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The Impact of Different Conditions on Protease Hydrolysis for Gliadin Degradation

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Abstract

α-gliadin has been proven to be the cause of gliadin's toxicity for celiac patients because it is highly immunogenic. Therefore, the effect of *Aspergillus niger* protease on gliadin degradation under different conditions (concentration, pH, incubation time, and temperature) was carried out. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) test revealed the ability of fungal protease to degrade gliadin. According to the results, the optimal conditions to completely degrade α-, β-, γ-, and ω-gliadin were 0.3 g fungal enzyme/100 g wheat flour under pH 3 for 16 hours at 55 °C..

Keywords: Celiac; Wheat gluten; Gliadin degradation; *Aspergillus niger;* protease; SDS-PAGE.

1. Introduction

Gluten, the main wheat flour protein is composed of two fractions: gliadins (α-, β-, γ- and ω-gliadin sub-groups) and glutenins [1]. Both fractions are important contributors to the rheological properties of wheat dough. However, several residues of the N-terminus of α-gliadin are immunologically active, e.g. amino acids at position 31–43, 62–75 and 57–89 in the protein [2]. In generally gliadin is the causative agent in wheat, while other prolamins are secalin in rye, avenin in oat, hordein in barley, zeins in maize, oryzins in rice and kafirins in sorghum [3]. Specifically, α-gliadin is responsible for gliadin toxicity which highly immunogenic to celiac patients [4].

Enzymatic degradation of gliadin by *Nigella sativa* seeds protease is considered a new treatment for celiac disease was investigated by Bellir et al. [5] at different pH and different temperature where, their results it appeared that *Nigella sativa* seeds protease degrade Triticum aestivum gliadin more efficiently than Triticum durum gliadin after 24 hr. of incubation. The activity of *Nigella sativa* seeds protease with gliadin as substrate, in pH 7,5 at 37 °C after 2 hr. of incubation, before and after partial enzymatic purification prove that the crude enzyme extract has a low activity with *Triticum durum* gliadin however it was important with *Triticum aestivum* gliadin.

A series of trials were investigated by Buddrick et al. [6] to evaluate the detoxification of gliadin by caricain enzyme incorporated directly into whole meal wheat dough, where the data from this study show that caricain is able to detoxify wheat gliadin to a level where it is likely to be less of a problem for coeliac and could be safely consumed without adverse effects providing that enzyme therapy is available as a safeguard.

A novel process for producing wheat gluten enzyme hydrolysates was developed by Kim [7] using combinations of enzymes (Flavourzyme, Alcalase 2.4L, Protamex, and Marugoto E) at the high pressure (300 MPa), and found that multiple-enzyme treatments increased the proportion of the electrophoretic bands less than 5 kDa in the hydrolysates greatly both at ambient pressure and 300 MPa compared with one-enzyme hydrolysis, where the free amino acids clearly indicated that the high pressure enzymatic process of this study is an efficient method for obtaining wheat gluten enzyme hydrolysates with increased degree of hydrolysis.

There is much conjecture as to the types of enzymes responsible in conjunction with studies of the types of peptides responsible for toxicity in celiac disease. As reported by Hartmann et al. [8] the proteases of germinating grains can rapidly hydrolyze gliadin peptides into fragments that no longer are toxic for celiac patients. Stepniak et al. [9] have identified a prolyl endopeptidase from *Aspergillus niger* that is able to degrade T-cell stimulatory peptides in vitro. It is also suspected that caricain, which contains

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endopeptidases, can achieve detoxification by digestion of immunoactive fragments of gluten [10].

The objective of this study is used the *Aspergillus niger* protease as enzymatic treatment to degrade the gliadin. Reduction of wheat dough gluten content was done to protect celiac patients' against immunosensitivity. Different degradation conditions, including enzyme concentration, pH, incubation time, and temperature, were performed to determine the optimal conditions.

Experimental

2.1. Materials: Commercial wheat flour 72% obtained from Amoun for milling Co. Giza, Egypt. Fungal protease *Aspergillus niger* with activity 500 U/g (DSM Food Specialties B.V., Delft, Netherlands).

2.2. Experimental design: Fungal protease from *A. niger* was used to degrade wheat gliadin with different condition (enzyme concentration, pH, incubation time, and incubation temperature) in four stages (Table 1) where, during the first stage only one parameter (incubation time) has been changed and the other parameters have been stabled, then the optimum condition of incubation time (based on gliadin) was select to be used as stable parameter in the second stage. During the second stage only pH parameter has been changed and the other parameters have been stabled, then the optimum condition of pH was select to be used as stable parameter in the third stage. During the third stage only incubation temperature has been changed and the other parameters have been stabled, then the optimum condition of incubation temperature was select to be used as stable parameter in the fourth stage. During the third stage only enzyme concentration has been changed and the other parameters have been stabled to obtain optimum condition for gliadin degradation. Then the obtained samples were freeze-dried to extract of gliadin for electrophoresis.

