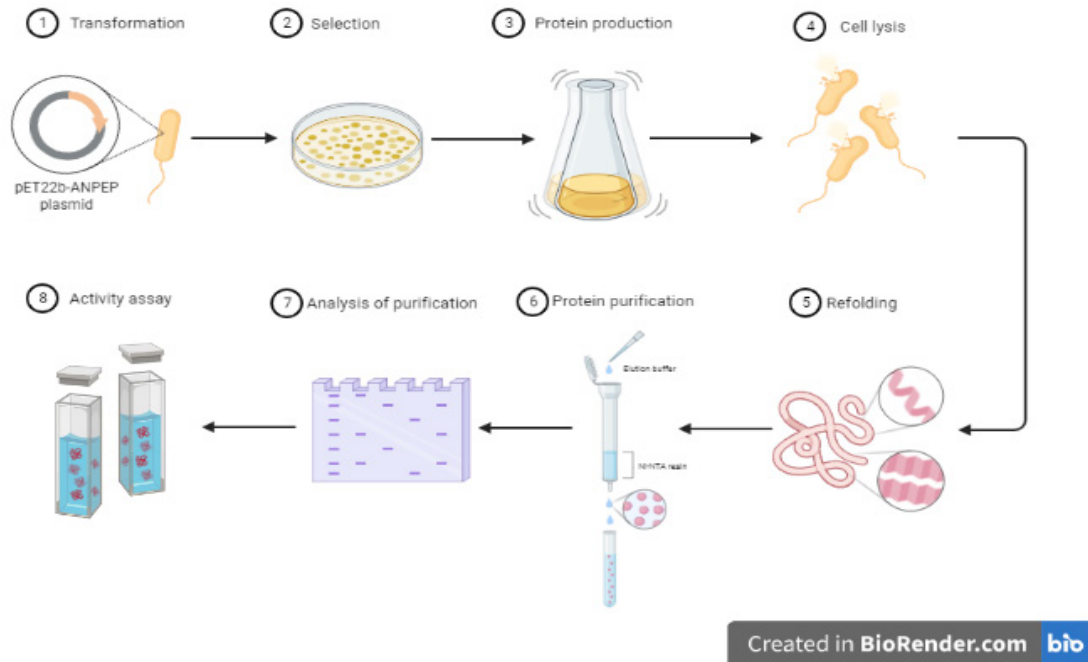


Figure 1. Experimental steps for recombinant production of AN-PEP protein (Created in BioRender.com)

Results and Discussion

Expression of AN-PEP enzyme in *E. coli* BL21(DE3) *pLysE*

E. coli BL21 (DE3) *pLysE* cells were transformed with the pET22b-ANPEP, plasmid mapped in Figure 2. *E. coli* cells incubated in a shaker incubator at 37°C at 240

rpm were induced with IPTG when OD₆₀₀: 0.7. It was analyzed whether AN-PEP protein was expressed by taking samples from the culture before and after induction with IPTG. As expected, a protein band of around 58 kDa was observed after IPTG induction (Figure 3).

