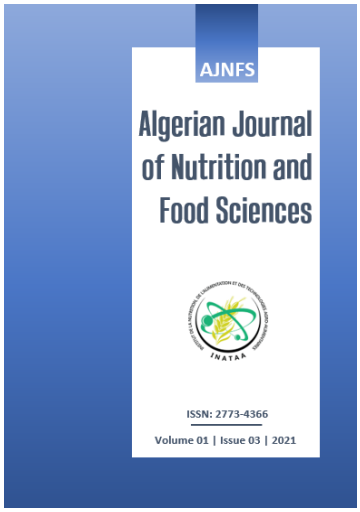

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Original Article

Production and characterization of proteases from *Aspergillus niger* strain isolated from dried fruit and its effect on gliadin

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Abstract *Fungal strain isolated from dried apricot was identified as Aspergillus niger by cultural and morphological characterization. Proteolytic enzyme production was carried out by solid state fermentation on wheat bran medium. The crude enzyme extract of Aspergillus niger has a proteolytic activity of 78.54 µg/ml/h (PU), with an optimum pH of 3.0 and temperature of 50°C. The protease activity was thermoresistant after 90 min of incubation. It has the capacity to degrade wheat gliadin (Triticum durum and Triticum aestivum). It may represent a potential therapeutic path in the treatment of celiac disease.*

Keywords *Aspergillus niger, protease, proteolytic activity, gliadin*

Introduction

Proteolytic enzymes, or proteases, are enzymes that catalyze proteins degradation. Due to the diversity of their applications, they represent one of the largest groups of industrial enzymes. In addition, proteases are ubiquitous: they are found in plants, animals and microorganisms (Kirti *et al.*, 2012). Microbial proteases are preferred over those of other sources because they have almost all the desired characteristics for the biotechnological applications. They are produced by a wide variety of bacteria, mold and yeast. They account for 40 % of the enzymes in the global industry (Raghunath *et al.*, 2010; Kiran *et al.*, 2012).

A wide variety of proteases are developed by molds. They are extracellular, produced in the fermentation medium which allows their separation from mycelium by simple filtration. They are active in a wide pH range (from pH 4 to 11) (Wu *et al.*, 2006; Jisha *et al.*, 2013).

Currently, there is no cure for Celiac Disease (CD). A strict gluten-free diet (GFD) is the only effective way to maintain the health of CD patients. In most patients with gluten sensitivity, the introduction of GFD leads to at least partial healing of the duodenal mucosa, improvement of most symptoms associated with gluten consumption, and a decrease in the titers of specific antibodies in gluten disease. However, in many patients, even with long-term strict adherence to GFD, symptoms may persist, including inflammatory and architectural changes in the small intestine mucosa and positive antibody levels. A number of factors may contribute to an incomplete response to a GFD. It is difficult to avoid cross contamination during food production because gluten is widely used in the food industry. Various therapeutic strategies are being developed to combat CD. Enzyme therapy is especially promising, as a supplement to food in the form of a peptidase preparation that efficiently degrades prolamins peptides. This approach is based on a direct effect on the pathogenic substance, namely, uncleaved peptides with a large number of proline and glutamine residues that are not digested by typical stomach enzymes (Dunaevsky *et al.*, 2021).

The search for alternate therapeutic pathways for the treatment of CD has become a topic of high importance for the celiac community and health care providers; food-grade proteases capable of detoxifying moderate quantities of gluten, are one of the most promising approaches (Wei *et al.*, 2020).

Throughout this work, we have tackled this therapeutic aspect, which opens up new perspectives in the field of the enzymatic approach aimed at eliminating the immunogenicity of gluten. The main objective of this study is the production of proteolytic enzyme from newly isolate *Aspergillus niger* and the study of their characteristics and effect on gliadin.

Materials and methods

Producer microorganism and inoculum preparation

Fungal strain, coded c17, was isolated from dried apricot previously exposed to ambient air. The identification of the isolate was mainly based on the cultural and microscopic characterization (Botton *et al.*, 1990). Spore

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recovery was performed on Sabouraud medium incubated at 30 °C for 5 days, fungal spores suspension was obtained by the addition of a sterile solution of 0.1% Tween 80 (10 ml/petri dish) (Paranthaman *et al.*, 2009; Bensmail *et al.*, 2015). After filtration, the estimation of the number of spores was carried out by measuring the absorbance at 650 nm. The photometric readings were transformed into the number of spores by using per Malassez cell under optical microscope. One milliliter of the spore suspension containing 2 x 10^6 spores/ml, was used as inoculum (Bensmail *et al.*, 2015).

