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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.

1. Introduction

Gliadins from gluten belong to a family of proteins whose members are insoluble in water. Besides this, 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 prolyl-specific enzymes are currently being tested as oral supplements to reduce gluten intake in celiac disease patients (Gass, Bethune, Siegel, Spencer, & Khosla, 2007; Stepniak et al., 2006). 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 use of selected lactic acid bacteria alone (Gerez, Dallagnol, Rollan, & Font de Valdez, 2012) or through the cooperative action of lactobacilli and fungal proteases during sourdough fermentations (De Angelis et al., 2010; Rizzello et al., 2007). However, only a handful of gliadinases have been described so far, while more than fifty gliadins exist that account for dozens of immunogenic peptides (Camarca, Del Mastro, & Gianfrani, 2012). It is, then, necessary to boost the finding of novel gliadinases in order to open new perspectives toward 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

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their insolubility and cohesiveness arises from hydrogen bonding involving amide groups on the numerous glutamine residues (Holme & Briggs, 1959; Vickery, 1923); 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, $FeCl_3$ and growth factors (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, KH_2PO_4, $MgCl_2$ and $CaCl_2$ were supplied by Merck (Darmstadt, Germany). Sodium Hydroxide (NaOH), Na_2HPO_4, Na_2SO_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 h 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 h, 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 solution of salts (Na_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, sodium acetate, NaCl, $MgCl_2$, $CaCl_2$, Na_2SO_4 and $FeCl_3$) plus glucose as described in Miladinov, Kuippers, and Topisirovic (2001). After autoclaving, 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) was added. 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) No. 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 polyethersulfone membrane (Sartorius Stedim Biotech 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 visualised 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 XTerra MS C18 5 μm, 4.6 × 150 mm column thermostatized 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 μl aliquots of the AHG and control F5 fractions were derivatized with DEEMM, filtered through a 0.2 μm nylon membrane (VWR), and separated and quantified by ultra-HPLC according to Redruello et al. (2013), without modifications. Briefly, an H-Class Acquity UPLC™ 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