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Journal of Cereal Science 43 (2006) 301–314

Journal of CEREAL SCIENCE

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Fermentation by selected sourdough lactic acid bacteria to decrease coeliac intolerance to rye flour

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Received 3 June 2005; received in revised form 24 November 2005; accepted 19 December 2005

Abstract

A pool of selected lactic acid bacteria was used to ferment suspensions of rye flour. Two-dimensional electrophoresis showed that 109 of the 129 ethanol-soluble rye polypeptides were hydrolysed almost totally by lactic acid bacteria. Matrix-assisted laser desorption ionization—time of flight mass spectrometry and reversed-phase high performance liquid chromatography analysis confirmed the hydrolysis of prolamins. After 48 h fermentation, no prolamin polypeptides were recognized by R5-Western analysis. HPLC analysis of glutelin polymers showed a very low bacterial proteolysis but a pH dependent hydrolysis probably due to activation of rye enzymes. Prolamins were extracted from rye flour and used to produce a peptic–tryptic (PT)-digest for in vitro tests with K 562 (S) sub-clone and Caco-2/TC7 cells of human origin. The PT-digest was also treated with lactic acid bacteria before assay. The Minimal Agglutinating Capacity increased ca. 8-times when K 562 (S) sub-clone cells were exposed to rye PT-digest treated with lactic acid bacteria. Hydrolysis of rye PT-digest by lactic acid bacteria decreased the toxicity of PT-digest itself towards Caco-2/TC7 cells as estimated by cell viability, caspase-3 activity and release of nitric oxide. Rye prolamins and glutelins were extracted from doughs and subjected to PT digestion. Compared to PT-digests from chemically acidified dough, coeliac jejunal biopsies exposed to the PT-digest from the dough fermented by lactic acid bacteria did not show an increase of the infiltration of $CD3^+$ intraepithelial lymphocytes. The same was found for epithelial cell Fas expression. Long-time fermentation of dough by selected lactic acid bacteria could be considered as a potential tool to decrease the risk of rye contamination of gluten-free products for coeliac patients.

Keywords: Prolamins; Lactic acid bacteria; Coeliac disease; Rye

1. Introduction

Coeliac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of wheat (*Triticum aestivum* and *T. durum*) gluten, and similar proteins of rye (*Secale cereale*) in genetically susceptible individuals. CD is associated with the human leukocyte antigen (HLA) molecules DQ2 (90–95%) and DQ8 (5–10%), and in the continued presence of gluten the disease is self-perpetuating (Fasano, 2005). Reports of CD date back to the first century AD, but it was not until 1888 that Samuel Gee gave the classical description of the disease (Gee, 1888), and it was only in 1930 that Dicke demonstrated that removal of wheat from diets alleviated symptoms and signs of CD (Van Berge-Henegouwen and Mulder, 1993). Currently, there is a growing interest in medical, nutritional, social and economic aspects of CD because the burden of this condition is undoubtedly higher than previously thought. Serological screening studies have shown the prevalence of CD in most European countries, South America, and the USA is 1 in 266 (Catassi et al., 1996; Not et al., 1998). Similar prevalence has been reported worldwide (Fasano and Catassi, 2001). CD is widespread in North Africa (Catassi et al., 1999), Iran (Shahbazkhani et al., 2000) and India (Sood et al., 2001).

Rye is the most important cereal crop after wheat, rice and maize. A substantial part of the rye crop is used for bread making, especially in Central, Northern and Eastern European countries where more than 3 million tonnes of rye per year are

Abbreviations: CD, coeliac disease; CFU, colony forming units; d.m., dry matter; HLA, human leukocyte antigen; LAB, Lactic acid bacteria; MALDI-TOF, matrix-assisted laser desorption ionization—time of flight; NO, nitric oxide; *p*NA, *p*-nitroanilide; PT, peptic–tryptic; RP-HPLC, reversed phase high performance liquid chromatography; 2DE, two-dimensional electrophoresis.

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0733-5210/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jcs.2005.12.008

used for bread making (FAOSTAT, 1998). During endoluminal proteolytic digestion, the prolamins of rye (secalins) as well as those of wheat α-, β-, γ- and ω-gliadin sub-groups, release a family of Pro- and Gln-rich polypeptides that are responsible for the auto-immunological response that underlies coeliac enteropathy (Silano and De Vincenzi, 1999).

CD is characterised by a variety of clinical presentations which include the typical malabsorption syndrome (classic symptoms) and a spectrum of symptoms potentially affecting any organ or body system (non-classic symptoms). Because CD is often atypical or even clinically silent, many cases go undiagnosed and are exposed to the risk of long-term complications (Fasano, 2005). At present, the only available treatment for CD is a strict exclusion diet. Foods disallowed in a gluten-free diet include: (i) breads, cereals or other foods made with wheat, rye, barley, triticale, dinkel and kamut flour or ingredients and by-products from those grains; (ii) processed foods that contain wheat, barley and rye and gluten-derivatives as thickeners and fillers, for example, hot dogs, salad dressings, canned soups/dried soup mixes, processed cheeses, cream sauces; and (iii) medications that use gluten or similar proteins as pill or tablet binders (Accomando and Cataldo, 2004). The Codex Standard adopted by the Codex Alimentarius Commission of the World Health Commission (WHO) and by the Food and Agricultural Organization (FAO) states that naturally gluten-free and wheat starch-based gluten-free products must contain no more than 20 and 200 ppm of gluten or similar proteins, respectively. Bona fide gluten-free products are not widely available and are usually more expensive than their gluten-containing counterparts. It is not surprising then that dietary compliance is, at best, imperfect for many patients, especially adolescent and adults. There is, therefore, an urgent need to develop safe and effective therapeutic alternatives which at least may protect patients from most forms of 'hidden gluten'.

Most studies relate to gluten from bread wheat but not rye flour (Fasano, 2005; Sollid and Khosla, 2005). In an earlier report a long-time (24 h at 37 °C) semi-liquid sourdough fermentation of wheat flour by selected lactic acid bacteria (LAB) was proposed (Di Cagno et al., 2002, 2004). After fermentation, wheat sourdough was mixed with tolerated flours (e.g. oat, millet and buckwheat) in a ratio 3:7, baker's yeast was added and the sourdough allowed to ferment for 2 h at 37 °C, and the breads baked at 220 °C for 20 min. The bread, containing ca. 2% of gluten, was tolerated by all coeliac patients tested, as shown by intestinal permeability challenge (Di Cagno et al., 2004). A similar protocol, with some technological modifications, was successful for the manufacture of Italian pasta from durum wheat (Di Cagno et al., 2005).

Here, we explore the capacity of selected sourdough LAB to hydrolyse rye flour prolamins, including polypeptides responsible for CD. Hydrolysis of prolamins was determined by complementary techniques such as two-dimensional electrophoresis (2DE), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, reversed-phase (RP)-high performance liquid chromatography (HPLC), and R5-Western analysis**.** In vitro assays on the K 562(S) sub-clone cells of human myelogenous leukaemia origin, human intestinal Caco-2/TC7 cells, infiltration of coeliac jejunal biopsies by $CD3^+$T lymphocytes and Fas molecules were used to show the decreased toxicity of partially hydrolysed rye proteins.

2. Experimental

2.1. Microorganisms and culture conditions

Lactobacillus alimentarius 15M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A and *Lactobacillus hilgardii* 51B previously selected on the basis of their capacity to hydrolyse gliadin fractions of wheat sourdoughs (Di Cagno et al., 2002) were used. The strains were routinely propagated for 24 h at 30 °C (*L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A) or 37 °C (*L. hilgardii* 51B) in MRS (de Man, Rogosa, Sharpe) broth (Oxoid, Basingstoke, Hampshire, England) with the addition of fresh yeast extract (5%, v/v) and 28 mM maltose at a final pH of 5.6. When used for rye sourdough fermentation, *Lactobacillus* cells were cultivated until the late exponential growth phase (optical density at 620 nm, ca. 2.5) was reached (ca. 12 h).

2.2. Sourdough fermentation

The characteristics of rye flour were as follows: moisture 10%; protein ($N\times5.70$) 10% of dry matter (d.m.); fat 2.5% of d.m.; ash 1.5% of d.m.

