

# Solubilization of gliadins for use as a source of nitrogen in the selection of bacteria with gliadinase activity

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## **Abstract**

For patients with celiac disease, gliadin detoxification via the use of gliadinases may provide an alternative to a gluten-free diet. A culture medium in which gliadins were the sole source of nitrogen was developed for screening for microorganisms with gliadinase activity. The problem of gliadin insolubility was solved by mild acid treatment, which renders an acid-hydrolysed gliadin/peptide mixture (AHG). This medium provided a sensitive and reliable means of detecting proteases compared to the classical spectrophotometric method involving azocasein. When a sample of fermented wheat (a source of bacteria) was plated on an AHG-based culture medium, strains with gliadinase activity were isolated. These strains' gliadinase profiles were determined using an AHG-based substrate in zymographic analyses.

**Keywords:** gluten, gliadin solubility, gliadinase, culture medium, zymography

Chemical compounds studied in this article

Gliadin (PubChem CID: 17787981); Diethyl ethoxymethylenemalonate (PubChem CID: 6871); Azocasein (PubChem CID: 39859); Cycloheximide (PubChem CID: 6197)

## 1. Introduction

Gliadins from gluten belong to a family of proteins whose members are insoluble in water. Besides, their high content in glutamine (35%) and proline (15%) favours the resistance of gliadins to complete gastrointestinal proteolysis; rather, the products of this reaction are peptides some 20-40 amino acids long that cannot be further broken down (Shan et al., 2005). Some of these peptides bear multiple epitopes that cause immunotoxic responses in the intestine of patients with celiac disease (Schuppan, Junker & Barisani, 2009). Gliadin detoxification by gliadinases from different microbial sources offers a potential alternative to a gluten-free diet for such patients. Some poly-specific enzymes are currently being tested as oral supplements to reduce gluten intake in celiac disease patients (Stepniak et al., 2006; Gass, Bethune, Siegel, Spence & Khosla, 2007). Problems with the stability of the enzymes in an acidic gastric environment and efficient mixing with gluten are frequently associated with oral administration. Other approaches may consider the use of gliadinases during food processing to eliminate the gluten toxicity before consumption. This strategy has been adopted through the selection of lactic acid bacteria alone (Gerez, Dallagnol, Rollan & Font de Valdez, 2012) or through the cooperative action of lactobacilli and fungal proteases during sourdough fermentation (Rizzi et al., 2007; De Angelis et al., 2010). However, only a handful of gliadinases have been described so far while more than fifty gliadins exist that account for tens of immunogenic peptides (Camarca, del Mastro & Gianfrani, 2011). It is then necessary boosting the finding of novel gliadinases in order to open new perspectives towards the elimination of gluten toxicity during food processing.

Selective culture media afford a reliable means of isolating bacteria with a targetable metabolic trait. A culture medium in which gliadin is the only nitrogen source could be used to

screen for microorganisms with gliadinase activity. However, the insolubility of gliadins is a major limitation to their use in this respect. Much of their insolubility and cohesiveness arises from hydrogen bonding involving amide groups on the numerous glutamine residues (Vickery, 1923; Holme & Briggs, 1959); hydrogen bonding between gliadin polymers greatly increases the stability of the overall matrix. Boiling of gliadins under acid treatment leads to the deamidation of glutamine (with the release of ammonia), as well as peptide bond hydrolysis (Vickery, 1922; 1923; Holme & Briggs, 1959). This eventually leads to the protein's complete hydrolysis. However, the products of this reaction cannot be used to detect gliadinase activity since no gliadin remains. The aim of the present study was to solve the problem of gliadin insolubility while retaining a substantial proportion of the gliadin matrix intact, thus allowing it to be used as a sole nitrogen source in a selective culture medium. Mild acidification of gliadin without heating produced a more soluble gliadin/peptide mixture with a small proportion of peptides and no free amino acids. Screening of a fermented wheat sample showed that a culture medium incorporating this mixture provides a simple, sensitive and selective means of isolating bacteria with gliadinase activity. The gliadinase profiles of isolated *Bacillus* strains and those of bacterial strains from collections/other sources were determined using this gliadin/peptide mixture as an enzyme substrate in zymography.