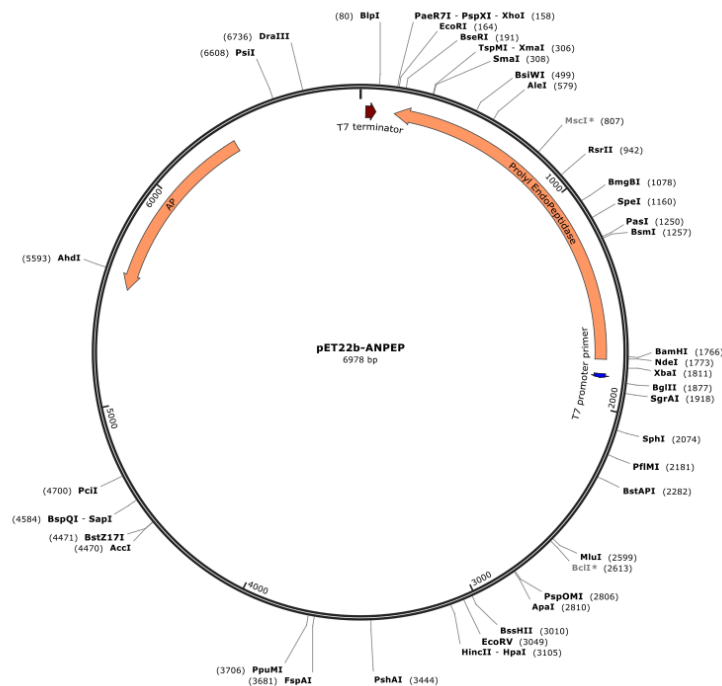


Figure 2. Plasmid map of the pET22b-ANPEP construct

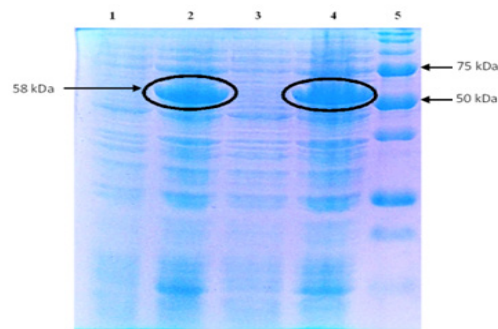


Figure 3. Analysis of recombinant expression of AN-PEP protein in 12% SDS-PAGE 1, 3. *E. coli BL21 (DE3) pLysE* cells containing the pET22b-ANPEP plasmid before induction with IPTG 2, 4. *E. coli BL21 (DE3) pLysE* cells containing the pET22b-ANPEP plasmid, after induction with IPTG. 5. Protein marker (SeeBlue® Plus2 Pre-stained Protein Standard)

Recovering from the inclusion body of rAN-PEP Enzyme

The rAN-PEP enzyme, which was highly expressed in *E. coli*, was in the form of the inclusion body. Two protocols was applied for the recovery of protein from the inclusion body. Although *protocol 1* took a relatively long time, the rAN-PEP enzyme could be obtained quite purely and

efficiently (Figure 4).

A relatively shorter protocol was used to recover the rAN-PEP enzyme from the inclusion body, in which the refolding procedure was performed on the column. Compared with *protocol 1*, relatively less protein was obtained in *protocol 2*. However, the rAN-PEP enzyme was very pure (Figure 5)

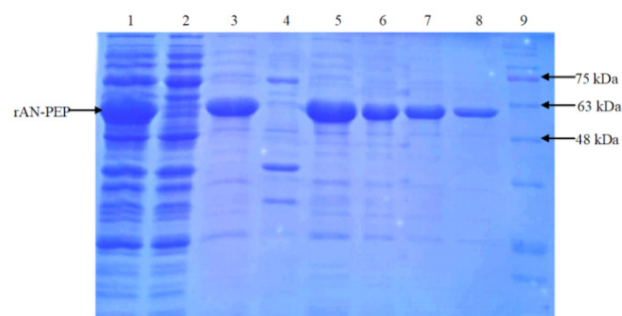


Figure 4. Analysis of rAN-PEP enzyme the recovery from inclusion body in 12% SDS-PAGE (*Protocol 1*) 1. *E. coli* cells lysed with a sonicator. 2, 3. Supernatant and pellet obtained after centrifugation at 16.000xg for 15 minutes, respectively. 4, 5. Supernatant and pellet obtained after two washes with resuspension buffer, respectively. 6. Supernatant after incubation with 8 M urea for 5 hours 7. The supernatant obtained after the dialysis procedure. 8. The obtained filtrate by passing the elution buffer containing 300 mM imidazole from the column. 9. Protein marker (NZYColour Protein Marker II)

