ENZYMATIC DEGRADATION OF GLIADIN BY NIGELLA SATIVA SEEDS PROTEASE: IMPLICATIONS FOR NEW TREATMENT OF CELIAC DISEASE

Nousseiba Bellir*a, Mohamed Nacer Bellirb, Leila Rouabaha

aLaboratory of Cellular and Molecular Biology, Faculty of sciences, Constantine 1 University, Route Ain El Bey., 25017 Constantine, Algeria.
bFaculty of Medicine, Department of Pharmacy, Constantine 3 University, Route Ain El Bey., 25017 Constantine, Algeria.

ABSTRACT
The protease was extracted from Nigella sativa seeds with 0.1 M citrate/phosphate buffer (pH 7.5), the crude enzyme extract showed maximum protease activity at pH 1.5 and optimal temperature at 50°C. After the partially purification of enzyme and analyses of RP-HPLC and SDS-PAGE results it appeared that Nigella sativa seeds protease degrade Triticum aestivum gliadin more efficiently than Triticum durum gliadin after 24h of incubation. The activity of Nigella sativa seeds protease with gliadin as substrate, in pH 7.5 at 37°C after 2h of incubation, before and after partial enzymatic purification prove that the crude enzyme extract have a low activity with Triticum durum gliadin however it was important with Triticum aestivum gliadin, this protease activity was increased in the same conditions using partially purified enzyme and it persist always higher with Triticum durum gliadin comparing with Triticum aestivum gliadin. On the bases of these results, Nigella sativa seeds protease represent the alternative means of treating celiac disease in the future using the detoxification of gliadin to eliminate the immunogenicity of gluten.

KEYWORDS: Gliadin, Celiac disease, Nigella sativa, Protease.
INTRODUCTION

Celiac disease (CD) is a chronic, immunologically determined form of enteropathy affecting the small intestine in genetically predisposed children and adults. This disorder is characterized by the presence of typical auto-antibodies and histological alterations of the small bowel mucosa caused by an inflammatory T-cell response to the storage proteins in wheat (gliadin), rye (secalin), and barley (hordein), which are collectively called “gluten”. Incomplete degradation of these dietary proteins in the gastrointestinal tract leads to the appearance of peptides, of which some are toxic and others immunogenic for celiac disease patients.

It has been proposed that some of these harmful gluten proteins could be detoxified by enhancing their hydrolysis during food processing.

*Nigella Sativa* Linn belongs to family Ranulaceae. The herb is widely known in different parts of the world and its seeds are used as condiment. In subcontinent it is known as “kalonji” and its Arabic name is “Habatul Sauda”. In the west it is known as “Black Cumin”. There is a Hadith of Hazrat Muhammad (PBUH) that, “black seed is treatment of every disease but death”.

In this study we hypothesized that the protease isolated from *Nigella sativa* seeds would be most efficient in detoxifying dietary gluten and could thus be suitable as enzyme supplement therapy in the treatment of celiac disease.

MATERIALS AND METHODS

Enzyme Extraction

The enzyme was extracted according to Lowry *et al.* (1951). 10 g of powder of *Nigella sativa* seeds was homogenized with 100mL of 0,1 M citrate/phosphate buffer (pH 7,5) in an ice bath. The homogenate was centrifuged at 1000 × g for 10 min at 4°C. The supernatant was used for protease activity assay and further purification and investigations.

Assay of Proteolytic Enzymes

Protease activity was assayed by the method of Lenoir and Aubgerer (1977) modified by Mechakra *et al.* (1999) by using casein as substrate. 1 ml of the crude enzyme extract, 1,5 ml (0,1M / 0,2 M) citrate - phosphate buffer, pH 6,8, and 2,5 ml of substrate (2,5% casein in 0,02 M citrate sodium buffer), The mixture was incubated at 40°C for 60 min. Reaction was
terminated by the addition of an equal volume of 4% (w/v) chilled trichloroacetic acid then the reaction mixture was allowed to stand in ice for 30 min to precipitate the insoluble proteins. The supernatant was separated by filtration; the acid soluble product in the supernatant was neutralized with 2.5 ml 2% Na2CO3 in (0.1N) NaOH which mixed with 0.5 ml of filtrate solution. The color developed after adding 0.25 ml of 3-fold diluted Folin–Ciocalteau reagent was measured at 750 nm.