Six grams of rye flour, 6 ml of tap water and 8 ml of a cellular suspension containing 5×10^8 colony forming units (CFU) of each strain/ml (ca. 10^8 CFU/g of dough) were used to produce 20 g of dough (dough yield, ca. 300 g). Sourdoughs were incubated at 37 °C for 24 or 48 h with stirring (ca. 200 rpm). Two controls were used: (i) dough, without bacterial inoculum, with the addition of erythromycin (0.05 mg/g), incubated for 24 h at 37 °C; and (ii) dough, without bacterial inoculum, chemically acidified to pH 3.9 with a mixture of lactic and acetic acids (molar ratio 4:1, v/v), incubated for 24 h at 37 °C.

2.3. Protein extraction

Protein fractions were extracted from doughs by the method of Osborne (1907) as modified by Weiss et al. (1993). Briefly, an aliquot of dough (12.75 g) was diluted with 30 ml of 50 mM Tris–HCl (pH 8.8), held at 4 °C for 1 h with vortex mixing at 15 min intervals, and centrifuged at 20,000 *g* for 20 min. The supernatant contained albumins and globulins. To minimise cross contamination among albumins, globulins and prolamins, the pellets were further extracted twice with the same buffer, and supernatants discarded. After washing with distilled water to remove buffer ions, the pellets were suspended in 30 ml of ethanol (75%, v/v), the suspension stirred at 25 °C for 2 h, and centrifuged as described above. The supernatant contained prolamins. The ethanol extraction was repeated twice. Residual ethanol was removed by suspending the pellets in distilled

water and centrifugation. Finally, the pellets were suspended with 4 ml of a urea–DTT mixture (6 M urea, 1% v/v Triton X-100, 0.5% w/v DTT and 0.5% v/v 2D Pharmalite pH 3–10), held for 2 h at room temperature with occasional vortex mixing and then centrifuged. The supernatant contained glutelins. The extracted proteins were used for further analyses. For 2DE analysis of rye flour, proteins were extracted by the method of Søndergaard et al. (1994). Briefly, 30 ml of ethanol (75%, v/v) were added to an aliquot of dough (12.75 g), stirred at 25 °C for 2 h, and centrifuged. The supernatant contained alcohol-soluble polypeptides.

For RP-HPLC analysis, prolamins were extracted as reported by Kruger et al. (1988). An aliquot of dough (10 g) was diluted with 30 ml of 0.5 M NaCl in 150 mM sodium phosphate, pH 6.8, and the suspension mixed thoroughly and incubated for 30 min at room temperature with stirring. After centrifugation at 20,000 *g* for 20 min, the supernatant, containing albumins and globulins, was recovered. The pellets were washed with 20 ml of distilled water to remove salts and residual organic acids, mixed with 30 ml of 1-propanol (50%, v/v) and stirred at room temperature for 30 min. After centrifugation, the prolamin extracts were stored at −20 °C in the dark until analysed.

For RP-HPLC analysis, glutelins were extracted as reported by Verbruggen et al. (1998). To remove secalins, doughs (100 g) were pre-extracted with 50% (v/v) 1-propanol (150 ml) at room temperature for 30 min. The suspension was centrifuged for 15 min at 2500 *g*. Glutelins were extracted (30 min at 60 °C) from the residue with 50% (v/v) 1-propanol containing 1% (w/v) DTT (150 ml).

Protein concentrations in extracted fractions were determined by the Bradford method (1976) with bovine serum albumin (BSA) as standard.

2.4. Two-dimensional electrophoresis (2DE)

2DE was performed with the immobiline-polyacrylamide system as described by Bjellqvist et al. (1993). Aliquots of 30 μg of rye proteins were used for the electrophoresis. Isoelectric focusing was performed on immobiline strips, providing a non-linear pH gradient from 3.0 to 10.0 (IPG strips; Amersham Pharmacia Biotech, Uppsala, Sweden) by IPGphore, at 20 °C. The voltages were: 0–300 V for 1 h, 300–500 V for 3 h, 500–2000 V for 4 h, and a constant 8000 V for 4 h. After electrophoresis, IPG strips were equilibrated for 12 min against buffer A (6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecylsulfate (SDS), 0.05 M Tris–HCl (pH 6.8), 2% (w/v) dithiothreitol) and for 5 min against buffer B (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris–HCl (pH 6.8), 2.5% (w/v) iodoacetamide, 0.5% Bromophenol Blue).

The second dimension was performed in a Laemmli system (1970) on 13.5% polyacrylamide gels (13 cm × 20 cm × 1.5 mm) at a constant current of 40 mA/gel and at 15 °C for approximately 5 h, until the dye front reached the bottom of the gel. Gels were calibrated with two molecular mass markers: (1) markers for two-dimensional electrophoresis (pI range, 7.6–3.8; molecular mass range, 17–89 kDa) from Sigma Chemical Co. and (2) human serum proteins in the molecular mass range from 200 to 10 kDa from the Department of Onco-Immuno-Dermatology, IDI-IRCCS, Rome. The electrophoretic coordinates used for serum were described by Bjellqvist et al. (1993). Gels were silver stained (Hochstrasser et al., 1998). For quantification of spots on each gel, the relative volume (% VOL) was used; the relative VOL was the VOL of each spot divided by the total VOL over the whole image. In this way, differences in the color intensities between the gels were eliminated (Appel and Hochstrasser, 1999). The hydrolysis factor for individual proteins was expressed as the ratio of the spot intensity of the protein in the selected lactobacilli fermented dough and in the chemically acidified dough. All the induction factors were calculated based on the average of the spot intensities of each of the three gels and standard deviation was calculated.

The protein maps were scanned with an Image Scanner (Amersham Pharmacia Biotech) and analysed with Image Master 2D v.3.01 computer software (Amersham Pharmacia Biotech). Three gels were analysed, and the spot intensities normalised as reported by Bini et al. (1997). Only hydrolysis factors with statistical significance where P value was <0.05, are reported.

2.5. Mass spectrometry MALDI-TOF analysis

Mass Spectrometry (MS) MALDI-TOF analysis of rye prolamins was performed at the Centro National de Biotecnologia, Gluten Unit, CNB using a Voyager De Pro Workstation (Perspective Biosystems, UK). The matrix-sample solution contained an ethanol-extract of prolamins (100 μl) added to 8 μl of 50 mM octyl-D-glucopyranoside detergent (ODGP) and 25 μl of saturated sinapic acid in 30% (v/v) acetonitrile solution, containing 0.1% (v/v) trifluoroacetic acid (TFA). The matrix-sample mixture was dried in a Speed-Vac centrifuge (30–35 min) and the residue dissolved in 6 μl of 60% ethanol, containing 0.1% TFA. Sample-matrix mixture (1 μl) was placed on a 100-sample stainless-steel probe and allowed to dry at room temperature for 5 min. Mass spectra were recorded in the linear positive mode at an acceleration voltage of 25 kV with a grid voltage of 93, 0.25% guide wire and 700 ns delay time by accumulating 100 spectra of single laser shots under threshold irradiance. Standards of European rye and wheat prolamins were also included in the analyses (Hernando et al., 2003).

2.6. RP-HPLC and determination of free amino acids

Rye prolamins and glutelins were separated using an ÄKTA Basic Instrument (Amersham Pharmacia Biotech). The elution system consisted of trifluoroacetic acid (TFA 0.1%, v/v) (A); and acetonitrile/TFA (99.9/0.1%, v/v) (B). For each sample, 100 μl of prolamin/glutelin extract (ca. 100 μg protein) were injected. HPLC conditions for secalin separation were: linear gradient of 0 min 25% B, 90 min 37% B; flow rate of 1 ml/min; column temperature set at 70 °C; and UV-detection at 210 nm. A linear gradient of 0–5 min 24% B and 5–40 min from 24 to

55% B was used for glutelin analysis (Thiele et al., 2004). The column was cleaned with 80% B (10 min) and equilibrated with 25% B (40 min).

