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Use of fungal proteases and selected sourdough lactic acid bacteria for making wheat bread with an intermediate content of gluten

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ABSTRACT

This study was aimed at combining the highest degradation of gluten during wheat flour fermentation with good structural and sensory features of the related bread. As estimated by R5-ELISA, the degree of degradation of immune reactive gluten was ca. 28%. Two-dimensional electrophoresis and RP-FPLC analyses showed marked variations of the protein fractions compared to the untreated flour. The comparison was also extended to in vitro effect of the peptic/tryptic-digests towards K562 and T84 cells. The flour with the intermediate content of gluten (ICG) was used for bread making, and compared to whole gluten (WG) bread. The chemical, structural and sensory features of the ICG bread approached those of the bread made with WG flour. The protein digestibility of the ICG bread was higher than that from WG flour. Also the nutritional quality, as estimated by different indexes, was the highest for ICG bread.

1. Introduction

Wheat is the most widely grown crop with more than 25,000 different cultivars (Sapone et al., 2012). The large part of this global production is consumed after processing into bread, other baked goods, pasta and noodles, or, as in the case of Middle East and North Africa, into bulgur and couscous. The functional properties of the gluten proteins make also the wheat flour as an irreplaceable ingredient for other foods (Sapone et al., 2012).

Gluten is the abundant structural protein complex of wheat, with equivalent proteins found in other cereals (e.g., rye and barley). Although the wide spread of gluten containing grains initiated ca. 10,000 years ago, more recently wheat breeding was addressed to massive selection of cultivars with an unusual and elevated content of gluten. The daily human exposure to such elevated levels of gluten suggested the possibility that this evolutionary challenge also created conditions for related human diseases (Sapone et al., 2012). Wheat allergy (WA) and celiac disease (CD), which are mediated by adaptive immune systems, are the most known diseases related to gluten (Sapone et al., 2012). Under both these conditions, gluten reaction occurs via T-cell activation at the gastrointestinal mucosa level. Cross-linking between immunoglobulin (Ig)E and gluten epitopes is responsible for WA, and it triggers the release of chemical mediators (e.g., histamine) from basophils and mast cells. CD is an autoimmune disorder, which mainly involves the response of serum anti-tissue transglutaminase (tTG) and anti-endomysial antibodies (EMA). Other cases of reaction to gluten are commonly described as gluten sensitivity (GS), and they do not involve allergic or autoimmune mechanisms. Intestinal (e.g., diarrhea, abdominal discomfort or pain, bloating) or extra-intestinal (headache, lethargy, attention-deficit/hyperactivity disorder, ataxia or recurrent oral ulceration) symptoms are often manifested during GS (Di Sabatino and Corazza, 2012). Since the clinical symptoms are somewhat overlapping, the correlation between irritable bowel syndrome (IBS), CD and GS recently received a marked interest.

It is well established that GS symptoms decrease or disappear after gluten is withdrawn from the diet (Di Sabatino and Corazza, 2012). The information about the level of gluten that is responsible for the disease and about the mechanisms that cause

Abbreviations: BV, biological value; CD, celiac disease; CS, chemical score; DY, dough yield; EAA, essential amino acid; EAAI, essential amino acid index; EMA, anti-endomysial antibodies; FAA, free amino acids; FHWF, fully hydrolyzed wheat flour; GS, gluten sensitive; IBS, irritable bowel syndrome; ICG, intermediate content of gluten; IEF, isoelectric focusing; IPG, immobilized pH gradient; MAC, minimal agglutinating capacity; NI, nutritional index; OPA, o-phtaldialdehyde; PER, protein efficiency ratio; PT-, peptic/tryptic; TFA, trifluoroacetic acid; TPA, texture profile analysis; TTA, titratable acidity; tTG, anti-tissue transglutaminase; WA, wheat allergy; WG, whole gluten.

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digestive problems is scarce. Removal of the immunological trigger (gluten) is the basis for treatment of all diagnosed manifestations. Furthermore, the daily consumption of wheat products with an intermediate content of gluten (significantly lower than the current one) may have a delaying effect on the susceptibility to GS or even cause the absence of symptoms of GS. Indeed, approaches targeting the uptake of toxic gluten peptides through enzyme breakdown, sequestering gluten or restoring the epithelial barrier function were developed at the level of clinical trials. Thermal and enzyme treatments to get hypoallergenic or low-gluten wheat flour were proposed for making modified-gluten products, which are tolerated by susceptible individuals (Sapone et al., 2012).

During the last decade, sourdough lactic acid bacteria were used as sources of proteolytic enzymes to markedly decrease the concentration of gluten during bread or pasta processing (Rizzello et al., 2007). In particular, a pool of selected lactic acid bacteria and fungal proteases, which are routinely used in bakery, caused the complete degradation of gluten to less than 10 ppm during sourdough fermentation (Rizzello et al., 2007). Fungal proteases liberated various sized polypeptides (e.g., 4–40 amino acids) from native proteins, which were subsequently transported inside the lactic acid bacteria cells to be hydrolyzed (De Angelis et al., 2010). A large number of intracellular peptidases (e.g., PepN, PepO, PEP, PepX, PepT, PepV, PepQ and PepR) were responsible for the complete hydrolysis of the 33-mer or other synthetic immunogenic polypeptides to free amino acids (Di Cagno et al., 2010). Two independent clinical challenges (Di Cagno et al., 2010; Greco et al., 2011) were carried out by daily administration of wheat flour baked goods, which contained the equivalent of 8–10 g of native gluten, to celiac patients under remission. After 60 days of challenge, all celiac patients completely tolerated the baked goods made with hydrolyzed wheat flour. Obviously, the fully hydrolyzed wheat flour had technology behavior and properties like the naturally gluten-free flours (e.g., corn), and the use of structuring agents was needed for making baked goods (Rizzello et al., 2007).