## **2. Materials and Methods**

### *2.1. Materials and reagents*

Gliadin, diethyl ethoxymethylenemalonate (DEEMM), bicine, tricine, azocasein, trichloroacetic acid (TCA), cycloheximide, Triton-X-100, sodium acetate, NaCl, FeCl<sub>3</sub> and vitamins (p-aminobenzoic acid, inosine, orotic acid, pyridoxamine-HCl, thymidine, D-biotin, 6,8-

thioctic acid, pyridoxine-HCl, folic acid, nicotinic acid, Ca-(D+) pantothenate, riboflavin, thiamine-HCl, and vitamin B12) were purchased from Sigma-Aldrich (Madrid, Spain). Ethanol, methanol, Tris (hydroxymethyl aminomethane), hydrochloric acid, acetic acid, Ringer tablets,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgCl}_2$  and  $\text{CaCl}_2$  were supplied by Merck (Darmstadt, Germany). Sodium Hydroxide (NaOH),  $\text{Na}_2\text{HPO}_4$ ,  $\text{Na}_2\text{SO}_4$  were from VWR (Barcelona, Spain), acrylamide:bis-acrylamide 37.5:1 solution (40%), ammonium persulphate and Coomassie brilliant blue R-250 were from Biorad (Hercules, CA, USA), and D-glucose, ammonium sulphate and sodium dodecyl sulphate (SDS) from USB (Cleveland, OH, USA). M17, de Man Rogosa and Sharpe (MRS) and Brain Heart Infusion (BHI) culture media, as well as tryptone and yeast extract, were from Oxoid (Hampshire, UK), while Plate Count Agar medium (PCA) was purchased from Scharlau (Barcelona, Spain). Pronase from *Streptomyces griseus* was supplied by Roche Diagnostics (Mannheim, Germany). All solutions were prepared with Milli-Q water (Millipore, Bedford, MA, USA).

## 2.2. Preparation of an acid-hydrolysed gliadin solution (AHG).

Gliadins were suspended at a concentration of 100 mg/mL in 3 mL of 2.5 N HCl (pH 2.0) and incubated for 1 hour at room temperature with occasional shaking. After adjusting to pH 6.5 with 2 N NaOH, the reaction products were dissolved to 15 mg/mL in 60% ethanol, and incubated at 37°C under agitation (1 hour, 250 rpm). The resulting whitish gliadin/peptide solution was termed acid-hydrolysed gliadin (AHG). A control suspension in which gliadins were not acid-treated before ethanol solubilization was also prepared.

### 2.2.1. Preparation of an acid-hydrolysed gliadin-containing culture medium (AHG-M)

An AHG-based culture medium (AHG-M; final volume 500 mL; pH 6.5) was made by adding the AHG solution (20 mL) described above to a freshly autoclaved, chemically defined medium composed of a salts solution ( $\text{Na}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ , sodium acetate,  $\text{NaCl}$ ,  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{Na}_2\text{SO}_4$  and  $\text{FeCl}_3$ ) plus glucose as described in Miladinov et al., 2001. After autoclaving, we added a growth factors' cocktail containing vitamins (pyridoxamine-HCl at 0.5 g/L, D-biotin at 0.25 g/L, pyridoxine-HCl at 0.2 g/L and folic acid, nicotinic acid, Ca-(D+) pantothenate, riboflavin, thiamine-HCl and vitamin B12 at 0.1 g/L each), nucleosides (inosine and thymidine, at 0.5 g/L each) and enzymatic cofactors (p-aminobenzoic acid at 1 g/L, orotic acid at 0.5 g/L and 6,8-thioctic acid at 0.25 g/L). The final gliadin concentration of the AHG-M was 0.6 mg/mL (i.e., 1200 ppm gluten equivalent, 60 times higher than the 20 ppm limit established for the content and labelling of "gluten-free" foodstuffs, according to international legislation [Commission Regulation (EC) N°41, 2009]).

#### *2.2.2. Preparation of a gliadin substrate for zymography (AHG-salts solution)*

A 1:4 mixture (v/v) of AHG-salts solution was prepared and used instead of water in a 10% SDS-polyacrylamide electrophoresis gel prepared as described in Gallagher (2007).