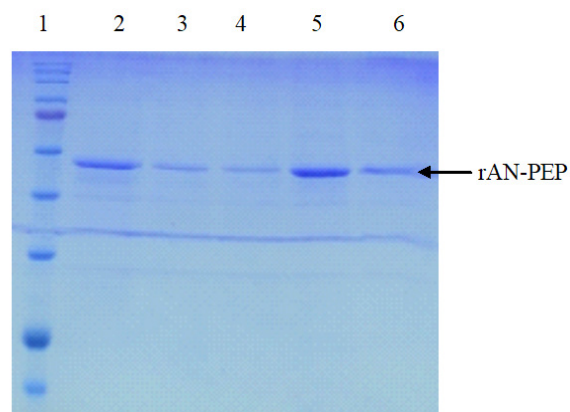


Figure 5. Analysis of rAN-PEP enzyme recovery from the inclusion body in 12% SDS-PAGE (*Protocol 2*) 1. Protein marker (Page Ruler Plus Prestained Protein Ladder) 2-6, the obtained filtrate by passing the elution buffer containing 300 mM imidazole from the column.

AN-PEP activity assays

Activity tests of the protein recovered from the inclusion body were carried out using the Z-Gly-Pro-pNA substrate following the protocol of Edens et al. (Edens et al., 2005). The optimum pH of the enzyme was determined as 6. The enzyme was found to have a wide range of activities, such as pH: 2.2-8.

The optimum working temperature of the enzyme was found to be 30°C, but it was also observed to show activity in the range of 25-40°C. In thermal stability experiments performed on rAN-PEP enzyme, it was observed that it preserves its activity by 30% after 30 minutes incubation at 60°C (Figure 6).

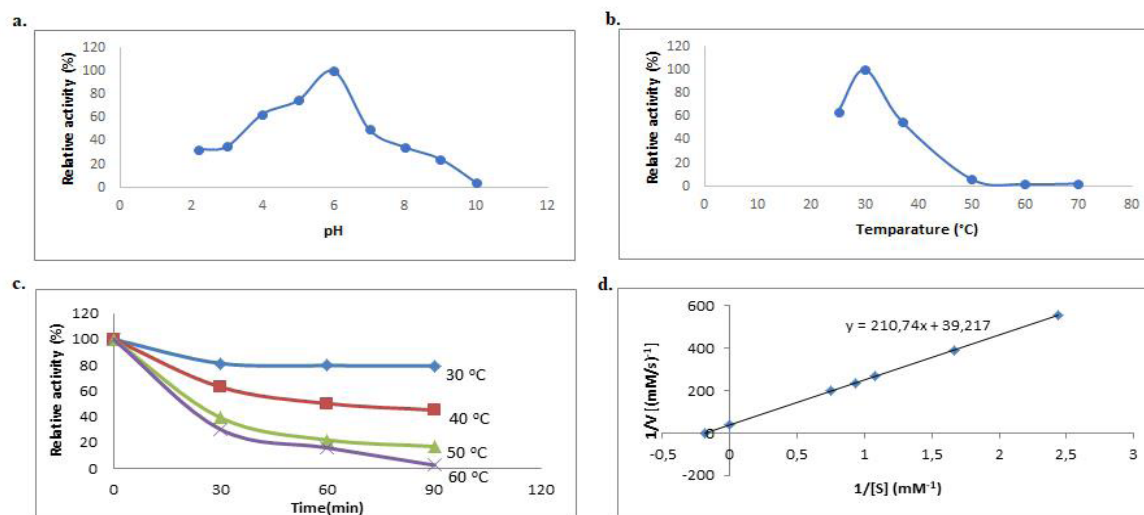


Figure 6. Characterization of the rAN-PEP (a) Relative activity of the rAN-PEP enzyme in the pH: 2.2-10 range (b) Relative activity of the rAN-PEP enzyme in the 25°C-70°C temperature range (c) Thermal stability of the rAN-PEP enzyme (Activity was determined at optimum temperature and pH) (d) Lineweaver-Burk model of the rAN-PEP enzyme (Activity was determined at optimum temperature and pH value.)

