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Proteolytic activity and reduction of gliadin-like fractions by sourdough lactobacilli

G. Rollán[1], M. De Angelis[2], M. Gobbetti[3] and G.F. de Valdez[1,4]

[1]Centro de Referencia para Lactobacilos (CERELA), CONICET, Chacabuco, Tucumán, Argentina, [2]Institute of Sciences of Food Production, CNR, Bari, Italy, [3]Dipartimento di Protezione delle Piante e Microbiología Applicata, Facoltà di Agraria, Università degli Studi di Bari, Bari, Italy, and [4]Cat.de Microbiología Superior, Universidad Nacional de Tucumán, Tucumán, Argentina

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ABSTRACT

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Aims: To characterize the peptide hydrolase system of *Lactobacillus plantarum* CRL 759 and CRL 778 and evaluate their proteolytic activity in reducing gliadin-like fractions.

Methods and Results: The intracellular peptide hydrolase system of *Lact. plantarum* CRL 759 and CRL 778 involves amino-, di- (DP), tri- (TP) and endopeptidase activities. These peptidases are metalloenzymes inhibited by EDTA and 1,10-phenanthroline and stimulated by Co^{2+}. DP and TP activities of *Lact. plantarum* CRL 759 and CRL 778, respectively, were completely inhibited by Cu^{2+}. *Lactobacillus plantarum* CRL 778 showed the highest proteolytic activity and amino acids release in fermented dough. The synthetic 31–43 α-gliadin fragment was hydrolysed to 36% and 73% by *Lact. plantarum* CRL 778 and CRL 759 respectively.

Conclusions: *Lactobacillus plantarum* CRL 759 and CRL 778 have an active proteolytic system, which is responsible for the high amino acid release during sourdough fermentation and the hydrolysis of the 31–43 α-gliadin-like fragment.

Significance and Impact of the Study: This work provides new information of use when obtaining sourdough starters for bread making. Moreover, knowledge regarding lactobacilli capable of reducing the level of gliadin-like fractions, a toxic peptide for coeliac patients, has a beneficial health impact.

Keywords: gliadin, *Lactobacillus*, proteolysis, sourdough.

INTRODUCTION

Sourdough fermentation by lactic acid bacteria (LAB) is necessary to render the flour suitable for baking, to control the development of characteristic flavour components, to achieve dough leavening with yeast and to inhibit undesirable fermentations by other bacteria and yeast, improving the shelf life of breads (Spicher 1986). A great variety of both homo- and hetero-fermentative lactobacilli and yeast has been isolated from spontaneously fermenting sourdough (Anonymous 1994). The predominant LAB belongs to the genus *Lactobacillus* such as *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus sanfranciscensis*, and their key role is well known.

During the bread dough fermentation, the main proteolytic activity was first attributed to endogenous flour enzymes, such as aminopeptidase (AP), carboxypeptidase and endopeptidase (EP) (Spicher and Nierle 1988). Later, proteolysis by sourdoughs has been found to be higher than in yeasted and unstarted doughs (Gobbetti *et al.* 1994). During dough fermentation, the proteolysis by LAB release small peptides and free amino acids, which are important for rapid microbial growth and acidification and as precursors for flavour development of leavened baked products (Gobbetti *et al.* 1994, 1996a; Gobbetti 1998; Rollán and Font de Valdez 2001). Furthermore, this proteolytic activity might be used as a tool to reduce certain allergen compounds

Correspondence to: Graciela Rollán, Centro de Referencia para Lactobacilos (CERELA), CONICET, Chacabuco 145, 4000 Tucumán, Argentina (e-mail: rollan@cerela.org.ar).

derived from gluten (e.g. gliadin), which are involved in coeliac disease and they are present in wheat-baked foods. During endoluminal proteolytic digestion, prolamins of wheat (α-, β-, γ- and ω-gliadin subgroups), rye (e.g. secalin) or barley (e.g. hordein) release a family of proline- and glycine-rich polypeptides causing an inappropriate T-cell-mediated response (Silano and De Vincenzi 1999a). Studies with fragments of α-gliadin clearly indicated that few short sequences very rich in glutamine and proline residues (e.g. P-S-Q-Q and Q-Q-Q-P sequences) are toxic to coeliac patients and they caused an inflammatory response of the small intestinal mucosa. The infusion of the 31–43 fragment of α-gliadin, which contains the sequence Q-Q-Q-P, directly into the jejunum of treated coeliac patients was shown to be toxic by mucosal biopsies (Marsh *et al.* 1995).