### *2.3. Protein analysis*

#### *2.3.1. Gliadin fractionation*

The AHG solution and control gliadin suspensions were prepared as described above. Both were then centrifuged at 3000 g for 10 min and 1 mL aliquots filtered through a 5000 Da cut-off polyethersulphone membrane (Sartorius Stedim Biothec GmbH, Goettingen, Germany). The eluent was termed the F5 fraction.

### 2.3.2. Determination of protein in AHG and control solutions, and in their derived F5 fractions

An AHG solution and a control gliadin suspension were prepared as above, and after centrifugation at 3000 g for 10 min the protein content in the supernatants was quantified by bicinchoninic acid assay (BCA; Pierce, Rockford, USA) using albumin as a calibration standard. The protein content in the F5 fractions was also determined in this way.

### 2.3.3. Electrophoresis of the AHG and control F5 fractions

The peptides in the AHG and control F5 fractions were separated and visualized by 16.5% Tris-tricine polyacrylamide electrophoresis gel using low range molecular weight markers as standards (Fermentas, Vilnius, Lithuania). The electrophoresis protocol followed was that of [Gallagher \(2007\)](#).

### 2.3.4. Reverse phase HPLC analysis of the AHG and control F5 fractions

Fifty microlitre aliquots of the AHG and control F5 fraction were subjected to reverse phase HPLC according to Alvarez-Sieiro et al. (2014), without modifications. Briefly, peptides were separated in a X Terra MS C18 5 Å, 4.6 x 150 mm column thermostated at 30°C within an Alliance 2795 chromatographic system (Waters, Milford, MA, USA). Peptides were detected by UV absorbance at 215 nm and 280 nm (PDA photodiode detector 2996 Waters) and quantified using Empower software (Waters).

### 2.3.5. Determination of ammonium and free amino acids in the AHG and control F5 fractions

Ammonium ions and free amino acids in 100  $\mu\text{L}$  aliquots of the AHG and control F5 fractions were derivatized with DEEMM, filtered through a 0.2  $\mu\text{m}$  nylon membrane (VWR), and separated and quantified by ultra-HPLC according to Redruello et al. (2013), without modifications. Briefly, an H-Class Acquity UPLC<sup>TM</sup> system (Waters) coupled to a PDA detector at 280 nm was used to separate the derivatized molecules. Separation was performed at 35°C in a Waters Acquity UPLC<sup>TM</sup> BEH C18 1.7  $\mu\text{m}$  column (2.1 mm x 100 mm). Data were acquired and analysed using Empower 2 software (Waters).

#### *2.4. Evaluation of AHG as an enzyme substrate*

Pronase is a general protease able to degrade gliadin (Bietz and Rothfus, 1971). Two-fold dilutions of pronase in water, ranging from 0 to 50  $\mu\text{g/mL}$ , were prepared. Thirty microlitres of each pronase dilution were added in triplicate to AHG-M plates in agar well diffusion tests, and incubated at 37°C for 19 hours. The radius of the halo around each well was measured and plotted against the dilution. The precision of the method was tested via intra-day (repeatability) as well as inter-day assays (reproducibility) with replicates of the same pronase concentration. Intra-day assays (same analyst, same agar plate batch and reagents) were performed by plating 12.5  $\mu\text{g/mL}$  of pronase 12 times on the same day; inter-day assays (same analyst but different agar plate batches) were performed with the same sample over six days. The quantification limit for the assay was taken to be the minimal pronase concentration that produced a halo around the wells that was distinguishable from a negative control (water instead of pronase).

The digestion of the AHG by pronase on the AHG-M plates, and of azocasein in a reference assay, was compared. The azocasein assay was performed following the protocol described by Secades and Guijarro (1999). The correlation coefficient between the azocasein-derived



absorbance results at 420 nm and the halo radius (in cm) in the AHG-M agar well diffusion test was calculated.

### *2.5 Preparation and screening of a fermented wheat sample for gliadin-positive bacteria*

Fifty grams of milled wheat grains were mixed with 50 mL of Ringer's solution and digested mechanically in a stomacher. The mixture was then left to ferment at room temperature in a tightly closed plastic bag for nine months. Ten-fold dilutions of the resultant material were plated on PCA medium containing 50 µg/mL of cycloheximide (an inhibitor of yeasts and moulds) and grown aerobically for 48 hours at 30°C to monitor the total bacterial growth. To detect bacteria capable of growing on AHG-M, an enrichment procedure was first followed. The fermented wheat material was first inoculated at 1% (v/v) into AHG-M broth containing cycloheximide and incubated at 30°C for 48 hours. A second round of enrichment in AHG-M broth was then performed. Both enriched cultures were then plated onto AHG-M plates and incubated for 48 hours at 30°C. The colonies that grew were collected for further analysis.