Table 1. Experimental design of gliadin degradation

Experiments	Protease Conc.	Time (hr.)	pH	Temperature (°C)
		Matrix 1		
1	0.20 g	8	4	50
2	0.20 g	16	4	50
3	0.20 g	24	4	50
		Matrix 2		
4	0.20 g	16	3	50
5	0.20 g	16	4	50
6	0.20 g	16	5	50
		Matrix 3		
7	0.20 g	16	3	45
8	0.20 g	16	3	50
9	0.20 g	16	3	55
		Matrix 4		
10	0.10 g	16	3	50
11	0.20 g	16	3	50
12	0.30 g	16	3	50

2.3. Methods:

2.3.1. Dehydration of treated wheat flour by Freeze-dryer technique:

All wheat flour samples which treated by protease was placed in an ice bath to maintain the temperature -50 °C. All samples were lyophilized after treatment in a freeze drier (CHRIST LSC plus, ALPA 1- 4, Germany) and stored at 4 ° C until the following experiments [11].

2.3.2. Gliadin extraction:

Gliadin was extracted from tested samples according to the method described by Bushuk and Zillman [12] with a slightly modifications. Briefly 0.05 g of sample (milled sample) weighted in a clean tube. About 200 ml ethanol 70% has been added to the sample then it was rested for 3 hrs. with vortex every 10 min. the tube was centrifuged for 15 min at 10000 rpm using Eppendorf centrifuge. The upper layer of aqueous solution was collected to new tube. The collected solution was mixed by 50% glycerin (100 µl) and rest for 15 min then it was cooled till it was running on gel.

2.3.3. Gel preparation:

The gel was prepared according to Dziuba et al. [13] with some modifications as follows:

A weight of 60 gm acrylamide was prepared, 250 mg ascorbic acid and 3 g Bis acrylamide was also weighted, All the materials were mixed using 150 ml double distilled water, about 20 ml of the buffer was added, the solution was completed up to 1 L solution. The whole solution was filtered after the complete dissolve. For each gel layer about 20 ml was mixed with 50 µL hydrogen peroxide (3%) and 4.0 µL ferric sulfate (hydrated 4%). After well mixing, it was poured to the gel plate and the comb has been added. The gel plate was then left to solidification, and the comb of solid gel was separated by buffer from the plate layers. The gel plates dipped in buffer solution of electrophoresis. In each well, about 20 µl of separated solution from the sample was injected. Using vertical BioRad apparatus™ the samples has been run in a present of the marker. The apparatus was adjusted at 40 mA for about four hours in two dimensional electrophoresis technique. The plates were dyeing use coomassie blue (20 hrs.), then it was washed in double distilled water for 2 hr. The buffer of running was consisting of 31.50 g aluminum lactic acid (pH= 3.0-3.1) after that completed to 500 ml by water.

2. Results and Discussion:

2.1. Degradation of wheat gliadin by fungal protease

Gluten proteins play active role in celiac disease, as ingestion of glutens lead to damage of villi of small intestine [14], where the toxic protein fractions of gluten are gliadin [15], which is responsible for development of celiac disease due to presence of celiac disease eliciting epitopes in gluten, particularly

highly immunogenic α-gliadins [4]. Thus, the enzymatic treatments of wheat gliadins seem to be an alternative method for decreasing of celiac activity. All protein fractions were analyzed by SDS-PAGE.

The experiment was conducted in four stages (Table 1). Where, this procedure is easy to use, highly reproducible and gives clear banding patterns of gliadin fraction this fraction was easily hydrolyzed and almost completely degraded to low molecular weight peptides of < 22 kDa.

2.1.1. Effect of incubation time on gliadin degradation:

During the first stage the effect of incubation time (8, 16 and 24 hr.) was studied. Effect of the specific condition of enzyme concentration (0.20 g/100 g flour), pH (4) and incubation temperature (50 °C) illustrates in Fig. 1.