All assays were done in triplicate. One protease unit is defined using a calibration curve of tyrosine (Fig.1), as the amount of enzyme that releases 1 mg of tyrosine per ml per minute under the above assay conditions.

![Figure 1: A calibration curve of tyrosine.](image)

**Effect of pH and Temperature**

Effect of pH on the crude enzyme activity was measured at various pH ranges (1 – 10). Reaction mixtures were incubated at 40°C for 60 min and the activity of the enzyme was measured as described previously at various pH levels (pH 1–10), (0.1M) Na-OH/HCL buffer (pH 1 à 3,5), (0.1 M / 0.2 M) citrate phosphate buffer (pH 4 à 7,5 ) and (0.1 M) glycine-NaOH buffer (pH 8–10). The optimal temperature of the enzyme was determined by measuring the enzyme activity at various temperatures (30–90 °C) in the optimal pH.

**Effect of Prolonged Heating**

The stability in the optimal temperature was studied by incubating the crude enzyme with the substrate in the optimal pH and temperature different intervals for times (10, 20, 30, 40, 50, 60, 70, 80 and 90 min).
Purification of Protease
Solid ammonium sulfate was added slowly to the crude enzyme extract to 30, 60, 80% saturation with gentle stirring in an ice bath.

The collected precipitate with the highest protease activity was dissolved in 10ml of enzyme extract buffer; the protein solution was extensively dialyzed against the same buffer at 4 °C, and loaded on a Sephadex G-200 column (2.5 cm× 90 cm) and eluted with enzyme extract buffer. The section with the highest activity was collected; the peaks from each fraction were monitored at 280 nm. The products of each step were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The determination of the activity of each step of the purification was determined using casein as substrate like described previously.

Extraction of Gliadin
Gliadin was extracted from wheat Triticum durum (Variety: WAHA) and Triticum aestivum (Variety: HD 1220) by a method of Singh et al.

Gliadin were extracted twice with 1 ml of 50 % (v/v) 1-propanol for 30 min at 65°C with brief vortexing every 10 min and centrifuged for 15 min at 10 000 rpm. The second extraction was without vortexing. The gliadin fraction present in the supernatant was aspirated and evaporated at 65°C for 12 h.

Incubation of Gliadin with the Crude Enzyme Extract
Gliadin (10mg) was incubated with the crude enzyme extract (1mL) for 2 h at 37°C in 1 ml (0,1M) citrate phosphate buffer, pH 7.5. The reaction was then stopped by addition of 3 ml 4% (w/v) chilled trichloroacetic acid. The determination of activity of the protease was determined like described previously.

Incubation of Gliadin with the Enzyme Purified
Gliadin (10mg) was incubated with or without the purified enzyme (0.5mL) for 2h, 4h, 8h and 24h at 37°C in 0,5 ml (0,1M) citrate phosphate buffer, pH 7.5. The reaction was then stopped by addition of 1 ml 4% (w/v) chilled trichloroacetic acid.

Afterward the samples were subjected to determine the protease activity, SDS-PAGE analysis and RP-HPLC.
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli performed. The separation conditions of the gel were for the separating gel 12% and the stacking gel 2, 88%. The buffer solution for the separating gel was Tris-HCl 1.5 M (pH 8.8). 2,125 ml of extraction solution (which prepared by addition of 20 mg SDS, 4ml glycerol, 0,8 ml Tris-HCL buffer, QSP=10 ml) was added to 5ml H2O qsp, 50 μL of this preparation was added to 100 μL of the gliadin degradation samples aliquots with different incubation duration and to the gliadin control samples.

The mixtures were after vortexing incubated at 65°C for 15 min and centrifuged for 5 min at 10000 rpm. 50 μL of each mixture were loaded onto the gel and electrophoresed with Tris-glycine buffer (pH 8.3). Electrophoresis was performed at 180 V. The protein bands were visualized by Commassie Brilliant Blue G-250

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

The HPLC procedure was performed according to the method of Lookhart et al (1986), with the exceptions noted below. 20μL were injected on a C18 RP column (TRACER EXTRASIL ODS2, 5μm, 250x 4,6 mm) at 1m/min. The elution conditions consisted of a multistep linear gradient; the starting solvent contained 25% acetonitrile concentration 75% water, each containing 0,1% trifluoroacetic acid. The acetonitrile concentration increased to 35% at 5min, 50% at 10 min, 75% at 17 min, 85% at 18 min, and then returned to initial conditions (25% acetonitrile) at 19 min. The total runtime, from injection to injection, was 30 min, which included an 11 min reequilibration step between runs. The absorbance was measured at 210 nm.