This study deals with the proteolytic system of *Lact. plantarum* strains (CRL 759 and CRL 778) isolated from sourdough and their ability to hydrolyse the toxic 31–43 α-gliadin fragment.

MATERIALS AND METHODS

Micro-organisms and preparation of cell fractions

Lactobacillus plantarum CRL 759 and CRL 778 used in this study were obtained from the Culture Collection of Centro de Referencia para Lactobacilos, Tucumán-Argentina. The strains were grown in MRS broth (De Man *et al.* 1960) at 37°C and subcultured at least twice prior to experimental use. Cells of active cultures (end of the exponential phase) were harvested by centrifugation (7000 *g* for 15 min at 4°C), washed twice with 10 mmol l^{-1} potassium phosphate buffer (pH 7·0) and resuspended in the same buffer. Cells were disrupted with a French press and cell-free extracts (CE) were obtained by centrifugation (9000 *g*, 15 min at 4°C) (model B-22M centrifuge; International Equipment Company, Needham Heights, MA, USA). The cell walls and cell membrane fractions were obtained by a second centrifugation (145 000 *g*, 60 min at 4°C) and resuspended in 10 mmol l^{-1} potassium phosphate buffer (pH 7·0).

Enzyme assays

AP activity. AP activity was measured by using chromogenic substrates *p*-nitroanilide (*p*-NA) derivatives of L-anomers of leucine, lysine, alanine, valine, proline, methionine, phenylalanine, glutamic acid and glycine, by the method of El Soda and Desmazeaud (1982). The assay mixture contained 50 μl of the substrate (20 mmol l^{-1} in methanol), 2·6 ml of potassium phosphate buffer (10 mmol l^{-1}, pH 6·0) and 25 μl of enzyme solution (CE). After incubation at 37°C for 15 min, the reaction was stopped with 30% (v/v) acetic acid, and the colour intensity of the *p*-nitroaniline produced was measured at 410 nm in a Cecil Model 2021 series 2000 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). The concentration of *p*-nitroaniline was calculated from the derived value of the molar absorption coefficient ($E_{410} = 8800$ mmol l^{-1} cm^{-1}). One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of *p*-nitroaniline per minute under the assay conditions.

X-prolyl-dipeptidyl aminopeptidase and EP activities. X-prolyl-dipeptidyl aminopeptidase (PDA) activity was measured on Gly–Pro–*p*-NA and EP activity on *N*-succinyl-L-phenyl-alanine-*p*-NA (Suphepa) and *N*-glutaryl-L-phenyl-alanine-*p*-NA (Gluphepa) as substrates. The hydrolysis of synthetic substrates was measured by the method of El Soda and Desmazeaud (1982). The enzyme units were defined as before.

Dipeptidase and tripeptidase activities. Dipeptidase (DP) activity was measured on Leu–Leu, Gly–Gly, Leu–Pro, Gly–Tyr and tripeptidase (TP) activity on Leu–Leu–Leu, Gly–Gly–Gly, DL-Leu–Gly-DL-Phe and Leu–Gly–Gly as substrates, which were chosen according to the major amino acids residues present in the wheat flour (Glu, Pro and hydrophobic amino acids: Leu, Iso, Ala, Gly and Val).

The hydrolysis of synthetic substrates was carried out at 37°C and the activities were determined by the estimation of α-amino groups by the Cd-ninhydrin method (Doi *et al.* 1981). The assay mixture contained potassium phosphate buffer 10 mmol l^{-1}, pH 7·0 (400 μl), substrate (20 mmol l^{-1}, 50 μl) and enzyme solution (50 μl). After incubation at 37°C for 15 min, the reaction was stopped by addition of Cd-ninhydrin reagent and further incubation at 85°C for 5 min; the reaction mixture was cooled and the enzyme activity determined by reading the A_{505}. An enzyme unit was defined as the amount of enzyme required to release 1 μmol of amino acid per minute. The specific activity was defined as enzyme units per milligram of protein.