### *2.6. Molecular identification of bacteria growing on AHG-M*

Isolated bacteria were identified at the species level by genomic DNA extraction (Ruiz-Barba, Maldonado & Jiménez-Díaz, 2005) followed by partial amplification and sequencing of the *gyrA* gene (Roberts, Nakamura & Cohan, 1994). These sequences were then compared against those for the *gyrA* gene in public databases using the BLAST algorithm (Altschul, Gish, Miller, Myers & Lipman, 1990). Isolated bacteria were typified by genomic DNA macrorestriction and subsequent analysis by pulsed-field gel electrophoresis (PFGE) according to

Herrero-Fresno et al. (2012), but using *Sma*I endonuclease (Fermentas) to digest the agarose-embedded genomic DNA.

### 2.7 Gliadinase activity of culture collection strains

The gliadinase activity of other bacteria was also examined. The strains investigated were *Enterococcus faecalis* CECT 481, *Enterococcus faecium* CECT 410, *Enterococcus durans* CECT 411, *Bifidobacterium bifidum* CECT 4549, *Bacillus subtilis* CECT 461, *Escherichia coli* CECT 423, *Enterobacter cloacae* CECT 194, *Citrobacter freundii* CECT 401 and *Lactobacillus brevis* CECT 5354 from the Spanish Type Culture Collection (CECT, Valencia, Spain), *Bacillus amyloliquefaciens* DSM7 from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and *Lactobacillus casei* BL23 (Mazé et al., 2010), *Lactobacillus plantarum* WCFS1 (Siezen et al., 2012), *Lactococcus lactis* subsp. *lactis* IL1403 (Bolotin et al., 2001), *Lactococcus lactis* subsp. *cremoris* MG1363 (Wegmann et al., 2007) and *Streptococcus mitis* B6 (Denapaite et al., 2010).

Lactobacilli were grown in MRS broth at 37°C while lactococci and enterococci were grown in M17 broth plus 1% glucose at 30°C. *S. mitis* was grown in BHI broth at 37°C. The enterobacteria (*E. coli*, *E. cloacae* and *C. freundii*) and bacilli were grown in Luria-Bertani medium (LB; 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl, pH 7.0) at 37°C with vigorous shaking (except for *C. freundii* which involved no shaking). All the strains were propagated under aerobic conditions except for *B. bifidum* which was grown in MRS broth supplemented with 0.25% (w/v) L-cysteine at 37°C in an anaerobic chamber (Whitley MG500 anaerobic workstation; DW Scientific, Shipley, UK) with a 10% CO<sub>2</sub>, 10% H<sub>2</sub>, and 80% N<sub>2</sub> atmosphere.

Overnight cultures of each strain were plated on AHG-M and incubated for 48 hours to test for their gliadinase activity. Cell viability was checked by parallel culturing on an optimal solid growth medium for each bacterial strain.

### *2.8. Separation of gliadinase enzymes by AHG-based zymography*

Overnight cultures (100 mL) of bacteria showing gliadinase activity, and gliadinase-negative controls, were centrifuged, the pellets discarded, and the cell-free supernatants filtered through a 0.2 µm nylon membrane (VWR). Ammonium sulphate was then added until 60% saturation was reached in order to precipitate the extracellular proteins. The mixture was stirred at 4°C for 3 hours and then centrifuged at 10,000 g for 30 min at 4°C. The pellets were resuspended in 100 µL of Tris HCl pH 6.8, and the solution concentrated 10 times in the same buffer by filtering through a 3000 Da cut-off cellulose membrane (Amicon Ultra, Millipore, Cork, Ireland). It was then immediately loaded onto a 10% polyacrylamide SDS-gel in which water was substituted for an equivalent volume of AHG-salts solution. The final gliadin concentration in the activity gel was 0.5 mg/mL. After running, the gel was re-natured for 1 hour in Tris-HCl 50 mM with 2.5% Triton X-100 followed by two brief washes in the salts solution, and the gel incubated in this solution overnight at 37°C. Finally, the gel was stained in 40% methanol/10% acetic acid/0.1% Coomassie brilliant blue R-250, with brief counterstaining in the same solution without the dye.