This study was aimed at manufacturing bread with an intermediate content of gluten. A biotechnology protocol, which used selected sourdough lactic acid bacteria and fungal proteases (Rizzello et al., 2007), was set up to, at the same time, decrease the largest level of gluten and maintain the texture and sensory features of traditional breads. The chemical characteristics of the wheat flour, and the structural, nutritional and sensory properties of the resulting bread were described.

2. Materials and methods

2.1. Microorganisms and enzymes

Lactobacillus sanfranciscensis 7A, LS3, LS10, LS19, LS23, LS38 and LS47, *Lactobacillus alimentarius* 15M, *Lactobacillus brevis* 14G, and *Lactobacillus hilgardii* 51B were previously selected based on peptidase activities (Di Cagno et al., 2002), and used in this study. Strains were propagated for 24 h at 30 °C in modified MRS broth (Oxoid, Basingstoke, Hampshire, United Kingdom), with the addition of fresh yeast extract (5%, vol/vol) and 28 mM maltose, at the final pH of 5.6 (mMRS). When used for sourdough fermentation, cells of lactobacilli were cultivated until the late exponential phase of growth was reached (ca. 12 h).

Fungal proteases from *Aspergillus oryzae* (E1; 500,000 hemoglobin units on the tyrosine basis/g) and *Aspergillus niger* (E2; 3000 spectrophotometric acid protease units/g), which are routinely used as improvers in bakery industry, were purchased from BIOCAT Inc. (Troy, VA).

2.2. Sourdough fermentation

The main characteristics of the wheat (*Triticum aestivum* cv. Appulo) flour used were the following: moisture, 10.2%; protein, 10.3% of dry matter (d.m.); fat, 1.8% of d.m.; ash, 0.6% of. d.m.; and total carbohydrates, 76.5% of d.m. Wheat flour and tap water containing ca. 10^9 cfu/g (cell density in the dough) of each lactic acid bacterium were used for sourdough fermentation at 30 °C, under stirring conditions (ca. 200 rpm). Sourdough fermentations were carried out varying, one by one, the following parameters: dough yield (DY, dough weight × 100/flour weight), 500, 333 and 250; time of fermentation, 15, 24 and 48 h; and fungal proteases E1 and E2 (ratio 1:1), 0, 50, 100 and 200 ppm.

2.3. Bread making

After fermentation, sourdoughs were freeze-dried to remove water. After milling, the resulting flour was analyzed and used for bread making. Breads (dough yield of 160) were manufactured at the pilot plant of the Department of Soil, Plant and Food Sciences. A wheat flour bread, which contained non hydrolyzed wheat flour, was manufactured according to the protocol routinely used for typical Italian bread, and used as the control.

For bread making, 241 g of non hydrolyzed or fermented flour, 150 ml tap water and 2% (wt/wt) of baker's yeast (corresponding to the final cell density of ca. 10^7 cfu/g) were mixed at 60 × *g* for 5 min, with a IM 5–8 high-speed mixer (Mecnosud, Flumeri, Italy). Fermentation was at 30 °C for 1.5 h. Before baking, pH and total titratable acidity (TTA) were measured. TTA was determined after homogenization of 10 g of sample with 90 ml of distilled water, and expressed as the amount (ml) of 0.1 M NaOH needed to get the value of pH of 8.3. All breads were baked at 220 °C for 30 min (Combo 3, Zucchelli, Verona, Italy). Moisture was determined according to the standard AACC method (AACC, 2003). Fermentations were carried out in triplicate and each bread was analyzed twice.

2.4. Immunological analyses

The concentration of gluten of the freeze-dried flours was determined through immunological analyses using the R5 antibody-based sandwich ELISA. Together with electrophoretic and chromatographic analyses described below, R5 sandwich ELISA was carried out to define the gluten proteolysis degree. The analysis was carried out with the Transia plate detection kit, following the instructions of the manufacturer (Diffchamb, Västra, Frölunda, Sweden). The R5 monoclonal antibody and the horseradish peroxidase-conjugated R5 antibody were used.

2.5. Characterization of the wheat flour

Proteins were selectively extracted from wheat flour according to the method of Osborne (1907), further modified by Weiss et al. (1993). The concentration of proteins was determined by the Bradford method (Bradford, 1976).

Two-dimensional electrophoresis (2-DE) was carried out with the immobiline-polyacrylamide system as described by Di Cagno et al. (2002). Aliquots of 30 μg of proteins were used for the electrophoretic run. Isoelectric focusing (IEF) was carried out on immobiline strips, providing a non-linear pH gradient from 3.0 to 10.0 (IPG strips; Amersham Pharmacia Biotech, Uppsala, Sweden), for albumin/globulin and glutenin fractions, or a linear pH gradient 6–11, for gliadin fraction, by IPG-phore at 20 °C. The second dimension was carried out in a Laemmli system on 12% polyacrylamide gels (13 cm by 20 cm by 1.5 mm) at a constant current of 40 mA/gel and at 15 °C for approximately 5 h, until the dye front