Effect of pH and temperature

The effect of pH on the activities of enzymes (AP, DP, TP and EP) was examined at 37°C in the pH range of 4·0–9·0. The buffers used were: citrate buffer (pH 4·0–6·0), potassium phosphate buffer (pH 5·6–8·0) and Tris–HCl buffer (pH 7·0–9·0). The effect of temperature was determined at pH 7·0 in the temperature range of 30–60° C. The enzyme mixture was equilibrated for 5 min at the test temperatures before the addition of the corresponding substrate.

Effect of chemical agents and metal ions on enzyme activities

A mixture containing CE, chemical reagents (0·1 mmol l^{-1}) or divalent cations (1 mmol l^{-1}) and 10 mmol l^{-1} potassium phosphate buffer pH 6·0 (AP) or pH 7·0 (DP and TP), was incubated at 37°C for 30 min. Reactions were initiated by adding the specific substrates.

Chemicals. The chemical reagents used were the following: ethylenediaminetetracetic acid (EDTA), *o*-phenanthroline, β-mercaptoethanol, L-cysteine, phenylmethylsulfonyl-fluoride (PMSF), dithiothreitol (DTT), *p*-chloro-mercuribenzoate (*p*-CMB). Divalent cations were added as chloride or sulfate salts. All the synthetic chromogenic substrates: L-Leu-*p*-NA, L-Ala-*p*-NA, L-Phe-*p*-NA, L-Pro-*p*-NA, L-Glu-*p*-NA, L-Lys-*p*-NA, Gly-*p*-NA, L-Met-*p*-NA, L-Val-*p*-NA, Gly-Pro-*p*-NA, *N*-succinyl-L-phenyl-alanine-*p*-NA (Suphepa), *N*-glutaryl-L-phenyl-alanine-*p*-NA (Gluphepa) and di- and tripeptides: Leu–Leu, Gly–Gly, Leu–Pro, Gly–Tyr; Leu–Leu–Leu, Gly–Gly–Gly, DL-Leu–Gly-DL-Phe and Leu–Gly–Gly were obtained from Sigma Chemical Company (St Louis, MO, USA).

Hydrolysis of the 31–43 α-gliadin fragment. The 31–43 fragment of the α-gliadin used in this study was chemically synthesized by the Neosystem Laboratoire (Strasbourg, France). The peptide has the following sequence: L-G-Q-Q-Q-P-F-P-P-Q-Q-P-Y. All enzyme preparations (cell-wall and intracellular extracts) used for the hydrolysis of the 31–43 α-gliadin fragment showed *c*. 80% hydrolysis on bradykinin substrate (P-P-G-F-S-P-F-R). Activity was determined by reverse-phase fast protein liquid chromatography (RP-FPLC) as described by Stepaniak and Fox (1995). The hydrolysis of the 31–43 α-gliadin fragment was evaluated in the reaction mixture contained 160 μl of 20 mmol l^{-1} phosphate buffer (pH 7·0), 75 μl of 4 mmol l^{-1} α-gliadin 31–43 fragment, 4 μl of NaN_3 (0·05% final concentration) and 100 μl of the enzyme preparation (cell-wall and intracellular extracts). The enzyme activity was stopped by the addition of 0·1% (final concentration, v/v) trifluoroacetic acid (TFA). Peptides were separated from the mixture by RP-FPLC using a PepRPC HR 5/5 column and FPLC equipment with a UV detector operating at 210 nm (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Elution was performed at a flow rate of 0·5 ml min^{-1} with a linear gradient (0–100%) of acetonitrile in 0·1% TFA.

Protein determination. The protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

Sourdough fermentation. Commercial wheat flour (moisture, 13·4%; protein, 10%) was used to prepare bread dough by mixing the following: flour, 200 g; water, 30 ml; and cell suspension, 89 ml, previously prepared by resuspending the cells into sterile tap water until a cell count of 10^9 CFU ml^{-1} of late log phase cells was reached (final cell count in the dough was 10^7 CFU ml^{-1}). The bread dough was prepared using a continuous high-speed mixer (90 rpm) (dough yield: 160). Dough produced with wheat flour and water, without adding LAB, was incubated under the same conditions and used as control. Sourdough fermentations were carried out in flasks at 30°C and samples were withdrawn aseptically at 0 and 6 h of fermentation.