Solid-state fermentation (SSF) and culture conditions

Wheat bran used as a substrate was crushed to reduce particle size (0.425 - 0.850 mm), then kept in sterile boxes before use (Sumantha *et al.*, 2005). Five grams (5 g) of crushed wheat bran supplemented with 4 ml of a mineral solution (NH_4NO_3 0.5; KH_2PO_4 0.2; $MgSO_4.7H_2O$ 0.1; NaCl 0.1, pH 3, (% p/v)) was introduced into 250 ml Erlenmeyer flasks (Initial Moisture Rate = 54%). After sterilization (121 °C for 20 min), medium was inoculated with 1 ml of the fungal spore suspension (Optical Density = 0.782), then incubated for 72 hours at 30 °C (Sumantha *et al.*, 2005; Bensmail *et al.*, 2015).

Protease extraction

The fermented medium was taken up in 50 ml of distilled water to a ratio of 1:5 (p/v) (Sandhy *et al.*, 2005), then slowly stirred for 45 minutes (stirring system: Stuart Scientific). The mixture was filtered and then centrifuged at 4000 rpm for 30 min at 4 °C (Kumar *et al.*, 2005). The recovered supernatant, representing the crude enzyme, has been kept cool at 4 °C (Tunga *et al.*, 2003; Sandhy *et al.*, 2005).

Proteolytic activity essay

Proteolytic activity was determined according to the method of Tsuchida *et al.* (1986). The reaction mixture contained 1 ml of 2% casein (prepared in phosphate buffer pH 7.0) and 1 ml of the crude enzyme solution. The mixture was incubated for 30 min at 40 °C. The enzymatic reaction was stopped by adding 2 ml of (0.4 M) trichloroacetic acid chilled, the mixture was centrifuged at 4500 rpm for 10 min at 4 °C. Then (1 ml) of the supernatant was transferred to another tube in which 5 ml of 0.4 M of Na_2CO_3 and 1 ml of 10% Folin Ciocalteu reagent, were added. After incubation for 20 min at 40 °C in obscurity, the absorbance of the mixture was measured at 750 nm. Control was prepared under the same conditions, except that the trichloroacetic acid was added before the substrate. Protease unit (UP) is equivalent to 1 µg of tyrosine released per ml per hour under essay conditions.

Proteolytic activity characterization

Optimal pH

The optimum pH was determined by performing the enzymatic reaction in buffer solutions at various pH ranges from 1.0 to 10.0: HCl–KCl buffer (0.2 M) (pH 1.0 - 2.0), citric acid - Na_2HPO_4 buffer (0.1M / 0.2 M) (pH 3.0, 4.0, 5.0 and 6.0), phosphate buffer (0.1 M) (pH 7.0 - 8.0) and glycine – NaOH buffer (0.2 M) (pH 9.0 - 10.0) (Bellir *et al.*, 2014).

Optimal temperature and thermostability

The optimum of temperature was determined by incubating the reaction mixture (crude enzyme - substrate) at different temperatures (30, 40, 50, 60, 70 and 80 °C) at optimal pH (Olajuyigbe and Falade, 2014; El-Khonezy *et al.*, 2015). The stability was determined by incubation of the mixture reaction (crude enzyme-substrate) at optimal pH and optimal temperature at different time intervals (10, 20, 30, 40, 50, 60, 70, 80 and 90 min) (Bellir *et al.*, 2014).

Action of crude enzyme extract on gliadin

Gliadin extraction

Gliadin extraction was carried out from the wheat seeds (*Triticum durum* and *Triticum aestivum*) according to the method described by Singh *et al.* (1991). 1 ml of 50 % propanol-1 was add to wheat (v/ v), after an incubation of 30 min at 65 °C with two intermediate vortex shaking every 10 min, a final centrifugation was then carried out at 10 000 rpm for 15 minutes. The second extraction was carried out without intermediate stirring. The gliadin fraction present in the supernatant was aspirated and evaporated at 65 °C for 12 h (Singh *et al.*, 1991).

