Significant Hydrolysis of Wheat Gliadin by *Bacillus tequilensis* (10bT/HQ223107): a Pilot Study

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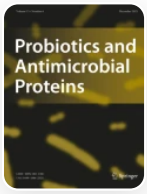

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Abstract

Peptidase therapy is suggested to be effective to minimize gliadin toxicity in celiac disease (CD). Hence, present study deals with gliadin-hydrolysing peptidases. The efficient peptidase from the *Bacillus tequilensis* was purified using ammonium sulfate fractionation and preparative electrophoresis. Analysis of in-solution and in-gel hydrolysis of gliadin using one and two-dimensional SDS-PAGE revealed nearly complete hydrolysis of gliadin peptides after 180 min of incubation with *B. tequilensis* protease. Purified peptidase was

found to be stable at acidic (pH 3.5) to neutral (pH 7.2) pH range. The molecular mass and isoelectric point of the peptidase were observed around 29 kDa and 5.2, respectively. The internal protein sequence obtained through mass spectrometric analysis suggested that peptidase might belong to peptidase S9 family known for prolyl-specific peptidases. This study recommends the possible applicability of this peptidase for elimination of immunotoxic gliadin peptides and may prove useful in CD treatment.

Introduction

Celiac disease (CD) is a small intestinal autoimmune disorder [1]. It is highly common (1:100) in most parts of the world [2]. CD is caused by storage proteins (gluten) from cereal grains in genetically predisposed persons [3]. Gliadin (α/β-, γ-, and ω-gliadins) is the main toxic component of gluten [4]. High content of proline and glutamine makes gliadin highly resistant to proteolysis [5]. Upon ingestion, incomplete hydrolysis of gluten results into the persistence of long gliadin peptide (33 mer fragment) in the gut epithelium, where this peptide gets deamidated by tissue transglutaminase (tTG) and becomes immunoreactive. Modulated peptides stimulate immune response of CD4+ T lymphocytes against tTG, which leads to the release of cytokines in the intestinal mucosa

causing villous atrophy [6, 7].The clinical symptoms of CD are abdominal pain, anemia, diarrhea, osteoporosis, and death if untreated [8, 9]. Furthermore, coexistence of CD with mental disorders such as schizophrenia has also been reported [10].

Currently, only treatment available for CD is strict elimination of gluten from the diet, which is difficult due to ubiquitous nature of gluten and that puts socioeconomic pressure on the patient [11,12,13,14]. One of the most potential therapeutic approaches for CD is to eliminate immunotoxic peptide domains through effective proteolysis [15,16,17,18]. Several gluten detoxifying peptidases (prolyl endopeptidases) have been isolated from probiotic preparations involving lactobacilli [19, 20], other microorganisms [18, 21], and germinating cereals [22]. However, their practical applications are limited due to slow reaction rate, high production costs, and irreversible inactivation by pepsin and acidic pH; in addition to that, these enzymes are released into cytoplasmic membrane, i.e., have intracellular expression and pH optima in between 7 and 8, ineffective at acidic pH found in stomach [3, 21, 23,24,25]. So, it necessitates search for the extracellular acid stable prolyl-hydrolysing enzymes.

An attempt was made in this study to purify and characterize an extracellular gliadin-hydrolysing peptidase from selectively isolated *Bacillus tequilensis* strain and to assess its stability and efficiency for possible application in celiac therapy.

Materials and Methods

Isolation and Selective Enrichment of Gluten-Utilizing Bacteria

The isolation of gluten-hydrolysing bacteria from soil sample was carried out using Luria Bertani (LB) medium (pH 6.9). For the enrichment, a selective isolation medium was used where tryptone was replaced with gluten (Sigma) 10.0 g l^{-1} as a source of nitrogen. The isolates obtained were further maintained on LB media supplemented with 1% gluten.

16S rDNA Molecular Identification

The molecular identification of bacterial isolate with the highest gluten utilizing activity was carried out using 16S rDNA gene sequencing. The genomic DNA was extracted from 48-h-old culture using Gene Elute Genomic DNA isolation kit (Sigma, USA), subjected to 16S rDNA amplification using a combination of universal primers [26, 27]. The amplified DNA was sequenced and compared with National Centre for Biotechnology Information (NCBI) GenBank entries by using the BLAST algorithm.