Conclusion

Prolyl endopeptidase is proline-specific endoprotease, also known as EC 3. 4. 21.26 and belongs to the serine protease family, the peptides can break down the inner proline residues (Shan et al., 2004). Today, many studies have been conducted to reduce or modify gluten to eliminate celiac immunoreactivity. Some researchers (Gessendorfer, 2011; Lopenen et al., 2009; Schwalb, 2012) have worked with enzymes that degrade gluten in plants such as *Triticum sp.*, *Carica papaya*, *Hordeum vulgare*, and *Secale cereale*. Gluten-degrading enzymes from bacteria such as *Myxococcus Xanthus*, *Bacillus sp.*, *Sphingomonas capsulata*, *Rothia mucilaginosa*, *Bifidobacterium sp.*, *Flavobacterium meningosepticum*, *Pseudomonas aeruginosa* and *Lactobacillus sp.* has been discovered, and its effectiveness has been studied in digesting gluten (Shan et al., 2004; Siegel et al., 2006; Wei et al., 2015; Zamakhchari et al., 2011). Gluten-degrading enzymes of fungi such as *Aspergillus niger* and *Aspergillus flavus* var *oryzae* have also been frequently studied (Edens et al., 2005; Lopez & Edens, 2005; Stepniak et al., 2006). The AN-PEP enzyme does not completely digest into amino acids but instead breaks down the peptide bond in the proline amino acid on the carboxyl side of the peptide (Edens et al., 2005). The optimum pH value of this enzyme with activity between pH 2.5 and 7.5 was determined as pH:4.2. This enzyme digests gluten and gliadin with high efficiency (Lopez & Edens, 2005; Stepniak et al., 2006). Studies have shown that AN-PEP is

capable of degrading gluten in all starches with a gluten content of up to 20mg/kg. The AN-PEP is obtained by fermentation of *Aspergillus niger* (Jiang et al., 2021; Walter, 2015).

In this study, AN-PEP enzyme, which has been obtained with *Aspergillus niger* fermentation so far, was produced recombinantly in *E. coli*. The production of proteins in *E. coli* provides a low cost, fast, and highly efficient production opportunity (Vallejo & Rinas, 2004). The AN-PEP enzyme was produced in *E. coli* at a high level and in the form of an inclusion body. When the proteins are produced in inclusion body form, it allows the protein to be obtained very pure and without degradation by proteases (Babaeipour et al., 2010). The rAN-PEP enzyme was recovered from inclusion bodies in a very pure and active form. We applied two different procedures for recovery from inclusion bodies and compared their efficiency. A very high amount of protein, such as 280 mg, was obtained from a 1 L bacterial culture. The optimum pH value of the rAN-PEP enzyme was 6, and the optimum working temperature was 30°C. This enzyme shows activity in a wide pH range such as pH: 2.2-8. Besides, even after incubation of this enzyme at 60°C for 30 minutes, it shows around 30% activity at pH: 6.

There are differences between the AN-PEP enzyme obtained by *Aspergillus niger* fermentation and the rAN-PEP enzyme produced in this study. The optimum pH value of the natural AN-PEP enzyme is 4.2, and the optimum temperature is 50°C (Edens et al., 2005). This difference may be due to



the lack of glycosylation in *E. coli*. Because, various post-translational modifications such as glycosylation occur in the natural AN-PEP enzyme. Sebela et al. reported that the AN-PEP enzyme contains high-mannose type N-glycans and is partially phosphorylated. It was also stated by Sebela et al. that AN-PEP enzyme with N-glycolysis was 63kDa, and its non-glycolysis form appeared to be around 58 kDa in SDS-PAGE (Sebela et al., 2009). Indeed, our results confirm this situation. Although glycolysis did not occur on the rAN-PEP enzyme, it showed high activity (Km: 5.37 Mm, Vmax: 0.025 mM/s). However, there is a shift in optimum pH and temperature values. Glycolysis and phosphorylation appear to be highly effective in AN-PEP enzyme activity. With this study, we presented a new methodology on the recombinant production and purification of the AN-PEP enzyme in *E. coli*. As a result, the rAN-PEP enzyme we produce in this study can be used in gluten-free food production.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Not applicable.