RESULTS

The protease activity (units/g) of the crude enzyme extract is estimated by 84,26 units/g UP with the casein as substrate in pH 6,8 and 40°C after 1h of incubation. The pH profile of Nigella sativa seeds protease is shown in Fig.2. Activity of the enzyme was determined at different pH ranging from 1.0-10.0. The optimum pH recorded was 1.5 for protease activity. Protease activity was found to be stable in al value of pH.
Figure.2: The pH profile of *Nigella sativa* seeds crude enzyme extract.

The temperature profiles of the protease of *Nigella sativa* seeds is shown in Fig. 3, the profile indicates that the temperature optimum of the enzyme is 50°C, at higher temperature it decreases slightly, but it persists stable.

Figure.3: The temperature profile of *Nigella sativa* seeds crude enzyme extract.

The effect of prolonged heating on protease activity of *Nigella sativa* seeds is shown in Fig.4. A proportional increase of protease activity was observed with the incubation time between 10 min and 30 min, between 30 min and 70 min stability is recorded, and it still increases proportionally with the incubation period, the highest activity was registered after 90 min of incubation.
Figure 4: Effect of Prolonged Heating on protease activity of *Nigella sativa* seeds crude enzyme extract.

**Purification of the Protease**

The results of purification of *Nigella sativa* seeds protease are summarized in Table 1 and figure 5. The overall activity yield of the purified protease was 14%. The protease activity measured by using the casein as substrate, calculated at each stage of purification is summarized in Table 1. The crude extract contains 84.26 units. The precipitate obtained after 30% saturation and dialyzed recovered nearly 49.2 units. And that of the partially purified enzyme recovered was 59.9 units.

Figure 5 shows that the fraction which corresponds to the peak of maximum protease activity represents the partially purified enzyme which used in further investigations.

**Table 1: Purification of *Nigella sativa* seeds protease.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Yield (%)</th>
<th>Protease activity (PU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme extract</td>
<td>50</td>
<td>100</td>
<td>84.26</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Precipitation, Dialyze (30%)</td>
<td>10</td>
<td>20</td>
<td>49.2</td>
</tr>
<tr>
<td>Chromatography on Sephadex G200</td>
<td>7</td>
<td>14</td>
<td>59.9</td>
</tr>
</tbody>
</table>

The activity of *Nigella sativa* seeds protease with gliadin as substrate, in pH 7.5 at 37°C after 2h for incubation, before and after partial enzymatic purification prove that the crude enzyme extract have a low activity with *Triticum durum* gliadin however it was important with *Triticum aestivum* gliadin, this protease activity was increased in the same conditions.
using partially purified enzyme and it persist always higher with *Triticum aestivum* gliadin comparing with *Triticum durum* gliadin. (Tab. 2)

![Elution profile of *Nigella sativa* protease from sephadex G200 column. (90cm x 2 cm) eluted with phosphate/citrate buffer 0.1M, pH 3.](image)

**Figure.5:** Elution profile of *Nigella sativa* protease from sephadex G200 column. (90cm x 2 cm) eluted with phosphate/citrate buffer 0.1M, pH 3.

**Table.2:** Protease activity of *Nigella sativa* seeds protease with gliadin as substrate, in pH 7, 5 at 37°C and after 2h for incubation.

<table>
<thead>
<tr>
<th>Protease activity (PU)</th>
<th>wheat</th>
<th><em>Triticum durum</em> (variety: WAHA)</th>
<th><em>Triticum aestivum</em> (variety: HD 1220)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme extract</td>
<td>19,7</td>
<td>69,3</td>
<td></td>
</tr>
<tr>
<td>Partially purified enzyme</td>
<td>73,1</td>
<td>101,8</td>
<td></td>
</tr>
</tbody>
</table>
SDS-PAGE analysis of the proteins at each step of purification shows that substantial qualitative difference between the proteins profiles of each purification step but the purified enzyme was also confirmed to not be homogeneous by SDS-PAGE (Fig. 6).