## **3. Results and Discussion**

### *3.1. Gliadin solubilization*

The protein content in the supernatant of the centrifuged AHG samples was  $14.3 \pm 4\%$  higher than in the control gliadin suspensions. A similar increase was seen ( $18.5 \pm 0.1\%$ ) in the AHG F5

fraction compared to the control. Indeed, low molecular weight peptides appeared in the AHG F5 fraction, as determined by gel electrophoresis (Figure 1A, arrow). This novel material was also detected in the acidified sample when F5 fractions were subjected to HPLC analysis (Figure 1B). These results support the idea that the appearance of smaller polypeptide units is a consequence of the exposure of gliadins to the acid treatment, as reported for classic acid boiling treatments of gliadin (Vickery, 1922; 1923) and wheat gluten (Wu, Nakai & Powrie, 1976). Gliadin insolubility arises from the hydrogen bonding involving the amide groups of its glutamine residues. Mild acid treatment is known to induce deamidation; it therefore increases gliadin solubility by reducing such interactions (Vickery 1923; Holme and Briggs, 1959). In the present work, ammonia ( $127 \pm 0.01$  mM per 100 g of gliadin) was observed in the AHG F5 fraction (Figure 1C), indicating that deamidation had taken place. Thus, the increases in gliadin solubilization would seem to be the result of both deamidation and peptide bond hydrolysis. The absence of free amino acids in the AHG (data not shown) means any bacteria that can use it as a nitrogen source must have gliadinase activity.

### *3.2. Evaluation of AHG as an enzyme substrate*

A good linear correlation ( $R^2=0.8858$ ) was found between the pronase concentration and the halo radius around the agar wells. The quantification limit for this assay was  $6.25 \mu\text{g/mL}$  of pronase, equivalent to  $0.044 \text{ U/mL}$ . The intra-day repeatability and inter-day reproducibility assays returned coefficients of variation of 3.1% and 8.2% respectively, within the permitted range (7.3 - 11.3%) for analytical method precision tests (Taverniers, De Loose & van Bockstaele, 2004) and the concentration used ( $12.5 \mu\text{g/mL}$  of pronase). Comparative analysis of the pronase digestion of AHG and azocasein yielded a correlation coefficient of 0.8543 (Figure

2), supporting the idea that AHG provides a suitable substrate for screening for gliadinase-positive bacteria.

### 3.3 Screening of the fermented wheat sample for gliadinase-positive bacteria

Plating the fermented wheat sample on PCA returned a total bacterial title of 6.04 log cfu/g. After the first round of enrichment and the plating of the fermented wheat sample on AHG-M, bacterial counts were two orders of magnitude lower than after plating on PCA (4.81 vs 6.04 log cfu/g). The second round of enrichment led to a further reduction by two orders of magnitude (2.30 vs 4.81 log cfu/g). Further, after the first round of enrichment, 21% of the colonies growing on AHG-M (7 out of 32) showed a surrounding halo; this figure reached 60% after the second round (6 out of 10). Bacteria producing a halo on AHG-M have the ability to degrade gliadin.

Since halos were visible without the need for staining with traditional protein dyes, positive colonies could be isolated directly from the plates, maintaining cell viability. Thirteen isolates showed clear gliadinase activity. In molecular analysis these were identified as belonging to *Bacillus* sp. PFGE clustered them into five types (data not shown) indicating that different strains had been selected. To our knowledge, no previous reports exist about the isolation of gliadin-degrading *Bacillus* strains, although it is well known the proteolytic capacity of this genus over a broad range of substrates such as casein, collagen or fibrin (Horikoshi, 1999). The use of *Bacillus* strains in the food industry is scarce and controversial due to the ability of some strains to cause food poisoning. As pathogenicity is a strain-specific trait, protocols have been designed to evaluate the safety of *Bacillus* representatives and their possible use as food and feed additives (EFSA, 2012). This is the case of the Natto variety of *B. subtilis*, traditionally used during soybean fermentation in eastern countries or the *Bacillus* strains often used as starters in

fermented African foods (Thorsen et al., 2011). A further evaluation on the safety of our *Bacillus* strains is needed pre-empting their use as food additives.