Funding

This work was supported by the Tokat Gaziosmanpaşa University Research Fund under Grant number:2018/32.

Data availability

Not applicable.

Consent for publication

Not applicable.

References

- Babaeipour, V., Abbas, M. P., Sahebazar, Z., & Alizadeh, R. (2010). Enhancement of human granulocyte-colony stimulating factor production in recombinant *E. coli* using batch cultivation. *Bioprocess Biosyst Eng*, 33(5), 591-598. Doi: <https://doi.org/10.1007/s00449-009-0380-3>
- Ciacci, C., Ciclitira, P., Hadjivassiliou, M., Kaukinen, K., Ludvigsson, J. F., McGough, N., Sanders, D. S., Woodward, J., Leonard, J. N., & Swift, G. L. (2015). The gluten-free diet and its current application in coeliac disease and dermatitis herpetiformis. *United European Gastroenterol J*, 3(2), 121-135. Doi: <https://doi.org/10.1177/2050640614559263>
- Comino, I., Fernandez-Banares, F., Esteve, M., Ortigosa, L., Castillejo, G., Fambuena, B., Ribes-Koninckx, C., Sierra, C., Rodriguez-Herrera, A., Salazar, J. C., Caunedo, A., Marugan-Miguelsanz, J. M., Garrote, J. A., Vivas, S., Lo Iacono, O., Nunez, A., Vaquero, L., Vegas, A. M., Crespo, L., Fernandez-Salazar, L., Arranz, E., Jimenez-Garcia, V. A., Antonio Montes-Cano, M., Espin, B., Galera, A., Valverde, J., Giron, F. J., Bolonio, M., Millan, A., Cerezo, F. M. (2016). Fecal Gluten Peptides Reveal Limitations of Serological Tests and Food Questionnaires for Monitoring Gluten-Free Diet in Celiac Disease Patients. *Am J Gastroenterol*, 111(10), 1456-1465. Doi: <https://doi.org/10.1038/ajg.2016.439>
- Di Sabatino, A., & Corazza, G. R. (2009). Coeliac disease. *Lancet*, 373(9673), 1480-1493. Doi: [https://doi.org/10.1016/S0140-6736\(09\)60254-3](https://doi.org/10.1016/S0140-6736(09)60254-3)
- Edens, L., Dekker, P., van der Hoeven, R., Deen, F., de Roos, A., & Floris, R. (2005). Extracellular prolyl endoprotease from *Aspergillus niger* and its use in the debittering of protein hydrolysates. *J Agric Food Chem*, 53(20), 7950-7957. Doi: <https://doi.org/10.1021/jf050652c>
- Ehren, J., Moron, B., Martin, E., Bethune, M. T., Gray, G. M., & Khosla, C. (2009). A food-grade enzyme preparation with modest gluten detoxification properties. *PLoS One*, 4(7), e6313. Doi: <https://doi.org/10.1371/journal.pone.0006313>
- Elli, L., Branchi, F., Tomba, C., Villalta, D., Norsa, L., Ferretti, F., Roncoroni, L., & Bardella, M. T. (2015). Diagnosis of gluten related disorders: Celiac disease, wheat allergy and non-celiac gluten sensitivity. *World J Gastroenterol*, 21(23), 7110-7119. Doi: <https://doi.org/10.3748/wjg.v21.i23.7110>
- Gass, J., Bethune, M. T., Siegel, M., Spencer, A., & Khosla, C. (2007). Combination enzyme therapy for gastric digestion of dietary gluten in patients with celiac sprue. *Gastroenterology*, 133(2), 472-480. Doi: <https://doi.org/10.1053/j.gastro.2007.05.028>
- Gessendorfer, B., Hartmann, G., Wieser, H., Koehler, P. (2011). Determination of celiac disease-specific peptidase activity of germinated cereals. *European Food Research and Technology*, 232, 205-209. Doi: <https://doi.org/10.1007/s00217-010-1375-7>
- Helmerhorst, E. J., Zamakhchhari, M., Schuppan, D., & Oppenheim, F. G. (2010, Oct 11). Discovery of a novel and rich source of gluten-degrading microbial enzymes in the oral cavity. *PLoS One*, 5(10), e13264. Doi: <https://doi.org/10.1371/journal.pone.0013264>
- Jiang, B., Wang, M., Wang, X., Wu, S., Li, D., Liu, C., Feng, Z., & Li, J. (2021). Effective separation of prolyl endopeptidase from *Aspergillus Niger* by aqueous two phase system and its characterization and application. *Int J Biol Macromol*, 169, 384-395. Doi: <https://doi.org/10.1016/j.ijbiomac.2020.12.120>
- Kaplan, Ö., İmamoğlu, R., Şahingöz, İ., Gökçe, İ. (2021). Recombinant production of *Thermus aquaticus* single-strand binding protein for usage as PCR enhancer. *Int. Adv. Res. Eng. J.*, 5(1), 42-46. Doi: <https://doi.org/10.35860/iarej.766741>
- Krishnareddy, S., Stier, K., Recanati, M., Lebwohl, B., & Green, P. H. (2017). Commercially available glutenases: a potential hazard in coeliac disease. *Therap Adv Gastroenterol*, 10(6), 473-481. Doi: <https://doi.org/10.1177/1756284817708888>