Enzyme Production and Culture Conditions

The production of enzyme was performed at 37 °C, pH 7.2 in liquid glucose yeast peptone (GYP) medium (glucose 10 g/l, yeast extract 10 g/l, peptone 10 g/l, and sodium chloride 5 g/l) supplemented with gluten (20 g/l). The inoculum was prepared by adding a loop full of cells from 24-h pure active slant culture into 25 ml of autoclaved medium in a 100-ml Erlenmeyer flask. The culture flask was incubated at room temperature in shaking condition at 180 rpm for 24 h (approx. 25–30 °C). Ten percent of inoculum from the above seed culture was added to 100 ml of the production medium in 250-ml Erlenmeyer flasks and incubated in an orbital shaker at room temperature at 180 rpm for 48 h (approx. 25–30 °C). The culture broth was subjected to centrifugation at 5000×*g* at 4 °C and supernatant was obtained as a crude source of enzyme.

Purification of Gliadin-Hydrolysing Enzyme

The crude supernatant was subjected to partial purification using ammonium sulfate precipitation. The 0–40, 40–80, and 80–100% fractionation was performed by addition of ammonium sulfate to the clear supernatant with constant stirring and it was incubated overnight at 4 °C. The precipitate was collected through centrifugation at 5000×*g* for 20 min at 4 °C. The collected precipitate was dissolved in 1 ml 50 mM Tris-HCl (pH 7.2). The desalting was performed using centrifugal membrane filter devices (Amicon Ultracell®-10K native molecular weight cutoff limit (NMWL) 10 kDa, Millipore). The fraction with high peptidase activity was further subjected to preparative electrophoresis using Laemmli discontinuous buffer system. About 3 mg of total protein in 2 ml of sample buffer was resolved onto 7 cm, 7% (T) preparative gel onto Prep-Cell system (Bio-Rad, USA). The continuous elution (1.5 ml/fraction) of resolved proteins was performed at a flow rate of 0.5 ml/min using Bio-Rad's Econo System and Bio-Rad's Fraction collector. Effective fractions were pooled together and concentrated using Amicon Ultracell®-10K membrane filtration. Determination of total protein content was performed using bovine serum albumin (SRL, India) as a standard [28].

Analysis of In-Solution Hydrolysis of Gliadin Using One-Dimensional and Two-Dimensional Gel Electrophoresis

For studying gliadin-hydrolysing activity, purified peptidase was incubated with 500 μl of reaction mixture containing 250 μg of gliadin from wheat (Sigma-Aldrich, USA). 5 mg/ml gliadin was prepared in 60% ethanol as previously described [29]. The pH of reaction was maintained using 50 mM Tris-HCl buffer (pH 7.2). The incubation was carried out in dry

bath at 37 °C with defined time interval of 30 min (0 to 180 min). Twenty microliters of reaction mixture for each time interval was resolved onto 10% one-dimensional (1D) SDS polyacrylamide gel electrophoresis (PAGE) and resulting gliadin hydrolysis was analyzed. Similarly, gliadin hydrolysis efficiency of purified enzyme was also investigated by varying pH of reaction mixture using buffers: 50 mM sodium citrate (pH 3.5) and 50 mM Tris-HCl (pH 7.2) with 180 min of incubation at 37 °C.

Two-dimensional electrophoretic analyses (2DE) of gliadin hydrolysis were performed using 7-cm Ready IPG strip (pH range 3–10; Bio-Rad, USA). The overnight rehydration of strip was carried out in a rehydration buffer containing 10 μl of reaction mixture: urea (5 M), thiourea (2 M), CHAPS (3-[(3-Cholamidopropyl) dimethyl ammonio]-1- propane sulfonate hydrate) (2% *w*/*v*), Bio-Lyte IPG buffer (0.2% *v*/*v*; Bio-Rad, USA), and trace amount of bromophenol blue. Isoelectric focusing of rehydrated strip was performed at 20 °C on Bio-Rad's Protean® IEF system as per the instructions manual (Catalog # 163-2099). It was followed by 30 min of equilibration under gentle shaking conditions in equilibration buffer containing glycerol (30%*w*/*v*), (2%*w*/*v*) SDS, Tris-HCl (50 mM, pH 8.8) as previously described [30]. The equilibrated strip was resolved onto 12% SDS-PAGE for second dimension and stained with silver staining. The isoelectric point (*pI*) of the purified peptidase was determined by comparing with standard molecular weight markers (Bio-Rad, USA).