#### 3.4 Gliadinase activity of the culture collection strains

Six of the 15 strains from the different collections/other sources (including the *B. subtilis* and *B. amyloliquefaciens* type strains) also grew on AHG-M, although only the two species of *Bacillus* degraded the gliadin (Figure 3). Another five strains showed a faint growth phenotype (indicated as +/- in Figure 3), and four failed to grow at all. These results confirm the possibility of using AHG-M as a selective medium for screening for gliadinase-positive bacteria. Only a few reports exist about the findings of bacteria with this activity. Some representatives of *Pediococcus* and *Lactobacillus* genera have been used to produce gluten-free foods (Rizzello et al., 2007; Gerez et al., 2012). And more recently, species from the genus *Rothia* have been isolated from oral human microbioma with demonstrated gliadinase activity (Helmerhorst, Zamakhchari, Schuppan & Oppenheim, 2010). Thus, our culture medium represents a cheap and quick tool to prospect microorganisms with this infrequent ability.

#### 3.5. Separation of gliadinase enzymes by AHG-based zymography

Nine bacterial strains were selected to separate the potentially different extracellular gliadinases responsible for halo formation. These strains included one representative of each *Bacillus* species PFGE type (from the wheat screening), the *B. subtilis* and *B. amyloliquefaciens* type strains, and *E. coli* and *L. lactis* (examples of non-growing, non-halo forming species) (Figure 3). Under zymography, clearing bands representative of gliadinase activity were recorded for all the *Bacillus* strains (Figure 4; asterisks), while none were recorded for *E. coli* or *L. lactis*

(neither of which grew on AHG-M). Clearing areas were also observed (vertical lines in Figure 4) indicating either the existence of various activities with similar molecular weights or a strong hydrolytic activity. Moreover, the different gliadinase-positive strains showed different profiles with a broad molecular weight range, from 75-80 kDa to less than 20 kDa. These results support the idea that AHG provides a reliable substrate for detecting different gliadinases. A further characterization of the gliadinases found in the present work should include their identification and purification, which may lead to the development of novel strategies of gluten processing and/or detoxification.

#### **4. Conclusion**

The results from the study shows that Gliadin solubilized by mild acid treatment could be used to provide the sole nitrogen source in chemically defined culture media designed to select bacteria with gliadinase activity. The AHG-based zymography could be used to separate the different gliadinase enzymes these bacteria produce.

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## Figures captions

**Figure 1.** Gliadin solubilization by mild acid treatment. (A) A silver-stained 16.5% tricine gel showing the small peptides produced by acid treatment (arrow). (B) HPLC analysis of the AHG F5 fraction and that of a control gliadin suspension: note the accumulation of more peptidic material in the AHG F5 fraction. (C) Ammonia levels increased in the AHG F5 fraction with respect to the control. The horizontal bar indicates the limit of quantification (LOQ) of the method for ammonia determination (3.91  $\mu\text{M}$ ).

**Figure 2.** Evaluation of AHG as a substrate for proteases. Correlation plot between pronase digestion of AHG (halo radius in cm) and azocasein (absorbance at 420 nm).

**Figure 3.** Behaviour of different bacterial strains on AHG-M. Individual colonies from the indicated bacterial strains were propagated in broth media and then plated on AHG-M and incubated for 48 h. The ability to grow and form a halo was then assessed. + positive phenotype; - negative phenotype; +/-, faintly positive phenotype.

**Figure 4.** AHG-based zymogram. Extracellular proteins were extracted from cultures of selected bacterial strains and subjected to zymography. Since the gel contained AHG as a substrate, hydrolysis was monitored as the appearance of clear bands against the background after Coomassie staining. Numbers indicate the strain used as the potential source of extracellular gliadinase: 1-5, isolated *Bacillus* sp. strains from the fermented wheat sample; 6, *Bacillus subtilis* type strain 461; 7, *Bacillus amyloliquefaciens* type strain DSM7; 8, *Escherichia coli* CECT 423; 9, *Lactococcus lactis* subsp. *lactis* IL1403. Asterisks located on the left of each lane mark

individual hydrolysis bands while vertical lines denote clearing areas where no individual bands can be clearly identified.