Free amino acids were determined in water- and salt-soluble fractions extracted from chemically acidified and fermented rye doughs. Total and individual free amino acids were analysed using a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd, Cambridge Science Park, England) with a cation-exchange column (20 × 0.46 cm inner diameter) in the sodium form. A mixture of amino acids at known concentration (Sigma Chemical Co., St Louis, MO, USA) was added with cysteic acid, methionine sulphoxide, methionine sulphone, tryptophan and ornithine, and used as standard. Proteins and peptides were precipitated by addition of 5% (v/v) cold solid sulfosalicylic acid, and held at 4 °C for 1 h before centrifuging at 15,000*g* for 15 min. The supernatant was filtered through a 0.22 μm pore size filter (Millex-HA; Millipore S.A., Saint Quentin, France) and diluited (1:5) with sodium citrate loading buffer (0.2 M, pH 2.2). Amino acids were derivatised post-column with ninhydrin reagent and detected by absorbance at 440 (proline and hydroxyproline) or 570 nm (all the other amino acids).

2.7. Western analysis

These analyses were performed at the Centro National de Biotecnologia, Gluten Unit, CNB. The R5 monoclonal antibody (from mouse cell line) and the horseradish peroxidase (HRP)-conjugated R5 antibody (R5-HRP) (Diffchamb, Västra Frölunda, Sweden) were used for analysis (Osman et al., 2001; Shewry et al., 1992). For R5-Western analysis the ethanol-soluble polypeptides separated by one-dimensional SDS-PAGE were electrotransferred onto polyvinylidene difluoride (PVDF) membranes, incubated directly with R5-HRP, and developed by ECL Western Blotting Analysis System (Amersham Bioscences, Bucks, UK) (Osman et al., 2001; Valdés et al., 2003).

2.8. Pepsin–trypsin digest

Glutelins and/or ethanol-extractable prolamins from rye flour were sequentially digested with pepsin–trypsin (PT) to simulate in vivo digestion (Auricchio et al., 1982). After digestion the PT-digest was heated at 100 °C for 30 min to inactivate enzymes. This peptide preparation was used directly, or was further incubated with the mixture of selected LAB used for sourdough fermentation. The reaction mixture contained selected LAB strain at a concentration of 10^9 CFU/ml and 70 mg of PT-digest in 7.16 ml of 5 mM phosphate buffer, pH 7.0. After incubation at 37 °C for 24 h with stirring at 150 rpm, the mixture was freeze dried and used for further analysis.

2.9. Agglutination test

K 562 (S) sub-clone cells of human myelagenous leukemia origin, from the European Collection of Cell Culture (Salisbury, United Kingdom), was used for the agglutination tests with PT-digests. Cells were grown in RPMI medium (HyClone, Cramlington, UK) supplemented with L-glutamine 0.2 mM, penicillin 50 U/ml, streptomycin 50 mg/ml, and foetal calf serum 10% (v/v) (Flow Laboratories, Irvine, Scotland), at 37 °C in a humidified atmosphere of 5% CO_2 in air for 96 h. After cultivation, cells were harvested by centrifugation at 900*g* for 5 min, washed twice with 0.1 M phosphate-buffer saline solution (Ca^{2+} and Mg^{2+} free; pH 7.4), and re-suspended at a concentration of 10^8 cells/ml in the same buffer. Cell suspension (25 μl) was added to wells of a microtiter plate containing serial dilutions (0.013 to ca. 7.0 g/l) of PT-digest. The total volume in the well was 100 μl, and the mixture was held for 30 min at room temperature. Following incubation, a drop of the suspension was applied to a microscope slide, agglutinated, and single cells counted. Agglutination tests were performed in triplicate.

2.10. Tests on Caco-2/TC7 cells

Human intestinal Caco-2 cells (TC7 clone) (Chantret et al., 1994) were kindly supplied by Dr Stammati, Istituto Superiore della Sanità, Rome, Italy. Cells were routinely grown and maintained in 25 ml culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI medium (Hyclon, UK) supplemented as described above. Experiments were performed using cells taken from passages 80 to 95 during cultivation in RPMI medium.

For cell viability determination, cells were seeded in 96 multiwell plates at 5×10^3 cells/well. After 24 h, the medium was removed and cells treated with the different PT-digests at a concentration of 1 mg/ml for 24–96 h. The same concentration of PT-digests was used in all assays. The decrease of the cell growth rate was determined by uptake of the vital dye Neutral Red (Silano et al., 2004). The dye present in viable cells was extracted and measured spectrophotometrically at 540 nm using a microplate reader (Novapath, Biorad, Hercules, CA).

For apoptosis, Caco-2/TC7 cells were seeded at a density of 3.2×10^3 cells/cm^2 and grown in 100 mm internal diameter plates (Falcon, Beckton-Dickinson, Labware, NJ). On the fifth day after plating, cells were treated with PT-digests from rye, for 24 h in complete culture medium supplemented with 5% (v/v) fetal calf serum. Untreated and treated cells with 1 mg/ml actinomycin D (A-1410, Sigma) were used as negative and positive controls, respectively, to determine the maximum level of apoptosis induction and spontaneous apoptosis in TC7 cell line. Apoptosis was evaluated using the Caspase-3 Colorimetric Assay Kit (CASP-3-C, Sigma), based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA) by caspase-3, resulting in the release of the *p*-nitroaniline (*p*NA) moiety which was measured spectrophotometrically at 405 nm ($\varepsilon_{mM}=10.5$). The concentration of *p*NA released was calculated using a *p*NA calibration curve. Nitric oxide production was determined by measuring the stable oxidation products, nitrite and nitrate, in samples of media from Caco-2/TC7 cells grown in 24 well plates. Samples were incubated with copper plated cadmium filings to reduce nitrate to nitrite. The total concentrations were

measured spectrophotometrically using the Griess reaction (Silano et al., 2004). After incubation at 37 °C for 24 h with PT-digests, 0.5 ml aliquots of the culture medium were taken from the plates containing the cell monolayers, mixed with an equal volume of Griess reagent (1% w/v sulphanilic acid in 0.5 M HCl and 0.1% w/v *N*′-1-naphthylethylendiamine hydrochloride) and the absorbance at 540 nm was measured after 30 min. The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

2.11. Processing of jejunal biopsies and in vitro organ culture

Jejunal biopsies were obtained from four female, untreated CD patients (age range: 35–40 years) who underwent gastrointestinal endoscopy for diagnostic purposes. Each patient suffered from symptoms suggestive of coeliac disease and returned a positive serum transglutaminase antibody. During the endoscopy, using a gastroscope, two samples of small intestine mucosa were obtained from each patient, one for diagnostic purposes and one for the experiments. Histological observations showing villous atrophy and crypt hyperplasia confirmed the clinical diagnosis of CD for each patient.

For culture each jejunal biopsy was divided into two parts. The mucosal sections were placed, villous surface uppermost, on stainless steel grids positioned over the central well of an organ culture dish. The sections were cultured for 24 h in RPMI supplemented with 10% FCS (Fetal Calf Serum) (Mazzarella et al., 2005) and PT-digests (1 mg/ml). PT-digests from unacidified and chemically acidified doughs were used as positive controls. RPMI medium alone served as the negative control. After incubation, the specimens were harvested, embedded in an 'optimal temperature' cutting compound (Bioptica, Milano, Italy) and stored at −80 °C. The biopsy samples were sectioned into 4-μm slices, fixed in acetone for 20 min and incubated for 30 min with normal rabbit serum (1:200, Dako, Carpinteria, CA) to prevent non-specific antibody binding (Auricchio et al., 2004). Sections were then incubated with anti $CD3^+$ (1:200, Dako) MAb and exposed to rabbit anti-mouse immunoglobulin for 30 min. The MAb was diluted in Ab dilution solution (Dako). After washing with Tris buffer, pH 7.4, the sections were incubated with monoclonal mouse APAAP (Dako) for 30 min and stained with New Fuchsin. Finally, sections were counterstained with Mayer's hematoxyllin (Dako) and mounted in Aquamount (Sigma). All procedures were performed at room temperature with incubations in a humidity chamber. The density of cells expressing $CD3^+$ was determined by counting the stained cells per millimeter of epithelium. Counting was repeated in two different sections for each sample. The data were compared by the Student's *t*-test with $P<0.05$ considered significant. Fas expression in epithelial cell was evaluated by intensity of staining using an arbitrary scale (1 = very weak to 4 = strong).

3. Results

3.1. Two-dimensional electrophoresis (2DE)

After fermentation at 37 °C for 24 h, the cell concentration of lactic acid bacteria in rye sourdough was ca. 10^9 CFU/g, for each species used in the mixture. The pH of the sourdough was ca. 3.8. The non-acidified dough with antibiotic had a pH of 5.5 and the total mesophilic bacterial count did not exceed 10^3 CFU/g.