- [org/10.1177/1756283X17690991](https://doi.org/10.1177/1756283X17690991)
- Kuduğ, H., Ataman, B., İmamoğlu, R., Düzgün, D., Gökçe, İ. (2019). Production of red fluorescent protein (mCherry) in an inducible *E. coli* expression system in a bioreactor, purification and characterization. *Int. Adv. Res. Eng. J.*, 3(1), 20-25. Retrieved from <https://dergipark.org.tr/en/pub/iarej/issue/44303/429547>
- Lopez, M., & Edens, L. (2005, Oct 5). Effective prevention of chill-haze in beer using an acid proline-specific endoprotease from *Aspergillus niger*. *J Agric Food Chem*, 53(20), 7944-7949. Doi: <https://doi.org/10.1021/jf0506535>
- Loponen, J., Kanerva, P., Zhang, C., Sontag-Strohm, T., Salovaara, H., & Ganzle, M. G. (2009). Prolamin hydrolysis and pentosan solubilization in germinated-rye sourdoughs determined by chromatographic and immunological methods. *J Agric Food Chem*, 57(2), 746-753. Doi: <https://doi.org/10.1021/jf803243w>
- Melissis, S. C., Papageorgiou, A. C., Labrou, N. E., & Clonis, Y. D. (2010). Purification of M-MLVH- RT on a 9-aminoethyladenine-(1,6-diamine-hexane)-triazine selected from a combinatorial library of dNTP-mimetic ligands. *J Chromatogr Sci*, 48(6), 496-502. Doi: <https://doi.org/10.1093/chromsci/48.6.496>
- Moreno, M. L., Cebolla, A., Munoz-Suano, A., Carrillo-Carrion, C., Comino, I., Pizarro, A., Leon, F., Rodriguez-Herrera, A., & Sousa, C. (2017). Detection of gluten immunogenic peptides in the urine of patients with coeliac disease reveals transgressions in the gluten-free diet and incomplete mucosal healing. *Gut*, 66(2), 250-257. Doi: <https://doi.org/10.1136/gutjnl-2015-310148>
- Ortiz, C., Valenzuela, R., & Lucero, A. Y. (2017). Celiac disease, non celiac gluten sensitivity and wheat allergy: comparison of 3 different diseases triggered by the same food. *Rev Chil Pediatr*, 88(3), 417-423. Doi: <https://doi.org/10.4067/S0370-41062017000300017>
- Palmer, I., & Wingfield, P. T. (2012). Preparation and extraction of insoluble (inclusion-body) proteins from *Escherichia coli*. *Curr Protoc Protein Sci*, Chapter 6, Unit6 3. Doi: <https://doi.org/10.1002/0471140864.ps0603s70>
- Rodrigues, M., Yonamine, G. H., & Fernandes Satiro, C. A. (2018). Rate and determinants of non-adherence to a gluten-free diet and nutritional status assessment in children and adolescents with celiac disease in a tertiary Brazilian referral center: a cross-sectional and retrospective study. *BMC Gastroenterol*, 18(1), 15. Doi: <https://doi.org/10.1186/s12876-018-0740-z>
- Schwalb, T., Wieser, H., Koehler, P. (2012). Studies on the gluten-specificpeptidase activity of germinated grains from different cereal species and cultivars. *European Food Research and Technology*, 235,1161–1170.