For the incubation time of enzyme effect, the results recorded a variation of impact of enzyme on gliadin degradation. After 8 hr. of incubation time all gliadin fragment still clearly appears while after 16 and 24 hr. ω-gliadin only partially disappear while α-, β- and γ-gliadin fragments are appeared. Consequently, the 16 hr. of incubation time was selected to be constant parameter during the next stages. In the study by Luoto and others [16], the addition of *Aspergillus niger* prolyl endoprotease was necessary to bring germinated wheat products below the threshold for gluten-free labeling of 20 ppm.

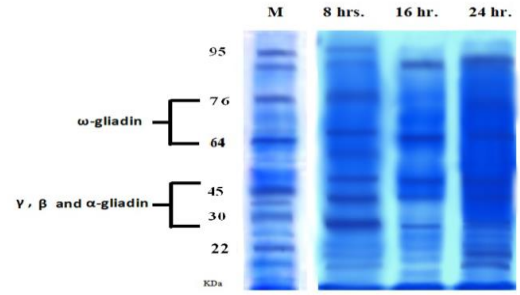

Figure 1. SDS pattern of gliadin degradation by fungal protease during three different incubation time (8, 16 and 24 hr.), pH (4), incubation temperature (50 °C) and enzyme concentration (0.2 g/100 g flour).

2.1.2. Effect of pH on gliadin degradation

Fig. 2 illustrates the second stage of gliadin degradation to study the effect of pH (3, 4 and 5) as a variable parameter with a specific condition of enzyme concentration (0.20 g/100 g flour), incubation time (16 hr.) and incubation temperature (50 °C). According to the obtained pattern α-, β- and γ-gliadin fragments were at pH 4 and pH 5 compared to the sample incubated at pH 3 under the same condition. Alternative enzyme derived from fungus *Aspergillus niger* (AN PEP) is active between pH 2-8, with an optimum activity at pH 4-5 [17]. On the other hand, Authors have argued that the most important contribution of sourdough fermentation is not the microbial protease activity, but lowering the pH to levels optimal for wheat endoprotease activity [18, 19]. Thus, cysteine proteases operate in a pH range of 3 to 6, with optimal gliadin hydrolysis at pH 4.25 [20].

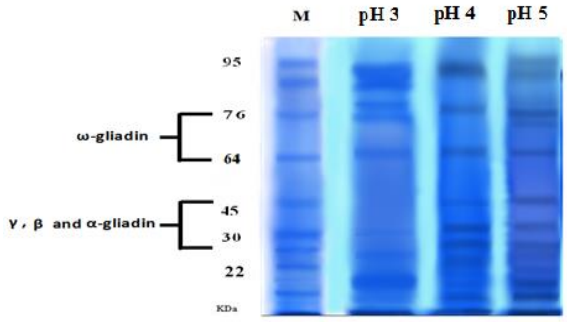

Figure 2. SDS pattern of gliadin degradation by fungal protease during three different pH values (3, 4 and 5), incubation time (16 hr.), incubation temperature (50 °C) and enzyme concentration (0.2 g/100 g flour).

A pH of 4.0 allowed more of the 33-mer degradation in wheat, emmer, einkorn, and rye, although degradation in barley was more efficient at pH 6.5 [20].

2.1.3. Effect of incubation temperature on gliadin degradation

Figure 3 illustrates the effect of incubation temperature (45, 50 and 55 °C) as a variable parameter with a specific condition of enzyme concentration (0.20 g/100 g flour), incubation time (16 hr.) and pH (3) where, α-, β- and γ-gliadin fragments were observed with 45 °C and still appeared in more fraction, while at 50 °C were collected in two fraction and collected to one fraction at 55 °C.

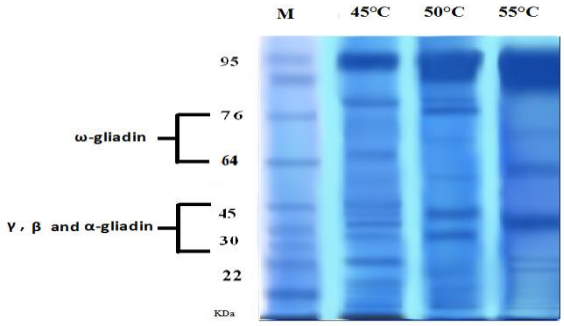

Figure 3. SDS pattern of gliadin degradation by fungal protease during three different incubation temperature (45, 50 and 55 °C), incubation time (16 hr.), pH (3) and enzyme concentration (0.2 g/100 g flour).