2DE analysis (Fig. 1A and Table 1) of the non-acidified dough resolved a total of 156 ethanol-soluble polypeptides with pIs from 3.8 to 9.4 and molecular masses from 20 to 75 kDa. This separation was substantially in agreement with electrophoretic analyses of rye flour made by Radzikowski et al. (2002). In particular, the ethanol-extract showed a group of proteins with molecular masses of ca. 75 kDa, corresponding to S-rich, γ-secalins, and at 45–40 and 30 kDa, corresponding to S-poor, ω-secalins, and S-rich, γ-secalins, respectively (Radzikowski et al., 2002; Shewry et al., 1983; Shewry and Tatham, 1990;). An additional group of proteins with molecular mass of 10–20 kDa was observed by Radzikowski et al. (2002). Using 2DE analysis a total of 129 ethanol-soluble polypeptides was identified in chemically acidified rye dough (Fig. 1B and Table 1). Several spots found in the unacidified doughs were not present in the acidified doughs. These spots were present in three clusters: four spots in the pI range 3.8–5.3 (70.5–68.7 kDa); three in the pI range 6.6–7.5 (58–50.5 kDa); and 20 in the pI range 3.8–8.5 (33.5–20.0 kDa). On the other hand, 12 spots were present in increased amounts in chemically acidified compared to unacidified doughs (Fig. 1A and B). Since, chemical acidification caused a modification of the 2DE polypeptide pattern with respect to non-acidified dough, the 2DE polypeptide pattern of the fermented dough was compared to chemically acidified dough to find variations due to bacterial proteolysis. Of the 129 polypeptides identified in the chemically acidified dough, 109, with molecular masses higher than 20 kDa, showed hydrolysis factors higher than 85% (Fig. 1C vs. B, and Table 1). Other polypeptides had hydrolysis factors in the range of 30–70%.

3.2. Mass spectrometry

The quantification of prolamins was also performed using MALDI-TOF M.S. (Hernando et al., 2003). Prolamin peaks detected in the European rye standard (var. Petkus) and chemically acidified dough, decreased markedly when the rye dough was fermented by LAB (Fig. 2A–C). The partial hydrolysis of secalins produced polypeptides mainly in the 30–39 *m/z* range. The low molecular mass peaks which increased in rye sourdough were presumptively attributed to hydrolysis end-products whose size may still be sufficient to contain toxic peptides.

3.3. RP- HPLC and determination of free amino acids

To confirm that extensive prolamin hydrolysis was occurring during sourdough fermentation, the extraction of

prolamins from rye was also performed as reported by Kruger et al. (1988). The secalins soluble in 1-propanol were analysed further by RP-HPLC and 2DE (Fig. 3A and B). The major peaks found in the hydrophilic part of the gradient (40–70 ml) of the chemically acidified rye dough disappeared totally in rye sourdough. This result was confirmed by 2DE analysis.

Compared to non-acidified doughs (Fig. 4), HPLC analysis showed that glutelin polymers were degraded during incubation of chemically acidified doughs at 37 °C for 24 h (Fig. 4). Wheat glutenin hydrolysis and gluten depolymerization during sourdough fermentation has been shown to be related mainly to pH dependent activation of cereal enzymes (Thiele et al.,

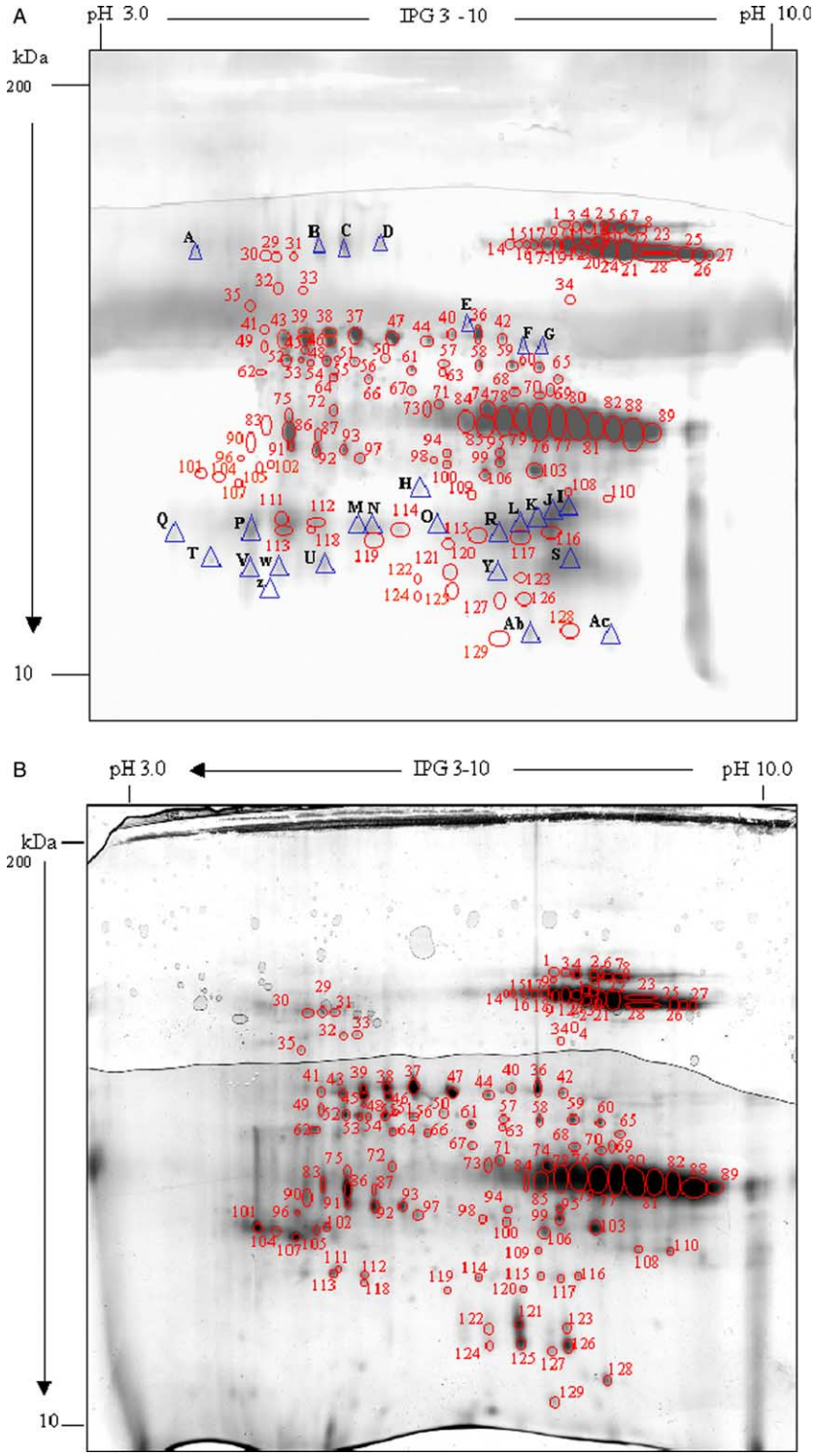

Fig. 1. 2DE analysis of ethanol-extract of rye polypeptides. Non-acidified dough (A), chemically acidified dough (B) dough fermented by selected lactic acid bacteria incubated for 24 h at 37 °C (C). Polypeptides were indicated by lettered triangles or numbered ovals. Lettered triangles indicate polypeptides present only in the non-acidified dough.