Determination of viable cell counts, pH and amino acids concentration. Fermented dough (10 g) plus sterile distilled water (90 ml) were homogenized in a Virtis homogenizer (The Virtis Company, Gardiner, NY, USA), the cell viability was determined in the sourdough aqueous extracts by plating appropriate dilutions on MRS agar and incubated at 37°C for 72 h. The pH was measured with a FC 200B short Teflon body spear tip electrode (Sartorius Portable pH/mV Meter; Sartorius, Goettingen, Germany).

The sourdough aqueous extracts were filtered through a 0·22-μm-pore size filter (Sartorius AG-W-3400; Sartorius), derivatized in *o*-phthaldialdehyde derivatives, and the amino acid concentrations was determined by reversed-phase high-performance liquid chromatography analysis. The chromatographic separation was carried out using an ISCO system (ISCO, Lincoln, NE, USA) on an Spherisorb ODS2 C18 column, 4·6 × 250 mm, with an elution flow rate of 1 ml min^{-1} (ISCO, Lincoln, NE, USA), and a UV detector operating at 210 nm was used. Identification and quantification of amino acids were carried out by comparison using a standard mixture of amino acids (Sigma Chemical Company).

Statistical analysis

Data are shown as mean ± SD and are the results of three independent experiments with three replicates each. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett *t*-test. The statistical analyses were performed with the Minitab-12 software (Minitab Inc., State College, PA, USA) to determine the statistical significance ($P < 0·05$).

RESULTS

Peptidase activities (AP, DP, TP and EP) of *Lact. plantarum* CRL 759 and CRL 778 were detected mainly in the intracellular fraction; however, *Lact. plantarum* CRL 759 also displayed only 39% of total DP activity associated to the cell membrane-wall fraction.

The relative activity of the strains on *p*-NA derivatives of a series of L-amino acids are presented in Table 1. Both *Lact. plantarum* CRL 759 and CRL 778 showed a wide spectrum of AP activity, which was active against all substrates assayed chosen according to the major amino acid residues present in the gluten of wheat flour. The activity on Lys-*p*-NA as substrate was 152% higher than that observed with Leu-*p*-NA, the *p*-NA derivative used as standard. From the evaluation of two strains, *Lact. plantarum* CRL 759 had higher EP activity, and *Lact. plantarum* CRL 778 showed higher AP activities, except when using Lys-*p*-NA as substrate. The enzymes had high affinity for positively charged amino acids such as Lys or for hydrophobic amino acids such as Leu but had low activity towards Glu and Pro-*p*-NA (Table 1). The results obtained with Gly–Gly and Gly–Gly–Gly as substrates for DP and TP activities, respectively, did not differ markedly from those determined with Leu–Leu and Leu–Leu–Leu (data not shown); however, maximum enzymatic activities was observed with the latter substrates and they were chosen further to characterize DP and TP activities.

The effect of pH and temperature on AP, DP, TP and EP activities are presented in Table 2. AP, DP, TP and EP activities were determined with Leu-*p*-NA, Leu–Leu, Leu–Leu–Leu, and Gluphepa-*p*-NA as substrates respectively. The optimum pH and temperature values for all the peptidase activities were 6·0–8·0 and 37–45°C, respectively, nevertheless, high peptidase activities at the pH (4·5–5·5) and temperatures (30°–35°C) of sourdough fermentation were observed.

Table 1 Hydrolysis of chromogenic synthetic substrates by cell-free extract of *Lactobacillus plantarum* CRL 759 and CRL 778

	Peptidase activity (μmol min^{-1} mg protein^{-1})	
Substrate	*Lact. plantarum* CRL 759	*Lact. plantarum* CRL 778
Leu-*p*-NA	12·52 ± 1·7	17·47 ± 2·3
Ala-*p*-NA	5·10 ± 0·9	7·95 ± 1·4
Pro-*p*-NA	0·93 ± 0·03	1·36 ± 0·8
Phe-*p*-NA	7·14 ± 0·5	7·16 ± 1·1
Glu-*p*-NA	2·60 ± 0·8	3·70 ± 1·3
Lys-*p*-NA	31·50 ± 3·5	25·68 ± 2·9
Gly-*p*-NA	1·95 ± 0·1	3·00 ± 0·6
Met-*p*-NA	5·0 ± 1·0	6·64 ± 0·9
Val-*p*-NA	2·20 ± 0·3	3·14 ± 0·7
Gly-Pro-*p*-NA	1·81 ± 0·8	9·43 ± 0·9
Gluphepa-*p*-NA	2·60 ± 0·5	1·05 ± 0·02
Suphepa-*p*-NA	3·30 ± 0·9	0·61 ± 0·02

p-NA, *p*-nitroanilide.
Values are expressed as mean ± SD of three independent measurements.