2.1.4. Effect of enzyme concentration on gliadin degradation

Figure 4 illustrates the effect of protease concentration (0.1, 0.2 and 0.3 g/100 flour) as a variable parameter with a specific condition of incubation temperature (55 °C), incubation time (16 hr.) and pH (3). The obtained pattern illustrates the positive effect of enzyme concentration where, with

lowest concentration of enzyme (0.1 g/100 g flour) gives more banding patterns of gliadin subunit, which more decreased with 0.2 g/100 g flour, while with the concentration of 0.30 g/100 g flour no gliadin subunit was observed. These results are in agreement with Socha et al. [17] they reported that the main sub-fraction of wheat gliadins is α-gliadin monomer with molecular weight of approx. 35 kDa and consisting from several celiac-active epitopes, and all fungal proteases coming from the same genus: Aspergillus, they show different pattern of proteolytic activity on wheat gliadins as a substrate, and most effective proteolytic activity was observed by using acid proteinase from *A. niger* since wheat gliadins and low molecular weight peptides were completely degraded. Also, Cornell and Stelmasiak [10] have identified a prolyl endopeptidase from *Aspergillus niger* that is able to degrade immunoactive fragments of gluten. As reported by Walter et al. [22] gluten-containing foods can be rendered gluten-free by enzymatic hydrolysis of gluten and gluten fragments. prolyl endopeptidase from *Aspergillus niger* appears to be the enzyme of choice because it manages to degrade even very high gluten concentrations (≈100,000 mg/kg) in different matrices at pH values between 1.0 and 4.0 and temperatures between 40 and 50 °C without any adverse effects. Also, Hartmann et al. [8] reported that, proteases have the ability to degrade gliadin peptides and it is shown for the first time that these proteases cleave these peptides rapidly into non-toxic fragments. Thus, fairly high concentrations of prolyl endopeptidase and prolonged exposure are needed to achieve complete digestion of gluten peptides [23].

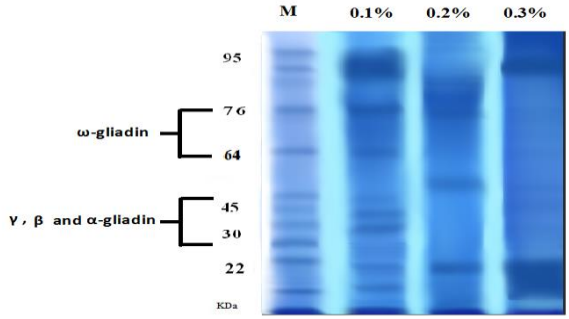

Figure 4. SDS pattern of gliadin degradation by fungal protease during three different enzyme concentration (0.1, 0.2 and 0.3 g/100 g flour), incubation time (16 hr.), pH (3) and incubation temperature (55 °C).

3. Conclusion

The breakdown of gliadins by *Aspergillus niger* protease shows the potential effect to degrade most of gliadin fraction after 16 hr. of incubation. On the other hand, the optimum pH values were between 3 to 4. Regards the effect of temperature, α-, β- and γ-gliadin fragments were collected to one fraction at 55 °C. Also, the positive effect of fungal protease concentration with lowest concentration of enzyme (0.1g /100 g flour) gives more banding pattern of gliadin subunit.

4. Conflicts of interest

The authors declare no conflict of interest.

5. References

1- Nagy-Réder D, Birinyi, Z, Rakszegi M. Békés F, Gell G (2021). The Effect of Abiotic Stresses on the Protein Composition of Four Hungarian Wheat Varieties. Plants 11(1): 1-23. https://doi.org/10.3390/plants11010001

2- Fasano A, Catassi C (2001) Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. Gastroenterology 120(3): 636-651. doi:10.1053 /gast.2001.22123

3- Badiu E, Aprodu I, Banu I (2014) Trends in the development of gluten-free bakery products. Food Technol 38(1): 21-36.

4- Sharma N, Bhatia S, Chunduri V, Kaur S, Sharma S, Kapoor P, Kumari A, Garg M (2020) Pathogenesis of celiac disease and other gluten related disorders in wheat and strategies for mitigating them. Frontiers in Nutrition 7(6): 1-26. https://doi.org/10.3389/fnut.2020.00006

5- Bellir N, Bellir MN, Khlifi D, Bellil I, Medouri A, Medoukali I, Rouabah L (2014) Enzymatic degradation of gliadin by *Nigella sativa* seeds protease: implications for new treatment of celiac disease. World Journal of Pharmacy and Pharmaceutical Sciences 3(11): 507-522.