In-Solution Gliadin-Hydrolysing Efficacy of Purified Enzyme, Trypsin, and Chymotrypsin

Experiment was designed as explained in above section, where gliadin was separately incubated with trypsin, chymotrypsin, and purified enzyme at 37 °C for 180 min and was loaded on 14% SDS-PAGE. After that, electrophoresis gel was processed as mentioned above.

In-Gel Analysis of Gliadinase Activity (Gliadin Zymography)

Gliadin-hydrolysing activity of the protein sample was analyzed on non-denaturing 10% SDS-PAGE [29]. Gel was prepared by incorporating 2 mg/ml of gliadin. Sample was prepared in non-reducing sample buffer. After electrophoresis, gel was renatured with 2.5% Triton X-100 (SRL, India) for 30 min followed by rinsing of distilled water. Gel was then subjected to overnight incubation in 50 mM Tris-HCl buffer (pH 7.2) and stained with Coomassie brilliant blue R-250. Similar process was carried out for zymogram

development in case of 2D electrophoresed gel also. The 2D electrophoresis was carried out as per the process mentioned by Gadge et al. [30].

Mass Spectrometry

The SDS-PAGE protein band of Prep-Cell purified protein was excised manually. The excised band was subjected to overnight in-gel digestion at 37 °C using sequencing grade trypsin. The mass spectrometry of resultant tryptic digest was carried out at Proteomics Facility of Molecular Biophysics Unit, IISC (Bangalore, India) using UltrafleXtreme MALDI-TOF/MS system (Bruker Daltonics, Germany). Comparison of obtained mass spectrum with the existing entries in the available databases was carried out using online Mascot software version 2.4.1 (http://www.matrixscience.com).

Results

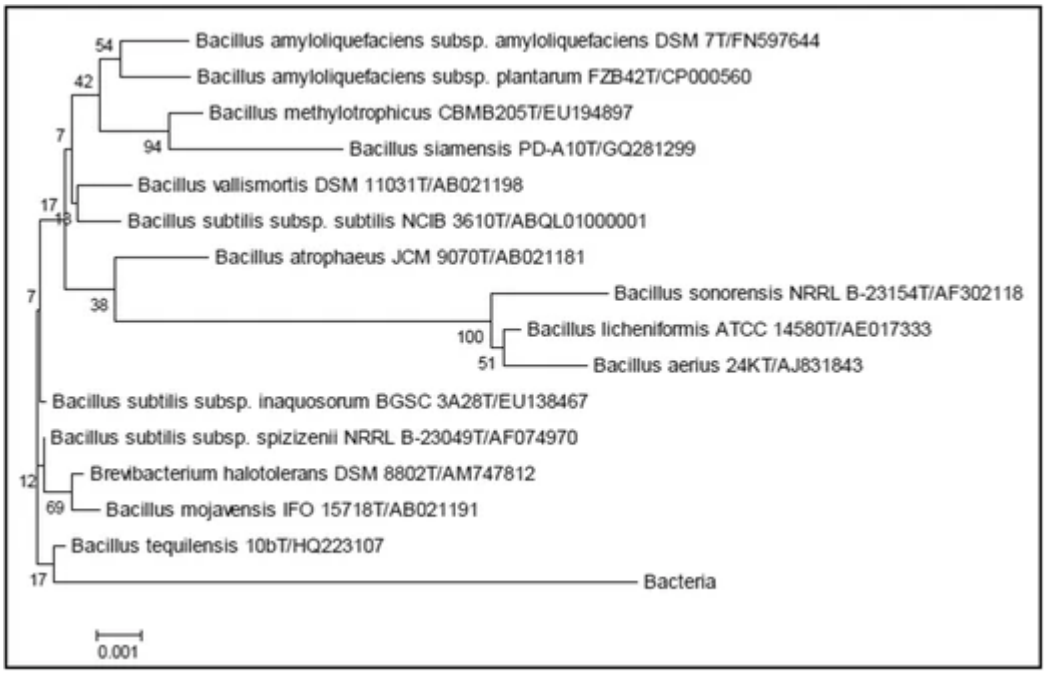
Enrichment and Selective Isolation of Gluten-Utilizing Bacteria