Incubation of crude enzyme extract with gliadin

Ten milligrams (10 mg) of gliadin were diluted in 0.5 ml of citrate phosphate buffer (0.1 M, pH 7.5). The mixture was added to 1 ml of the crude enzyme extract and then incubated for 2 h at 37 °C (Bellir *et al.*, 2014). The reaction was stopped by adding 2 ml of chilled trichloroacetic acid (0.4 M). The proteolytic activity of the mixture was measured according to the same protocol described previously.

Results and discussion

Fungal strain identification

The cultural and microscopic characteristics of the strain are shown in Figure 1. Cultural development on Sabouraud medium showed that the studied strain has grown rapidly (colony diameter 15-20 mm that can extend to 40 mm). It was white at the beginning, changed to yellow and then went entirely black after sporulation with a granular aspect. The reverse of the colonies was colorless to pale yellow. The results obtained from the microscopic examination are mentioned as follows: the strain presented septate mycelia, smooth, unbranched, long, large and brown conidiophores. Aspergillar head radiate with dark brownish color and globose vesicle, phialid of small size compared to metula, globose conidium, brown and radiate, rough around the vesicle. All these structures characterize the species *Aspergillus*

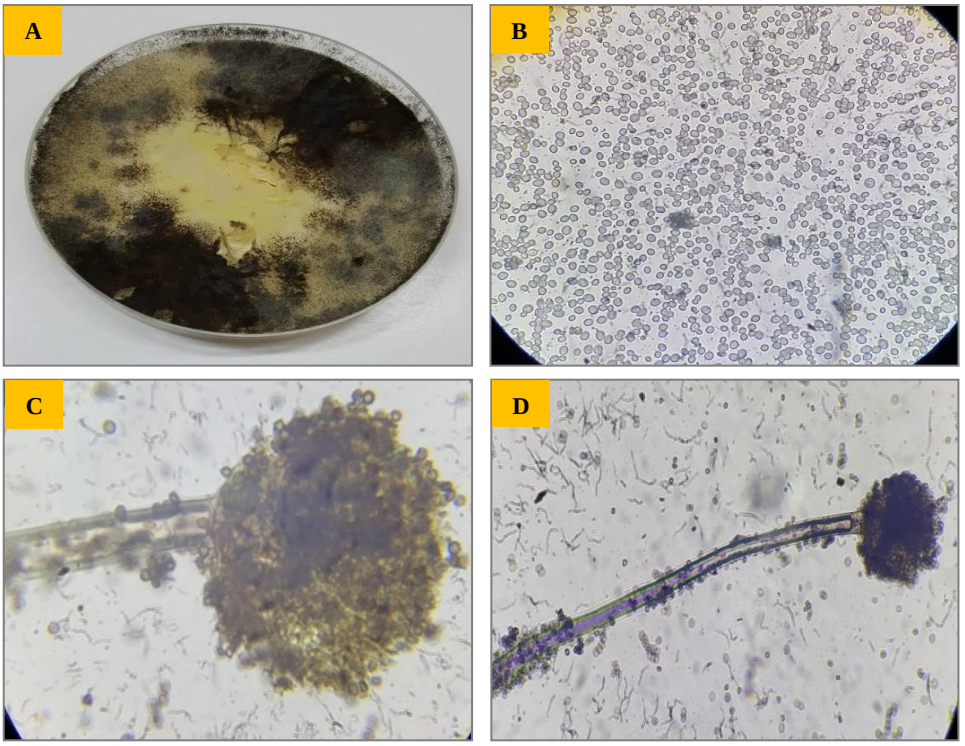

Figure 1. Macroscopic and microscopic aspect of strain c17 on Sabouraud medium
A. Macroscopic aspect on Sabouraud medium, after 5 days of growth; B. Microscopic aspect of spores (40× magnification); C. Conidial head radiate (100× magnification); D. Conidiophore aspect (40× magnification)

niger (Botton *et al.*, 1999; Jernejc and Cimerman, 2001; Pitt and Hoecking, 2009).