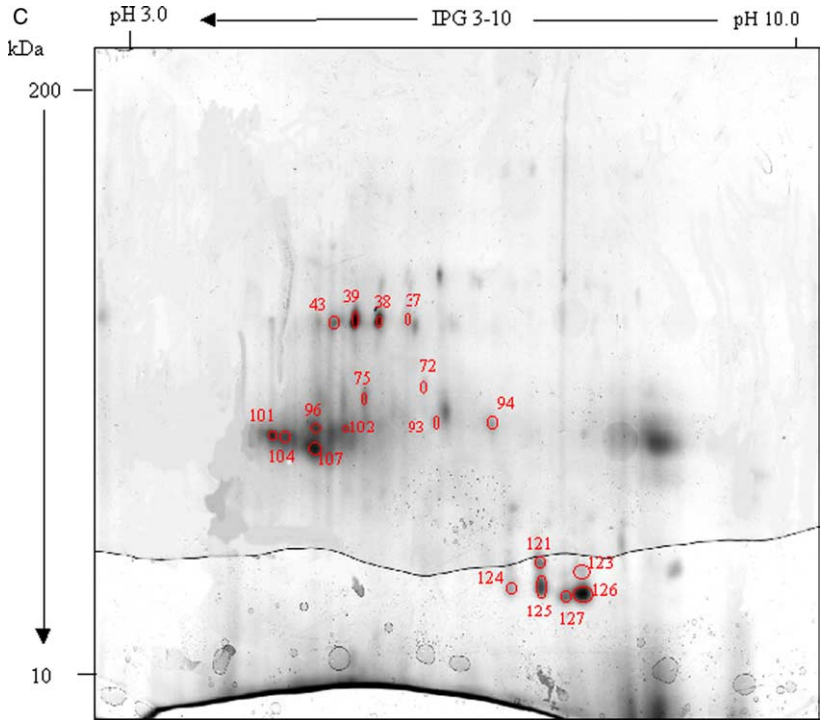

Fig. 1 (*continued*)

2004). Incubation of doughs with lactic acid bacteria did not substantially modify the rye glutelin profile compared to chemically acidified dough, except for the disappearance of some polypeptides found in the hydrophobic zone of the acetonitrile gradient.

Evidence for proteolysis during sourdough fermentation was also found by determination of free amino acids. Fermentation by lactic acid bacteria increased the concentration of free amino acids ca. 40-fold compared to chemically acidified rye dough (204.87 vs. 5.36 mg/kg of dough). Ala, Leu, Val, Glu, Ile, Phe and Pro showed the highest increases.

3.4. Immunological analysis by R5-western blot

The R5 monoclonal antibody recognizes the consensus amino acid sequence QXPW/FP present in multiple immuno-reactive epitope repeats, which occur in wheat α-, γ- and ω-gliadins, and also in related rye prolamins (Osman et al., 2001; Shewry et al., 1992). Greatest reactivity is associated with the QQPFP sequence, but homologous repeats such as LQPFP, QLPYP, QLPTF, QQSFP, QQTFP, PQPPP, QQPYP and PQPFP are also recognized but with weaker reactivity (Osman et al., 2001). When compared with the European secalin standard (var. Petkus), the Western blot of chemically acidified rye dough showed an almost

Table 1
Properties of rye ethanol-soluble polypeptides hydrolysed by selected lactic acid bacteria during rye sourdough fermentation

Spot numbers[a,b]	Estimated pI range	Estimated molecular mass range (kDa)	Hydrolysis factor range (%)
1–4, 7–11	6.62–6.90	75.00–72.45	90.0–95.0
5–6	6.74–6.82	73.80–73.75	85.0–87.0
12–18	6.39–6.72	72.00–70.75	85.0–95.0
19–36, 40–42	5.20–7.90	70.60–43.00	85.0–90.0
44–71	5.41–6.83	42.80–37.60	85.0–95.0
73–74, 76–92	5.41–8.30	37.00–33.20	85.0–92.0
95–100, 103, 105–106			
108–120	5.10–7.60	32.50–27.50	85.0–90.0
122–123	6.22–6.69	25.80–25.60	90.0
128–129	6.60–6.65	20.00–15.00	90.0

Analyses were performed with Image Master software (Pharmacia). Three gels of independent replicates were analysed. For spot quantification and hydrolysis factor calculation, see Section 3.1. All of the hydrolysis factors were calculated on the basis of the average of the spot intensities of each of three gels.

[a] Spot designations correspond to those of the gels in Fig. 1.

[b] Spots reported were clustered in function of the range estimated molecular mass and hydrolysis factor higher than 85%.

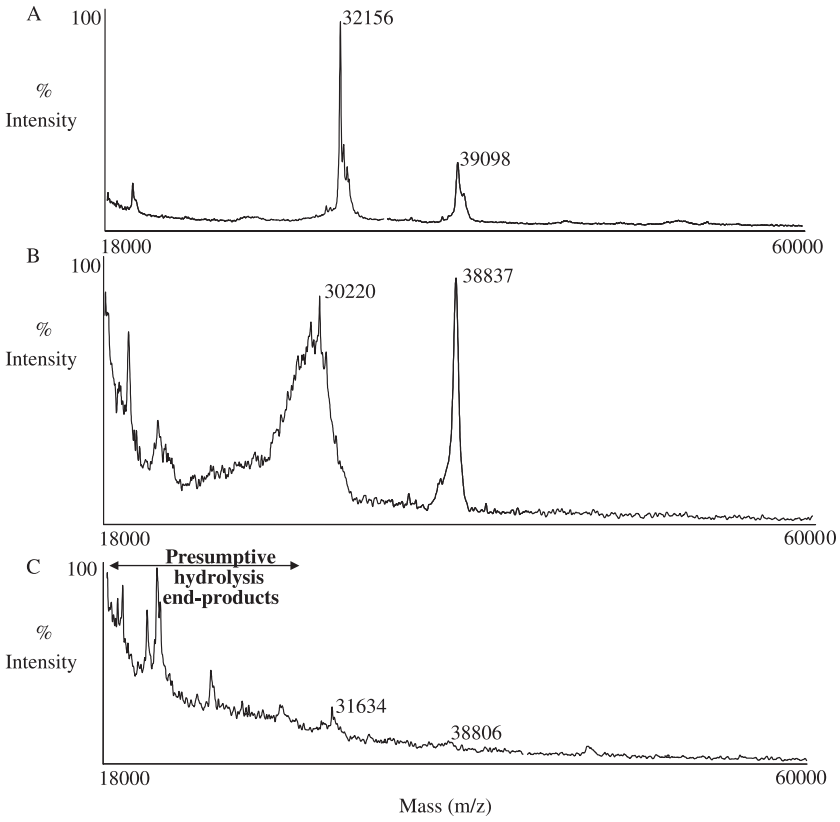

Fig. 2. MALDI-TOF mass spectra of rye prolamins. European secalins standard, var. Petkus (A), chemically acidified dough (B) and dough fermented by selected lactic acid bacteria (C). The doughs were incubated for 24 h at 37 °C.

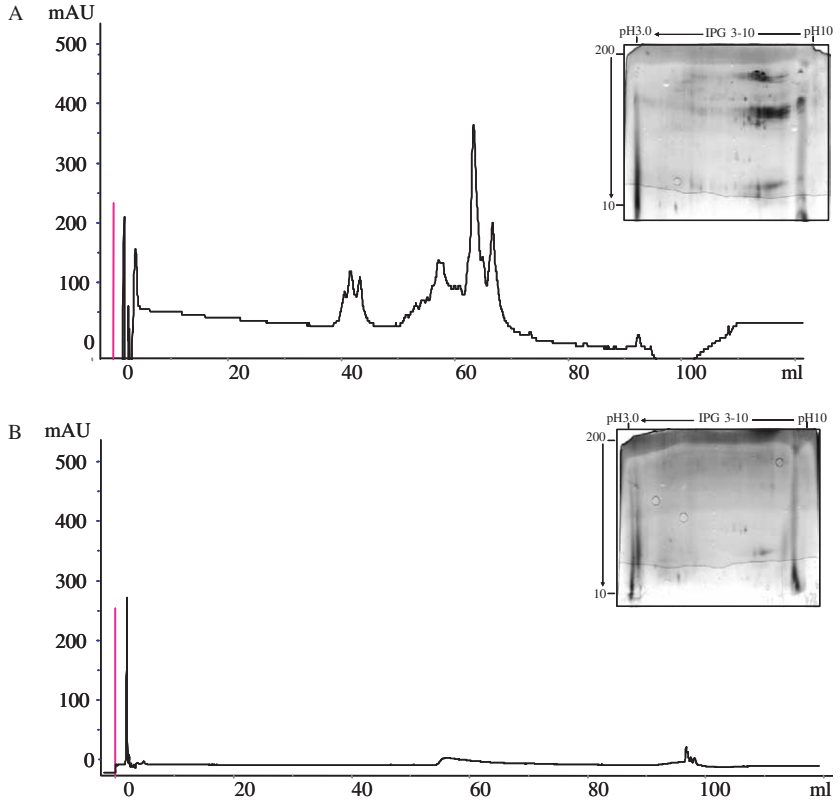

Fig. 3. RP-HPLC analysis of 1-propanol extract of rye prolamins and related 2DE. Chemically acidified dough (A) and dough fermented by selected lactic acid bacteria (B). The doughs were incubated for 24 h at 37 °C.