- Sebela, M., Rehulka, P., Kabrt, J., Rehulkova, H., Ozdian, T., Raus, M., Franc, V., & Chmelik, J. (2009, Nov). Identification of N-glycosylation in prolyl endoprotease from *Aspergillus niger* and evaluation of the enzyme for its possible application in proteomics. *J Mass Spectrom*, 44(11), 1587-1595. Doi: <https://doi.org/10.1002/jms.1667>
- Shan, L., Marti, T., Sollid, L. M., Gray, G. M., & Khosla, C. (2004). Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. *Biochem J*, 383(Pt 2), 311-318. Doi: <https://doi.org/10.1042/BJ20040907>
- Siegel, M., Bethune, M. T., Gass, J., Ehren, J., Xia, J., Johannsen, A., Stuge, T. B., Gray, G. M., Lee, P. P., & Khosla, C. (2006). Rational design of combination enzyme therapy for celiac sprue. *Chem Biol*, 13(6), 649-658. Doi: <https://doi.org/10.1016/j.chembiol.2006.04.009>
- Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., van Veelen, P., Edens, L., & Koning, F. (2006). Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. *Am J Physiol Gastrointest Liver Physiol*, 291(4), G621-629. Doi: <https://doi.org/10.1152/ajpgi.00034.2006>
- Tye-Din, J. A., Anderson, R. P., Ffrench, R. A., Brown, G. J., Hodsmann, P., Siegel, M., Botwick, W., & Shreeniwas, R. (2010). The effects of ALV003 pre-digestion of gluten on immune response and symptoms in celiac disease in vivo. *Clin Immunol*, 134(3), 289-295. Doi: <https://doi.org/10.1016/j.clim.2009.11.001>
- Vallejo, L. F., & Rinas, U. (2004). Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. *Microb Cell Fact*, 3(1), 11. Doi: <https://doi.org/10.1186/1475-2859-3-11>
- Walter, T., Wieser, H., Koehler, P. (2015). Degradation of gluten in rye sourdough products by means of a proline-specific peptidase. *European Food Research and Technology*, 240, 517–524. Doi: <https://doi.org/10.1007/s00217-014-2350-5>
- Wei, G., Helmerhorst, E. J., Darwish, G., Blumenkranz, G., & Schuppan, D. (2020). Gluten Degrading Enzymes for Treatment of Celiac Disease. *Nutrients*, 12(7). Doi: <https://doi.org/10.3390/nu12072095>
- Wei, G., Tian, N., Valery, A. C., Zhong, Y., Schuppan, D., & Helmerhorst, E. J. (2015). Identification of Pseudolysin (lasB) as an Aciduric Gluten-Degrading Enzyme with High Therapeutic Potential for Celiac Disease. *Am J Gastroenterol*, 110(6), 899-908. Doi: <https://doi.org/10.1038/ajg.2015.97>
- Zamakhchari, M., Wei, G., Dewhirst, F., Lee, J., Schuppan, D., Oppenheim, F. G., & Helmerhorst, E. J. (2011). Identification of *Rothia* bacteria as gluten-degrading natural colonizers of the upper gastro-intestinal tract. *PLoS One*, 6(9), e24455. Doi: <https://doi.org/10.1371/journal.pone.0024455>