Proteolytic activity of the crude enzyme extract

According to the results obtained, the crude extract of strain c17 had 78.54 PU. It is a very important activity compared to that recorded by Sandhya *et al.* (2005), Paranthman *et al.* (2009) and Bhatnager *et al.* (2010) obtained with the same species, these authors noted 31.2 PU, 67.7 PU and 54.89 PU respectively. However, the activity is relatively close to that found by Syed and Vidhale (2013) with the species *Fusarium oxysporum* (70.5 PU).

Proteolytic activity characterization

Optimal pH

Proteolytic activity at different pH is shown in figure 2. The optimal pH was detected at 3.0, the results indicated that the selected strain also had proteolytic activity at neutral pH, but it was less important, it may be suggested that the strain produced at least two proteases, one acid with optimal pH 3.0 and another alkaline at pH 7.0. Similar results were also obtained by many authors (Edens *et al.*, 2005; Bhatnagar *et al.*, 2010; Negi and Banergee, 2010). Other research gave optimal pH at 4.5 from *Aspegillus niger* species (Mukhtar and Ul-Haq, 2009; Bensmail *et al.*, 2013; De Castro *et al.*, 2014).

Optimal temperature and thermostability

The effect of temperature on the proteolytic activity is illustrated in figure 3. The maximal activity was obtained at 50 °C. Beyond this temperature a decrease was observed. The enzyme becomes almost inactive, demonstrating the instability of its structure (Kumar *et al.*, 2008).

The optimal temperature obtained was higher than that found by Bensmail *et al.* (2015) which was 45°C with *Aspergillus niger* proteases and was lower than that given by Negi and Banerjee (2009) and Vishwanatha

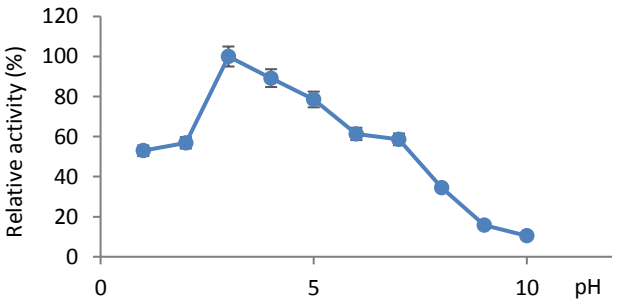

Figure 2. Relative proteolytic activity at different pH

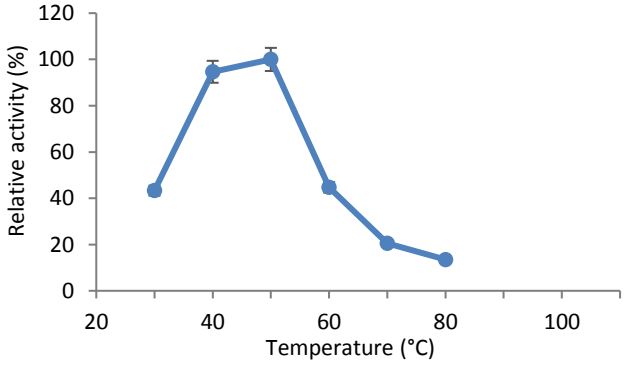

Figure 3. Relative proteolytic activity at different temperature

et al. (2009), which were both at 55 °C with *Aspergillus awamori* MTCC 6652 and *Aspergillus oryzae* MTCC5341 proteases. The stability of the proteolytic activity at the optimal temperature is shown in figure 4. The enzyme extract presented maximal activity after 90 min of incubation; therefore, the enzyme extract was thermostable. It is probably due to the presence of numerous disulfide bonds in the protease oligomeric structure (Bellir *et al.*, 2014). This thermostability can also be explained by strain c17 origin (dried fruits) that gives it this characteristic in order to withstand environmental conditions.

Effect of the proteolytic activity on gliadin

Compared with casein, the proteolytic activity of strain c17 presented an interesting capacity to degrade gliadin. After 2 hours of incubation, the enzyme extract was able to degrade 58.17 and 64.04% of *Triticum durum* and *Triticum aestivum* gliadin respectively (fig. 5).