Figure 1

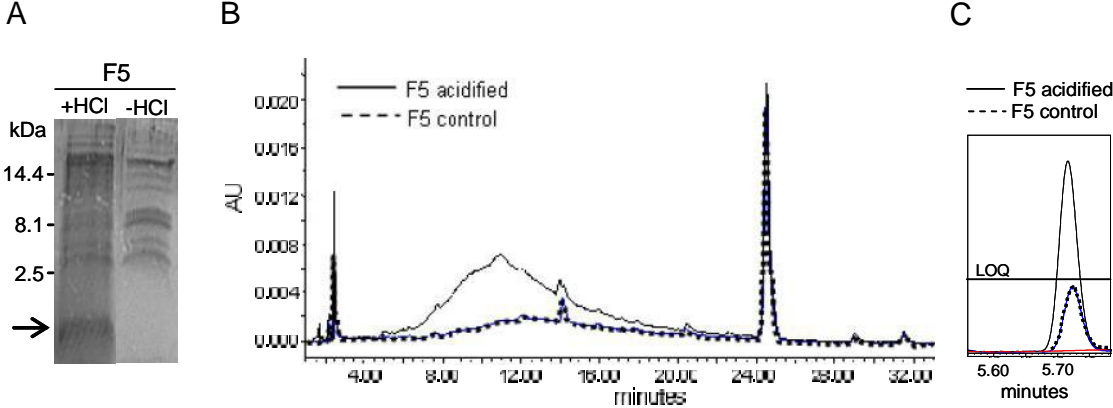


Figure 2

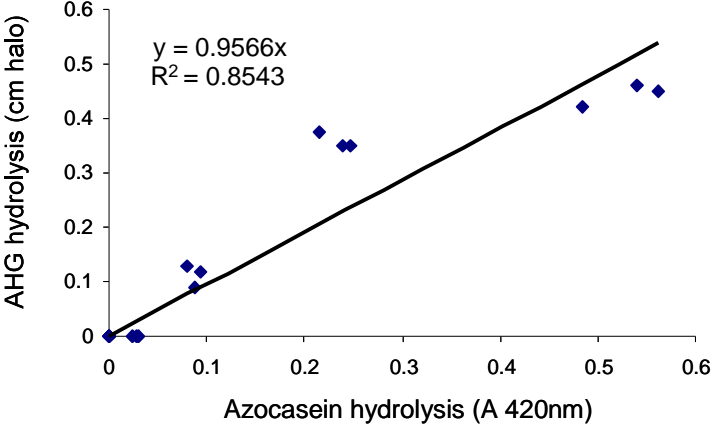


Figure 3

Strain name	Growth	Halo	Image
<b>Gram(+)</b>			
<i>Lactobacillus casei</i> BL23	+	-	
<i>Lactobacillus plantarum</i> WCFS1	+/-	-	
<i>Lactobacillus brevis</i> CECT 5354	+	-	
<i>Lactococcus lactis</i> sbs. <i>lactis</i> IL1403	-	-	
<i>Lactococcus lactis</i> sbs. <i>cremoris</i> MG1363	+/-	-	
<i>Enterococcus faecalis</i> CECT 481	-	-	
<i>Enterococcus faecium</i> CECT 410	+/-	-	
<i>Enterococcus durans</i> CECT 411	-	-	
<i>Streptococcus mitis</i> B6	+/-	-	
<i>Bifidobacterium bifidum</i> CECT 4549	+	-	
<i>Bacillus subtilis</i> CECT 461	+	+	
<i>Bacillus amyloliquefaciens</i> DSM7	+	+	
<b>Gram(-)</b>			
<i>Escherichia coli</i> CECT 423	-	-	
<i>Enterobacter cloacae</i> CECT 194	+	-	
<i>Citrobacter freundii</i> CECT 401	+/-	-	

	Growth No halo
	Growth No halo
	Growth No halo
	No growth No halo
	Faint growth No halo
	Faint growth No halo
	Faint growth No halo
	No growth No halo
	Faint growth No halo
	Growth Halo
	Growth Halo
	Growth Halo
	No growth No halo
	No growth No halo
	No growth No halo

Figure 4