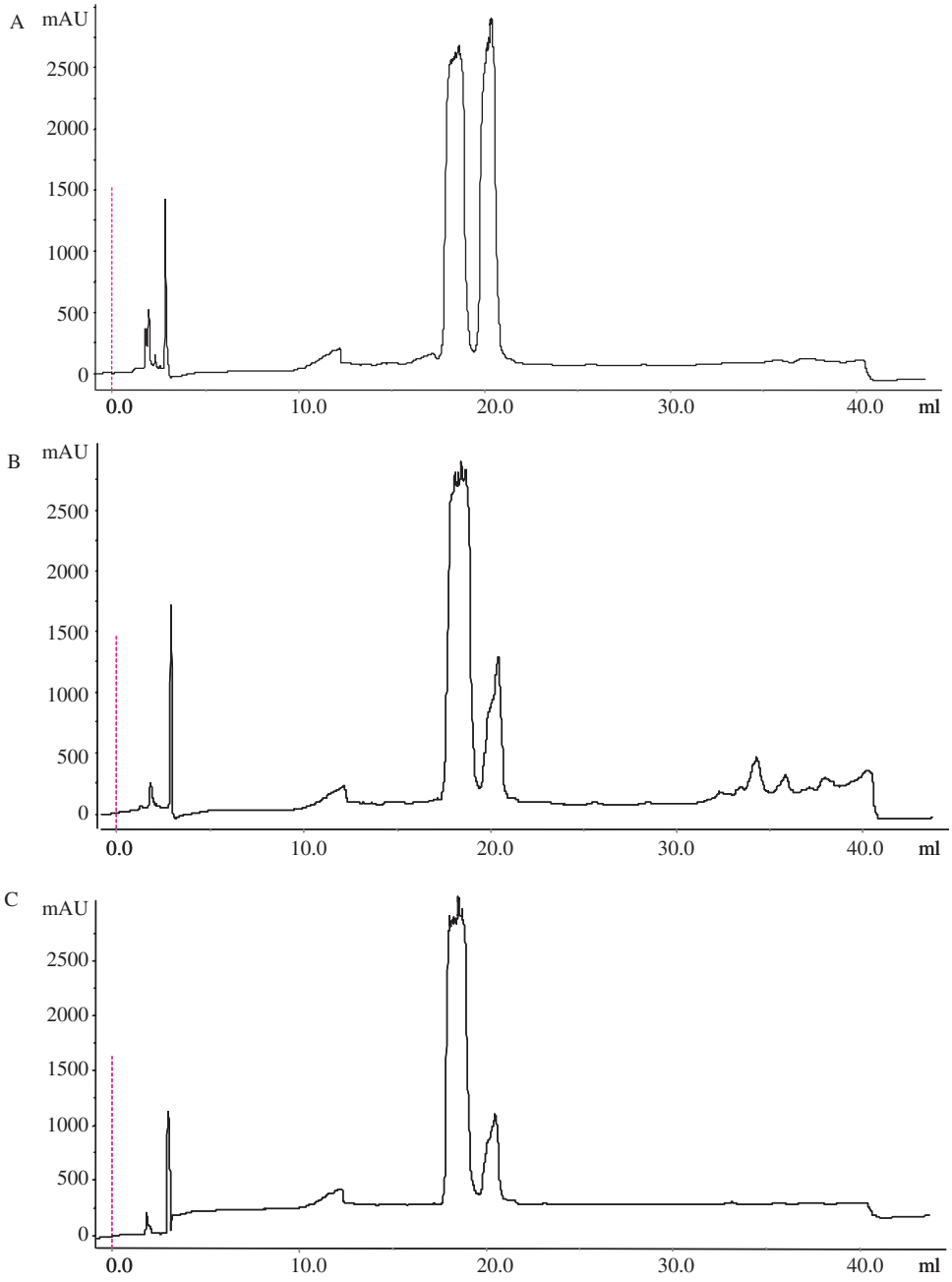

Fig. 4. RP-HPLC analysis of rye glutelins extract. Unacidified dough (A), chemically acidified dough (B), dough fermented by selected lactic acid bacteria incubated for 24 h at 37 °C (C).

complete degradation of the polypeptide band at 70 kDa (Fig. 5). The same was found for rye sourdough which, in addition, showed the disappearance of two other polypeptide bands of ca. 39 and 32 kDa, respectively. A new polypeptide of ca. 35 kDa was also generated. After prolonged incubation of rye sourdough (48 h at 37 °C), no polypeptide signals were recognized by the R5 monoclonal antibody.

3.5. Agglutination test

Prolamins were extracted from rye flour and subjected to PT degradation to mimic in vivo protein digestion (Di Cagno et al., 2002). No significant evidence of cell clustering was found for the undifferentiated K562 (S) cells in the untreated controls. On the contrary, rye PT-digest caused 100% cell agglutination at a Minimal Agglutinating Capacity (MAC) of 0.58 g/l. Before use, rye PT-digest was further treated with LAB for 24 h at 37 °C also. When assayed alone, the lactobacilli mixture was ineffective in causing cell agglutination (data not shown). The MAC of the rye PT-digest treated with selected LAB increased to 4.66 g/l.

3.6. Tests on Caco-2/TC7 cells

Caco-2/TC7 cell death may occur either by necrosis or by apoptosis. The Neutral Red uptake assay measured the whole

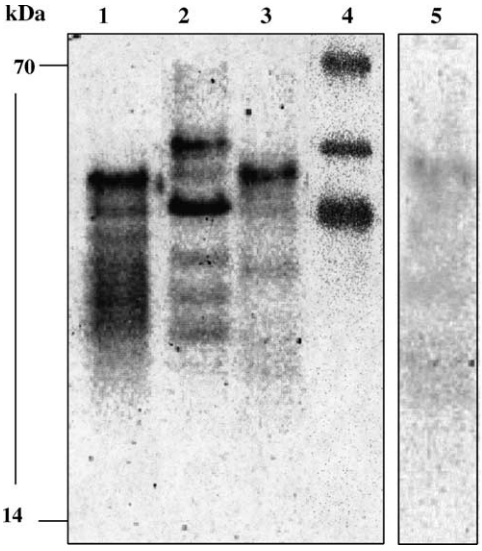

Fig. 5. Western blot/R5 analysis of prolamin fraction. European gliadin reference (lane, 1); rye chemically acidified dough (2); rye dough fermented by selected lactic acid bacteria (3); secalin reference var. Petkus (4). The doughs were incubated for 24 h at 37 °C. Rye dough fermented by selected lactic acid bacteria for 48 h at 37 °C (5).

cell death process without discrimination between necrosis and apoptosis. Prolonged incubation (72–96 h) with rye PT-digest (1 mg/ml) significantly ($P<0.01$) decreased the cell viability with respect to the control (untreated cells) (Fig. 6). When cells were exposed to PT-digest treated with LAB, the cell viability was higher than that found with untreated rye PT-digest.

The activity of caspase-3 was measured to quantify the apoptosis in Caco-2/TC7 cells. After 24 h of treatment with rye PT-digest, the caspase-3 activity increased ca. fourfold with respect to control (Fig. 7). This finding showed that PT-digest induced the apoptosis pathway as shown for a wheat gliadin

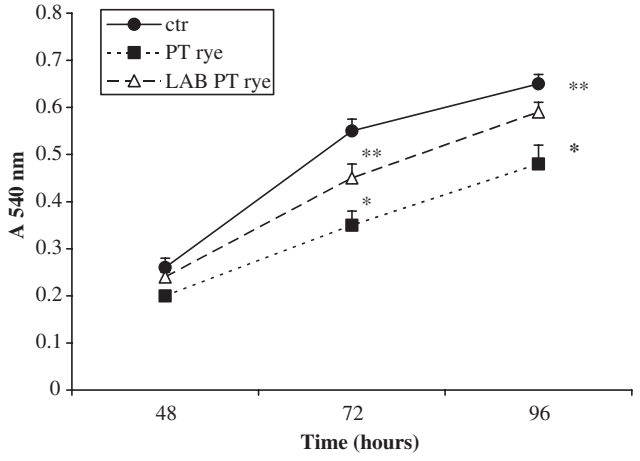

Fig. 6. Viability of Caco-2/TC7 cells measured as Neutral Red uptake after 48, 72 and 96 h of incubation with rye PT-digest (PT rye) and rye PT-digest (1 mg/ml) treated with selected lactic acid bacteria (LAB PT rye). ctr, control. Data are the means + SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Student's *t*-test. One asterisk indicates a significant difference ($P<0.01$) with respect to the control; two asterisks indicate a significant difference ($P<0.01$) with respect to cells incubated with rye PT-digest.