Paper electrophoresis of the crude enzyme gave seven different bands (Lane 1), witch after ammonium sulfate precipitation and dialyze (30%) gave six bands (Lane 2), and the isolated protease after Sephadex G200 filtration (Lane 3) migrated as divers bands. The purification steps were little effective but the quantities of the partially purified protease were insufficient to complete the purification.

![Image of SDS-PAGE analysis]

**Figure 6:** Electrophoretic patterns after each purification step of *Nigella sativa* seeds protease.

Lane1: crude extraction; Lane2: after ammonium sulfate precipitation and dialyse (30%); Lane 3: after Sephadex G-200 filtration.

SDS-PAGE pattern of the gliadin from two variety of wheat (*Triticum durum* (variety: WAHA) and *Triticum aestivum* (variety: HD 1220) before and after incubation with *Nigella sativa* seeds protease is shown in Fig. 7 and Fig.8.

After proteolysis, the electrophoretic pattern of the resultant hydrolysate showed a lot of differences from that of unhydrolysed gliadin, all gliadin sub-fractions were decreased.
The incubation of *Triticum durum* gliadin with *Nigella sativa* seeds protease partially purified (Fig.7) resulted in a decrease in total gliadins proportionally with the duration incubation with the enzyme (Lane2, Lane3, Lane4) comparing with the control (Lane1). After the final incubation (Lane 5) the β gliadin bands were lost completely, however α gliadin bands was present but with slim apparition translated the incomplete degradation.

![Figure 7: SDS Page results of gliadin from *Triticum durum* (Variety: WAHA).](image)

Control (Lane1); after incubation with *Nigella sativa* seeds protease for 2h (Lane2); 4h (Lane 3); 8h (Lane 4) and 24h (Lane 5).

In the case of wheat gliadin from (*Triticum aestivum* (variety: HD 1220)) (Fig.8) the majority of bands region were apparent slightly proportionally with the incubation duration with the enzyme partially purified (Lane2, Lane3, Lane4) comparing with the control (Lane1). and it were lost definitively after 24h for incubation (Lane 5), regarding the regions correspondent of the β, Υ and ω gliadin.

The region of α gliadin was apparent as a slight band. Finally, the results of RP-HPLC illustrates clearly this observations, It appeared that *Nigella sativa* seeds protease degrade *Triticum aestivum* gliadin more efficiently than *Triticum durum* gliadin.
Figure 8: SDS Page results of gliadin from *Triticum estivum* (variety : HD 1220).

Control (Lane 1) ; after incubation with *Nigella sativa* seeds protease for 2h (Lane 2) 4h (Lane 3) 8h (Lane 4) and 24h (Lane 5).

This is evidenced by Fig. 9 & Fig.10, showing the peptide profiles of gliadin extracted from two variety of wheat before (Fig. 9a, Fig.10 a) and after incubation with *Nigella sativa* seeds protease.

This can be confirmed by diminution or disappearance of picks correspondents of gliadin fractions in comparison with the control (Fig. 9a, Fig.10 a) concerning and by the increasing formation of peptides from gliadin detected after 2, 4, 8 and 24 h of incubation.

It appeared a disappearance of the first pick of the *Triticum durum* gliadin chromatogramme control (Fig. 9a) (between 2.5 and 2 min min) after 2 h incubation of gliadin with partially purified protease (Fig. 9b), and the reduction of the second pick in (Fig. 9a) (between 3.5 min and 4.5 min) after 2 h and 4 h of incubation with the enzyme (Fig. 9b and Fig. 9c respectively), until its disappearance after 8 hours of incubation (Fig . 9d). In addition to the appearance of a new pick between 2 and 4 min after 4 h incubation (Fig. 9c). While the disappearance of the two picks of the *Triticum aestivum* gliadin chromatogramme control (Fig. 10a) was complete after only 2 hours of incubation (Fig. 10b).

We can observe the formation of a new concentrated pick corresponding to the resulting peptides degradation after 4h incubation of gliadin with the protease (Fig. 10c). The figures (Fig. 9, Fig.10 e) represent two completely different chromatograms compared to controls (Fig. 9a, Fig.10 a) with a complete disappearance of the two picks reflecting the
complete degradation of the protein fractions followed by formation of new picks corresponding to peptides resulting from the degradation after 24 hours of incubation.

