The Impact of Different Conditions on Protease Hydrolysis for Gliadin Degradation


*Food Tech. Dept., Food Industry and Nutrition Research Division, National Research Centre, 33 El Buhouth St., 12622 Dokki, Giza, Egypt.
bFood Sci. Dept., Fac. of Agric., Ain Shams Univ., 68 Hadayek Shoubra P.O. Box 11241, Cairo, Egypt

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 δ-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 δ-gliadin subgroups) 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 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
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**


2.2. 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**

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Protease Conc.</th>
<th>Time (hr.)</th>
<th>pH</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrix 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.20 g</td>
<td>8</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>0.20 g</td>
<td>16</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>0.20 g</td>
<td>24</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td><strong>Matrix 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.20 g</td>
<td>16</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>0.20 g</td>
<td>16</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>0.20 g</td>
<td>16</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td><strong>Matrix 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.20 g</td>
<td>16</td>
<td>3</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>0.20 g</td>
<td>16</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>0.20 g</td>
<td>16</td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td><strong>Matrix 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.10 g</td>
<td>16</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>11</td>
<td>0.20 g</td>
<td>16</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>0.30 g</td>
<td>16</td>
<td>3</td>
<td>50</td>
</tr>
</tbody>
</table>

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 gluten leads 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. α-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.

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) 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.

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.

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
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

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