A total of 14 bacterial colonies were obtained from the enrichment using selective isolation procedure. Gluten-hydrolysing ability was observed as a zone of clearance around the colony. After repetitive experimentation, isolate showing the most potent gluten-hydrolysing ability was subjected to fermentation and molecular identification.

16S rDNA Molecular Identification

The bacterial isolate with the highest gluten-hydrolysing ability was identified using 16S rDNA gene sequencing. Sequence analysis indicated that isolated bacteria is homologous at 98% with *Bacillus tequilensis* 10b (T) 16S ribosomal RNA gene, partial sequence (HQ223107). A phylogenetic tree has been constructed using neighbor-joining method [31] shown in Fig. 1.

Fig. 1



Phylogenic tree analysis of *Bacillus tequilensis* based on 16 rDNA sequences

Production and Purification of Gliadin-Degrading Enzyme

The crude enzyme was obtained after incubation of bacteria in media at shaking conditions at 180 rpm at 37 °C. The maximum enzyme production was observed after 48 h of fermentation. The enzyme in the crude extract was separated by ammonium sulfate fractionation. The maximum gliadin-hydrolysing activity was observed in the 40–80% fraction after zymographic analysis. Partially purified enzyme was then subjected to preparative electrophoresis. The successful purification of enzyme to homogeneity was obtained through preparative electrophoresis (data not shown). Approximate molecular weight of the native purified peptidase was observed to be 29 kDa (data not shown). In-gel activity results were also compared with the molecular markers and molecular weight was found to be the same as above.

Analysis of In-Solution Hydrolysis of Gliadin Using 1D and 2D SDS-PAGE

The incubation of gliadin with the purified enzyme obtained from *B. tequilensis* was carried out at different time intervals at 37 °C. The resulting gliadin hydrolysis after time interval was examined by 1D and 2D SDS-PAGE (data not shown).

The in-solution gliadin-hydrolysing efficacy of these enzyme was compared on gel with trypsin and chymotrypsin, which revealed almost complete hydrolysis of gliadin by the

enzyme studied while only partial hydrolysis of gliadin was observed with trypsin and chymotrypsin (data not shown).

Linear increase in the gliadinase activity was observed with increased incubation time. The effective digestion of gliadin peptides was observed after 180 min of incubation as evidenced in electrophoretic analysis (data not shown). The hydrolysis of gliadin peptides in the range of 33–55 kDa was observed within 30 min as hydrolysis of gliadin peptides in the range of 20–29 kDa took almost 180 min (data not shown). These results were also evident in 2DE analysis performed after 180 min of incubation (data not shown), which revealed a complete disappearance of gliadin peptides in the range of 33–55 kDa along with significant reduction of the gliadin spots in the range of 20–29 kDa. The in-solution gliadin-hydrolysing efficiency of the purified enzyme was also compared at pH 3.5 and 7.2 after 180 min of incubation at 37 °C. Comparatively, more hydrolysis of gliadin peptides at acidic pH was observed in the SDS-PAGE analysis of the resultant reaction mixture (data not shown).

In-Gel Analysis of Gliadin-Hydrolysing Activity (Gliadin Zymography)

Gliadin zymography was performed to conclude in-gel hydrolysis of gliadin by *B. tequilensis* enzymes obtained after 48 h of fermentation. Zymogram revealed clear zone of gliadin hydrolysis after 12 h of incubation at 37 °C. The activity profile of gliadin-hydrolysing enzymes present in the culture supernatant was obtained through 1DE zymography (data not shown) which revealed the presence of gliadin-hydrolysing peptidases. Further, 2DE zymography performed for the Prep-Cell purified gliadinase isoform revealed that enzyme had *pI* 5.2 (data not shown). Collective electrophoretic analyses along with 2DE zymography showed monomeric nature of the enzyme (data not shown).