Figure 9: RP-HPLC results of degraded wheat gliadins extracted from *Triticum durum* (variety: WAHA). Control (a); after incubation with *Nigella sativa* seeds protease for 2h (b) 4h (c) 8h (d) and 24h (e).
Figure.10: RP-HPLC results of degraded wheat gliadin extracted from *Triticum estivum* (variety: HD 1220). Control (a); after incubation with *Nigella sativa* seeds protease for 2h (b) 4h (c) 8h (d) and 24h (e).
DISCUSSION
The results of this study show that this medicinal seeds analyzed have high protease activity and can be effectively used to cure the gastrointestinal disorders as these acts, as digestive aids. The pH optimum of *Nigella sativa* seeds protease is around 1.5 after this value of pH protease activity was found to be stable in al values of pH. Therefore the protease present in *Nigella sativa* seeds is a mixture of acidic, neutral and alkaline proteases. It may be effective both in stomach where the medium is acidic and in small intestine where the medium is alkaline. From the temperature profiles, it is evident that *Nigella sativa* seeds protease show higher temperature optimum at 50°C. Most of plant proteases generally denature between 40 - 50°C as described by Khan *et al.* [10] and Jilani and Khan. [11]

The proteases of *Nigella sativa* seeds exhibit relatively higher temperature optimum. This may be due to their binding with the cells in powder form as this binding increases thermostability. It is also confirmed by the effect of prolonged heating on the activity of protease of *Nigella sativa* seeds which shows that it are thermostable even at 90°C and it still increases proportionally with the incubation period, the highest activity was registered after 90 min of incubation.

The major objective of this study reported here is to find the enzymatic degradation of gliadin by *Nigella sativa* seeds protease to reduce gluten immunogenicity and the Implications of this seeds for new treatment of celiac disease on the basis of their protease activity, as the proteases are capable of degrading proteins.

Recently Matysiak-Budnik and colleagues [12] showed that fairly high concentrations of bacterial PEP and prolonged exposure are needed to achieve complete digestion of gluten peptides. A further difficulty in using bacteria-derived PEP as an alternative therapy in celiac disease is that it does not completely function in conditions found in the stomach and it is broken down by pepsin. [13] After the study of the effect of gastric and intestinal enzymes on the activity of this protease, these results indicate the possibility functional role of this enzyme in vivo in participation in the degradation of storage proteins. *Nigella sativa* seeds protease used in the current study provide an alternative means to overcome the above-mentioned problems. *Nigella sativa* seeds protease are active over a large pH range, being thus effective both in the stomach and in the small intestine. Further benefits of using this enzyme in detoxifying gluten are that they have a good safety profile as they are derived from a naturally safe source; the isolation of these proteases is simple and inexpensive.
In addition the breakdown of gliadins by *Nigella sativa* seeds protease shows the potential of these proteases to degrade all of the gliadin fractions after 24h of incubation. The degradation of gliadin extracted from *Triticum durum* occurred to a lower extent when compared to gliadin extracted from *Triticum aestivum*.

Although the 24-hour incubation time applied in the current study is far from a physiological timeframe in the gastrointestinal tract, it is important to note that we did not enrich the proteins responsible for the gliadin hydrolysis but instead used simple water-soluble *Nigella sativa* seeds protease preparations. Thus it is very likely that the active enzyme concentrations can be increased many-fold by optimizing the extraction process, and hence the incubation times can be shortened respectively.

Therefore, the present study is the initial point for further analysis of wheat storage proteins by *Nigella sativa* seeds protease with biochemical methods and it documents the eligibility of these enzymes as a prospective degrading tool with regard to celiac disease.

**CONCLUSIONS**

Thus, the data obtained in the present study confirm the presence of proteases in *Nigella sativa* seeds and its ability to be applied in many industrial areas instead of other protease enzymes which are produced from animals, microorganisms or tropical plants.

Furthermore; from the results of our research, we concluded that the protease enzyme which was partially purified from *Nigella sativa* seeds can hydrolyze the storage protein so it could be used for new treatment of celiac disease. We conclude that gluten detoxification by *Nigella sativa* seeds protease offers a promising alternative means of treating celiac disease in the future. Such enzymes could be administered at meal-times to detoxify ingested gluten, or they could be utilized in food processing in order to eliminate gluten toxicity and to develop high-quality celiac-safe food products. This proof-of-concept study provides a platform for future exploratory investigations.

**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest related to this article.
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