6- Buddrick O, Cornell HJ, Small DM (2015) Reduction of toxic gliadin content of wholegrain bread by the enzyme caricain. Food chemistry 170: 343-347. DOI:10.1016/j.foodchem.2014.08.030

7- Kim N (2017) Production of wheat gluten hydrolyzates by enzymatic process at high pressure. Food Science and Biotechnology 26(6): 1587-1593. doi: 10.1007/s10068-017-0152-9

8- Hartmann G, Koehler P, Wieser H (2006) Rapid degradation of gliadin peptides toxic for coeliac disease patients by proteases from germinating cereals. Journal of Cereal Science 44(3): 368-371. DOI: 10.1016/j.jcs.2006.10.002

9- Stepniak D, Spaenij-Dekking L, Mitea C, Moester M, de Ru A, Baak-Pablo R, Koning F (2006) Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. American Journal of Physiology-Gastrointestinal and Liver Physiology 291(4): G621-G62. doi: 10.1152/ajpgi.00034.2006.

10- Cornell HJ, Stelmasiak TA (2007) A unified hypothesis of coeliac disease with implications for management of patients. Amino Acids 33(1): 43-49. doi: 10.1007/s00726-006-0420-0
11- Azab DE, Heikal YA, Salaheldin TA, Hassan AA, Abu-Salem FM (2019) Nano formulated soy proteins for improvement of beef burgers quality. Egyptian Journal of Chemistry 62(7): 11671184. **DOI:** 10.21608/ejchem.2019.6867.1573
12- Bushuk W, Zillman RR (1978) Wheat cultivar identification by gliadin electrophoregrams. I. Apparatus, method and nomenclature. Canadian Journal of Plant Science 58(2): 505-515.
13- Dziuba M, Nałęcz D, Szerszunowicz I, Waga J (2014) Proteomic analysis of wheat α/A- and β-gliadins. Czech Journal of Food Sciences 32(5): 437-442. DOI:10.17221/600/2013-CJFS
14- See JA, Kaukinen K, Makharia GK, Gibson PR, Murray JA (2015) Practical insights into gluten-free diets. Nat Rev Gastroenterol Hepatol 12: 580-591. doi: 10.1038/nrgastro.2015.156
15- Shewry PR, Tatham S (2016) Improving wheat to remove coeliac epitopes but retain functionality. Journal of Cereal Science 67: 12-21. https://doi.org/10.1016/j.jcs.2015.06.005
16- Luoto S, Jiang Z, Brinck O, Sontag-Strohm T, Kanerva P, Bruins M, Edens L, Salovaara H, Loponen J (2012) Malt hydrolysates for gluten-free applications: autolytic and proline endopeptidase assisted removal of prolamins from wheat, barley and rye. J Cereal Sci 56: 504–9. (2012). DOI: 10.1016/j.jcs.2012.06.004
17- Socha P, Mickowska B, Urminská D, Kačmárová K (2015) The use of different proteases to hydrolyze gliadins. Journal of Microbiology. Biotechnology and Food Sciences 9(5): 101-104. DOI: 10.15414/jmbfs.2015.4.special2.101-104
18- Ganzle MG, Loponen J, Gobbetti M (2008) Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. Trends Food Sci Technol 19: 513–21. https://doi.org/10.1016/j.tifs.2008.04.002
19- Loponen J, Kanerva P, Zhang C, Sontag-Strohm T, Salovaara H, Ganzle MG (2009) Prolamin hydrolysis and pentosan solubilization in germinated-rye sourdoughs determined by chromatographic and immunological methods. J Agric Food Chem 57: 746–53. https://doi.org/10.1021/jf803243w
20- Bottari A, Capocchi A, Fontanini D, Galleschi L (1996) Major proteinase hydrolysing gliadin during wheat germination. Phytochemistry 43: 39–44. DOI: 10.1016/0031-9422(96)00193-8
21- Nilsson U, Oste R, Jagerstad M (1987) Cereal fructans: Hydrolysis by yeast invertase, in vitro and during fermentation. J Cereal Sci 6: 53–60. doi:10.1016/S0733-5210(87)80040-1
22- Walter T, Wieser H, Koehler P (2014) Degradation of gluten in wheat bran and bread drink by means of a proline-specific peptidase. J Nutr Food Sci 4(5): 2-15. DOI: 10.4172/2155-9600.1000293
23- Matysiak–Budnik T, Candalh C, Cellier C, Dugave C, Namane A, Vidal–Martinez T, Heyman M. (2005) Limited efficiency of prolyl-endopeptidase in the detoxification of gliadin peptides in celiac disease. Gastroenterology 129(3): 786-796. doi: 10.1053/j.gastro.2005.06.016.