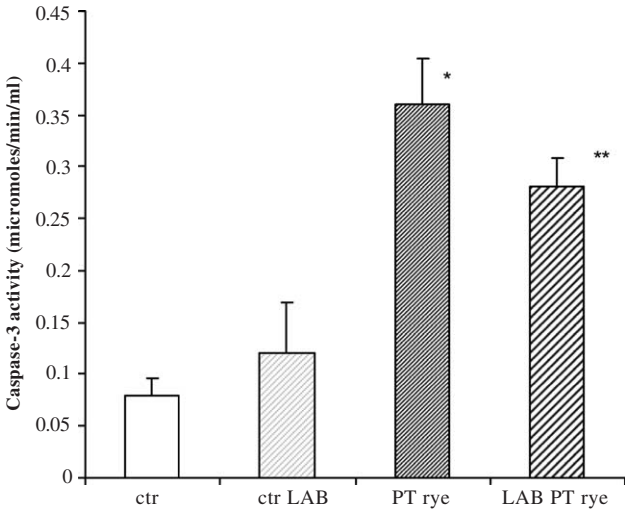

Fig. 7. Caspase-3 activity of Caco-2/TC7 cells after 24 h of incubation with lactic acid bacteria preparation (ctr LAB), rye PT-digest (PT rye) and rye PT-digest (1 mg/ml) treated with selected lactic acid bacteria (LAB PT rye). ctr, control. Data are the means (+SD) of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Student's *t*-test. One asterisk indicates a significant difference ($P<0.01$) existing between PT rye and the control. Two asterisks indicate a significant difference ($P<0.01$) existing between rye PT-digest treated with selected lactic acid bacteria and rye PT-digest.

PT-digest by Giovannini et al. (2000) and Silano et al. (2004). When Caco-2/TC7 cells were treated with rye PT-digest hydrolysed by LAB, caspase-3 activity was still higher than the control, but decreased significantly ($P<0.01$) by 15.8% with respect to untreated PT-digest.

Caco-2/TC7 cells have the capacity to produce nitrogen oxides (NO_2^-, NO_3^- and NO) in the presence of inhibitors and natural toxins (Vignoli et al., 2001) including exposure to a wheat gliadin PT-digest (Maiuri et al., 2003a,b). Treatment with rye PT-digest for 24 h increased the release of nitrogen oxides with respect to the control (Fig. 8). The release of nitrogen oxides decreased significantly ($P<0.01$) when Caco-2 cells were exposed to PT-digest treated with lactic acid bacteria.

3.7. $CD3^+$ cell infiltration and Fas epithelium expression

Organ culture of small intestine biopsies is a valuable model for the study of immunological events occurring in coeliac mucosa following contact with toxic epitopes. In vitro challenge systems reproduce many features of the mucosal immune response occurring in the established coeliac lesion (Maiuri et al., 1996). Compared to exposure to medium alone (negative control), coeliac jejunal biopsies cultured with PT-digest of rye prolamins and glutelins extracted from chemically acidified dough (positive control) showed a significant increase of the infiltration of $CD3^+$T lymphocytes (Figs. 9 and 10). The PT-digest obtained from rye sourdough showed a $CD3^+$T lymphocyte response similar to that found by incubating coeliac jejunal biopsies in medium alone. Unacidified dough behaved similarly to chemically acidified dough (data not

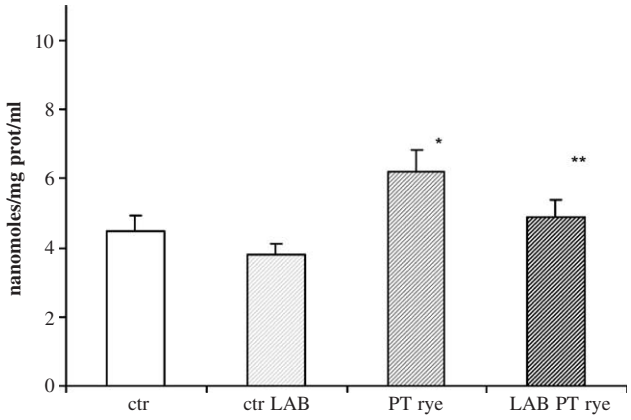

Fig. 8. Nitrogen oxide production by Caco-2/TC cells after 24 h of incubation with lactic acid bacteria preparation (ctr LAB), rye PT-digest (PT rye) and rye PT-digest (1 mg/ml) treated with selected lactic acid bacteria (LAB PT rye). ctr, control. Data are the means (+SD) of three separate experiments performed in duplicate. Statistical differences between mean values were determined with Student's *t*-test. One asterisk indicates a significant difference ($P<0.01$) with respect to the control; two asterisks indicate a significant difference ($P<0.01$) with respect to cells incubated with rye PT-digest.

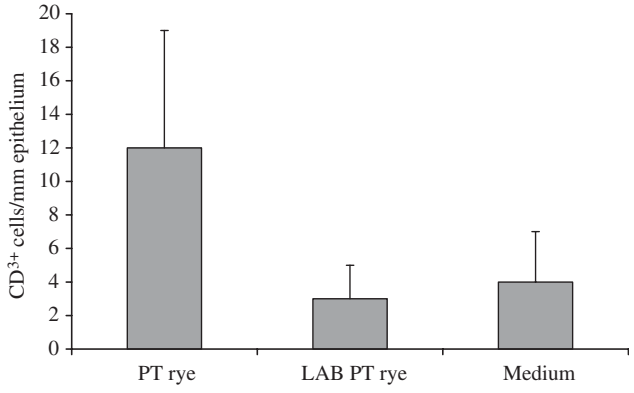

Fig. 9. Organ culture studies in CD mucosa specimens: $CD3^+$ intraepithelial lymphocytes. Bars indicates the means and SD in biopsies cultured in vitro with PT-digests from secalin and glutelin proteins extracted from doughs chemically acidified (PT rye) or fermented with selected lactic acid bacteria for 24 h (LAB PT rye). A control with RPMI medium alone was reported (medium).

shown). Epithelial expression of Fas, particularly evident in basolateral membranes of enterocytes, increased after 24 h challenge with a PT-digest of rye prolamins and glutelins from the chemically acidified dough (Fig. 11). No significant differences with respect to the negative control were found when Fas expression was determined in biopsies cultured with PT-digest from rye sourdough fermented by lactic acid bacteria.

4. Discussion

Rye is a cereal grain that forms part of the daily diet of many populations. The nutritional benefits of high rye diets include positive effects on digestion and decreased risk of heart disease, hypercholesterolemia, obesity, and non-insulin dependent diabetes and some hormone-dependent cancers (Aldercreutz, 1990; Kritchevsky, 2001; Zhang et al., 1990). For coeliac patients the exclusion of rye from the diet is not only a nutritional problem; adherance to a strict gluten-free diet seems to be difficult, if not impossible, to maintain, due to contamination with non-tolerated cereals. A recent study by Størsrud et al. (2003) showed that 30% of oat products in the market had a gluten content over 200 ppm. Also most starch-based, gluten-free wheat flours are contaminated with gluten at concentrations ranging from 10 to over 200 ppm (Collin et al., 2004). Although, occasional intake of small amounts of gluten or similar proteins (e.g. secalins) may not cause damage to the intestinal mucosa of CD patients on gluten-free diets, care should be taken if gluten or related proteins are ingested more frequently. Silent forms of the disease may expose people to long-term complications (Robbins and Howdle, 2004). Several therapeutic options are currently under investigation: (i) provision of prolyl-endopeptidase which complements gastrointestinal proteolytic processes; (ii) provision of tissue transglutaminase inhibitors (e.g. cystamine) to block the proliferative capacity of gluten-responsive T cells; (iii) blocking the binding sites of HLA-DQ2 to prevent the presentation of disease-inducing gluten peptides; and (iv) provision of therapeutic agents used for other chronic inflammatory diseases such as cytokine therapy and selective adhesion molecule inhibition (Sollid and Khosla, 2005). In addition to these treatments, the use of commensal bacteria has been suggested (Yan and Brendt Plok, 2004).