Mass Spectrometry

The tryptic digest of purified *B. tequilensis* gliadinase was analyzed by MALDI-TOF MS. Comparison of experimentally determined peptide masses with the existing entries in the available databases was carried out using MASCOT-PMF. Search revealed that 12 peptide masses matched to the peptidase S9 from *Geodermatophilaceae bacterium* URHA0031 having 29% of protein sequence coverage shown in Table 1.

Table 1 Similarity of experimentally determined peptide masses of gliadinase with the existing entries (peptidase S9 from *Geodermatophilaceae bacterium*URHA0031) in the available databases using MASCOT-PMF search

Discussion

The present study was aimed at isolating extracellular gliadin-hydrolysing peptidase from the bacteria. Gliadins are proline-rich immune toxic peptides present in gluten, responsible for uncontrolled immune response called as celiac disease [32].

We have isolated and characterized extracellular peptidase from a *Bacillus* strain selectively isolated from enrichment. *Bacillus* genus is known for its broader range of proteolytic capacity [33].The capability of several *Bacillus* species to produce extracellular gliadinases has also been demonstrated very recently [34]. The isolated strain was identified as *Bacillus tequilensis* 10b (T). The *Bacillus tequilensis* has been reported as a good candidate for probiotic application [35].The *B. tequilensis* is also suggested as a starter culture for the superior preparation of traditional Indian rice based fermented food [36]. *B. tequilensis strain* (10bT/HQ223107) identified in present study showed some features similar to probiotics such as acid tolerance, bile salt tolerance and antibiotic resistance (data not shown). But these tests are not sufficient to prove probiotic nature of the strain. The zymography analysis of partially purified active fraction revealed the presence of multiple peptidases capable of gliadin hydrolysis. Similar pattern of multiple peptidases capable of gliadin hydrolysis has also been shown in case of sourdough lactic acid bacteria [37]; this is necessary in a way to hydrolyze all the iminopeptide bonds present in the gliadin [38].

The molecular weight of the native purified gliadinase was observed around 29 kDa. The intracellular gliadin-hydrolysing peptidases are usually larger in size about 80 kDa [39]; whereas, extracellular gliadinases are shown to have broad molecular weight range, from 70 to 80 kDa to less than 20 kDa [34]. The acidic isoelectric point (pH 5.2) of the purified gliadinase is in correlation with the observation of Helmerhorst et al. [29] who have reported acidic nature of the most prominent gliadinases produced by dental plaque bacteria.

The evidences obtained in the in-solution and gliadin zymographic analyses suggest that the gliadin/gluten are suitable substrates for the purified extracellular enzyme. Usually, typical gliadin polypeptides have characteristic mass pattern between 33 and 55 kDa and characteristic isoelectric points at *pI* 6–9 [19, 40]. As evidenced in our results, purified gliadinase completely hydrolysed the gliadin polypeptides in the abovementioned range after 180 min of incubation. The studied enzyme showed good in vitro gliadinase activity, but in vivo enzyme has to work in presence of gut proteases like trypsin, chymotrypsin, and pepsin in the presence of bile salt. Hence, attempts were made to provide this environment in vitro. Our studied enzyme showed gliadinase activity even in presence of gut enzymes and bile salt (data not shown).

The identification of purified peptidase using mass spectrometric approach revealed that peptidase may belong to peptidase S9 family. However, further primary sequence determination is necessary which may reveal more features of this enzyme. This family consists of peptidases which cut only peptide substrates [41]. Along with S9, extracellular peptidase of *Aspergillus niger* belonging to S28 family of serine peptidase is also shown to have prolyl-specific activity [42]. As *B. tequilensis* produces a pattern of extracellular gliadinases, further characterization and identification of these enzymes may prove more useful.

B. tequilensis and its extracellular gliadinases may prove useful in food processing for elimination of toxic peptides to reduce risk and improve healthy living of CD patients. For this purpose, further in vivo studies and structure elucidation is necessary.

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Ethics declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This article does not contain any studies on human or animals performed by any of the authors.

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