Aspergillus niger proteases are used in several industrial fields, such as the pharmaceutical industry, where they are involved in the development of new therapeutic agents (Azadeh *et al.*, 2014). In this study the crude extract seems to be a mixture of neutral and acid proteases that could be effective in stomach and also in intestines. Shaukat *et al.* (2003) have recommended the use of proteolytic enzymes from *Carum copticum* which are mixed proteases with optimal pH at 3.0 and 7.0 for the treatment of gastrointestinal problems.

Also, the activity was more important on gliadin from *Triticum aestivum* than that from *Triticum durum*. The complexity of the wheat gliadin molecule makes the degradation procedure very difficult (Shan *et al.*, 2004, Matysiak-Budnik *et al.*, 2005). A combination of different enzymes, rather than a specific protease, is probably needed (Siegel *et al.*, 2006; Gass *et al.*, 2007). Oral supplementation with enzymes could be a valuable method to fight this disease. Ideally, gluten degradation should occur in the stomach before gluten fragments or gluten can reach the duodenum. However, a problem seems relevant in the use of proteolytic enzymes as an alternative therapy in celiac disease, is that these enzymes would not function completely under the conditions of the stomach and could be degraded by pepsin (Shan *et al.*, 2004).

The use of oral prolyl peptidases from bacterial sources such as *Flavobacterium meningoseptica*, *Sphingomonas capsulata*, *Myxococcus xanthus* and *Lactobacillus helveticus* have shown some efficiency limits due to incomplete degradation of gluten, allowing the release of immunogenic peptides. In addition, it has been reported that these bacterial enzymes were inactivated by pepsin and low gastric pH (Matysiak-Budnik *et al.*, 2005). whereas, the protease characterized in our study showed optimal proteolytic activity at pH 3.0 and therefore would be functional in gastric pH and active in the stomach, if not degradable by gastrointestinal enzymes. For this purpose, proteases from *Aspergillus niger* isolates aim to prevent the contact of immunogenic fragments of gluten with the immune system through oral supplementation which could allow the digestion of gluten peptides rich in proline (gliadin) into fragments entirely devoid of immunogenic properties (Shan *et al.*, 2002; Sollid and Khosla, 2005). The major advantage of using this protease in the detoxification of gluten, is that the production from fungi is simple and inexpensive, but requires advanced purification at high costs to eliminate toxins before being used in vivo.

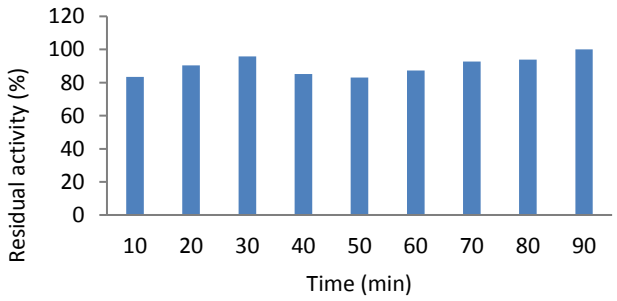

Figure 4. Thermostability at 50 °C

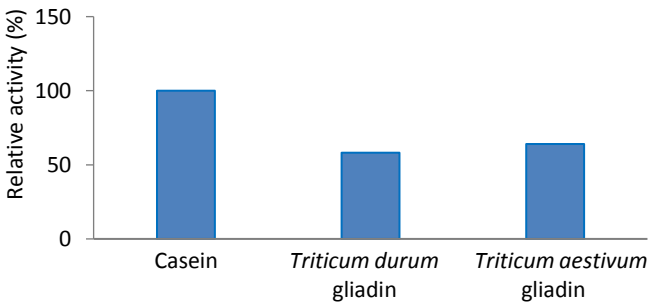

Figure 5. Proteolytic activity with different substrates (casein and gliadin)

Conclusion

This study showed the possibility of using proteolytic enzymes in the treatment of celiac disease and serves as starting point to clinical studies, since we have demonstrated that the protease of an *Aspergillus niger* isolate were effective in conditions similar to those found in the gastrointestinal tract and showed some ability to degrade gluten. In vivo the efficiency of this protease on gluten detoxification should be demonstrated in clinical studies involving celiac patients.

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