Previously, we showed that the same pool of sourdough lactic acid bacteria used in this study had a complement of specialized cytoplasmic peptidases capable of hydrolysing Pro-rich polypeptides which are responsible for CD after their uptake into the bacterial cells (Di Cagno et al., 2004). Here, we have shown, using several complementary techniques, that this

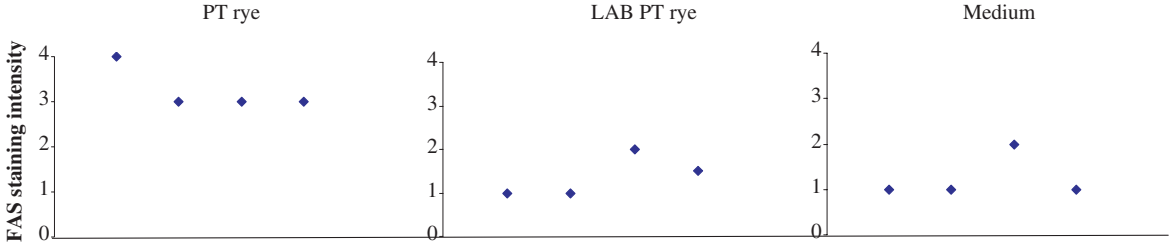

Fig. 10. Organ culture studies in CD mucosa specimens: epithelial cell Fas expression. Bars indicates the means and SD in biopsies cultured in vitro with PT-digests from secalin and glutelin proteins extracted from doughs chemically acidified (PT rye) or fermented with selected lactic acid bacteria for 24 h (LAB PT rye). A control with RPMI medium alone was reported (medium).

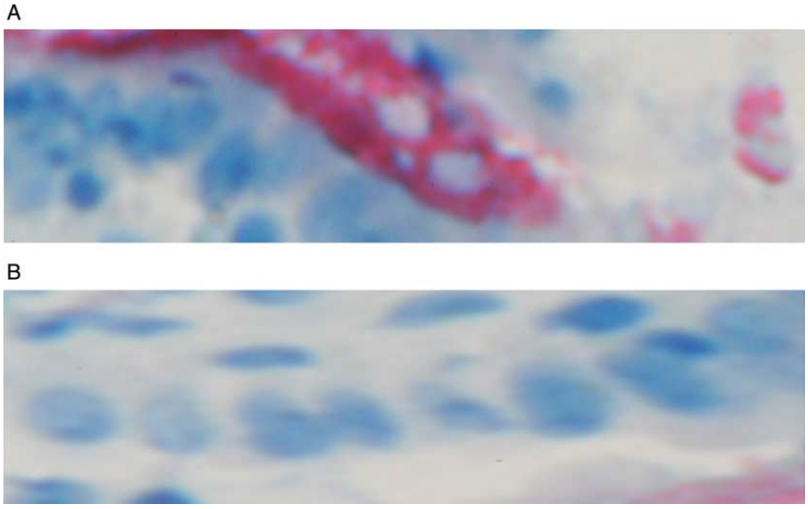

Fig. 11. Fas expression in a CD specimen treated with PT-digests from secalin and glutelin proteins extracted from doughs chemically acidified (panel A) or fermented with selected lactic acid bacteria for 24 h (panel B). Original magnification 200×; immunohistochemistry; Red Fuchsin APAAP staining technique (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article).

pool of selected lactic acid bacteria has the capacity to extensively hydrolyse rye prolamins. Nevertheless, further studies are needed to show their potential for degrading high concentrations of rye flour and, especially, the sequences of residual peptides and to show that their toxicity is absent or decreased.

The lack of animal models of CD and the ethical constraints on the use of human intestinal tissues severely limit the capability of screening, with a high throughput, protein or peptide epitopes potentially toxic to CD patients. The use of intestinal human cell lines as a tool to investigate CD has been corroborated in several studies (De Angelis et al., 1998; Giovannini et al., 2003; Pinto et al., 1983; Zucco et al., 1994). As observed in vivo, the data reported in this study for the enterocyte-like in vitro system suggest that secalin polypeptides can be intrinsically toxic to immature or undifferentiated intestinal cells by triggering apoptotic cellular events. Toxicity seemed to be higher than that found for gliadin polypeptides (Silano et al., 2004). In agreement with the analytical results, the treatment of the rye PT-digest with selected lactic acid bacteria decreased the toxicity of the PT-digest towards Caco-2/TC7 cells, evaluated through uptake of Neutral Red (cell viability), caspase-3 activity (apoptosis) and release of nitrogen oxides. Similar findings were made using K 562(S) cells in agglutination tests; the Minimal Agglutinating Capacity (MAC) of the PT-digest treated with lactic acid bacteria increased ca. 8-times. A number of investigations have shown the ability of the wheat gliadin PT-digest to prevent in vitro recovery of coeliac mucosa biopsy specimens, thus causing disorganisation of crypt architecture, reduced height, and deformation of enterocytes and crypt cells (Auricchio et al., 1982; Falchuk et al., 1974; Silano and De Vincenzi, 1999). Overall, a relatively high correlation is found between the agglutination activity of cereal components on K 562(S) cells and their toxicities in clinical and in vitro trials on the basis of biopsy samples of intestinal mucosa from CD patients (Silano and De Vincenzi, 1999). Studies on intestinal T cell clones from the mucosa of CD patients have led to the identification of immunogenic gliadin epitopes. $CD3^+$T intraepithelial lymphocytes increased after treatment of gluten in coeliac patients (Mazzarella et al., 2005; Troncone et al., 2005). Indeed, small intestine T cell activation by anti-$CD3^+$ antibodies in explants of human fetal gut induced a significant increase of intraepithelial lymphocytes, suggesting that it might be a consequence of lamina propria T cell activation (Monk et al., 1988).

Rye prolamins and glutelins were extracted from doughs and subjected to PT digestion. Compared to PT-digest from chemically acidified dough, coeliac jejunal biopsies exposed to the PT-digest from rye sourdough did not show an increase of the infiltration of $CD3^+$T intraepithelial lymphocytes. Fas expression on epithelial cells is a measure of apoptosis (Maiuri et al., 2001). Gliadins inducing apoptosis of enterocytes play a pivotal role in causing villous atrophy, the main hystological feature of the small intestine mucosa of CD patients (Maiuri et al., 2003a,b). Fas expression in biopsies cultured with PT-digest from rye sourdough did not show significant differences with respect to the negative control.

Although the findings of this study were focused on secalins from which the major part of the CD epitopes are derived (Rocher et al., 1996; Silano and De Vincenzi, 1999), glutelins are also involved in CD pathogenesis (van de Wal et al., 1999; Ciclitira et al., 2005). Compared to activity on secalin, a very low effect of selected lactic acid bacteria was found towards glutelins. Nevertheless, it was shown that acidification (chemical or biological) led to a substantial degradation of glutelins due to the probable activation of endogenous rye flour proteolytic enzymes. These findings could be in agreement with the coeliac jejunal biopsy experiments which showed neither infiltration of $CD3^+$ cells nor Fas expression when subjected to PT-digests from fermented secalins and glutenins.

The compliance to a gluten-free diet is an extremely challenging task, given the problems related to cross contamination, lack of clear food labeling policies, and poor information on minimal toxic amounts of prolamins in CD

subjects. Therefore, a biotechnology which includes the use of selected sourdough lactic acid bacteria that would eliminate any traces of toxic rye peptides in processed foods, would minimise the long-term risks and improve life quality of a multitude of individuals affected by CD worldwide. Based on the results of this study, long-time fermentation by selected sourdough lactobacilli could be used to eliminate traces of gluten contamination in processed and naturally gluten-free products. The potential for the addition of hydrolysed, non-toxic rye flour to gluten-free products will be examined in future studies which will also involve the sequencing of residual peptides and the assessment of improvement of the taste and texture of the product.

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