Table 2 Effect of pH and temperature on peptidase activities of *Lactobacillus plantarum* CRL 759 and CRL 778

	pH optimum		Temperature optimum (°C)	
Enzyme*	CRL 759	CRL 778	CRL 759	CRL 778
Aminopeptidase	7·0	6·0	37	37
Dipeptidase	8·0	6·0	45	37
Tripeptidase	8·0	8·0	37	37
Endopeptidase	6·5	6·0	37	37

*The enzyme activities were determined as described in Materials and methods.

AP and DP activities of *Lact. plantarum* CRL 759 and CRL 778 were strongly inhibited by 0·1 mmol l^{-1} of EDTA and *o*-phenanthroline (metal-complexing reagents) (Table 3). TP activities of *Lact. plantarum* CRL 759 and CRL 778 were inhibited by *o*-phenanthroline 28% and 83% respectively. DTT, a thiol group reducer, had no effect on AP activity, but showed a dual effect on DP activity as it stimulated (27%) and inhibited (38%) the enzyme activity of CRL 759 and CRL 778 respectively. DTT inhibited the TP activity by 61% in both strains.

Table 4 shows the effect of several divalent cations on the enzyme activities. Under the conditions used, AP, DP and TP activities of both *Lact. plantarum* CRL 759 and CRL 778 were strongly inhibited (73–100%) by Fe^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+} and Cd^{2+}. The ion Co^{2+} was the only one having a pronounced stimulatory effect on peptidase activities of *Lact. plantarum* strains. The Mg^{2+} ion stimulated DP and TP activities of *Lact. plantarum* CRL 759 by 42–43% and TP activity of *Lact. plantarum* CRL 778 by 71%. *p*-CMB, a sulfhydryl-blocking reagent, inhibited AP (61%) and DP (52%) activities of *Lact. plantarum* CRL 778 (Table 3), which were also severely inhibited (96–97%) by the heavy metal Hg^{2+} (Table 4) indicating that these enzymes are metalloenzymes and at least one sulfhydryl group is essential for the enzyme activity.

Amino acid evolution during sourdough fermentation. Individual amino acids were grouped according to their chemical structures as dicarboxylic acids (Asp and Glu), aliphatic (Leu, Ala, Val, Gly and Ile), basic (Arg, Lys and His), sulfur-containing (Met and Cys), hydroxy (Ser and Thr) and aromatic (Phe and Tyr) for easier discussion of results. Contents of amino acid groups in the dough were determined by the sum of individual amino acid concentrations. The results obtained showed an increase of basic (30%) and aromatic (55%) amino acids in dough fermented by *Lact. plantarum* CRL 759 while in dough containing *Lact. plantarum* CRL 778 an increase of basic (166%), aliphatic (73%), dicarboxylic (20%) and aromatic (95%) amino acids was observed after 6 h of incubation, with respect to

Table 3 Influence of chelating agents and group-specific inhibitors on enzyme activities of *Lactobacillus plantarum* CRL 759 and CRL 778

Chemical reagents (0·1 mmol l^{-1})	Relative activity (%)					
	Aminopeptidase*		Dipeptidase*		Tripeptidase*	
	CRL 759	CRL 778	CRL 759	CRL 778	CRL 759	CRL 778
Control	100	100	100	100	100	100
EDTA	12 ± 0·9†	10 ± 0·4	7 ± 0·5	3 ± 0·2	3 ± 0·3	4 ± 0·2
o-Phenanthroline	23 ± 1·0	17 ± 0·8	7 ± 0·3	8 ± 0·8	72 ± 2·1	17 ± 0·9
β-Mercaptoethanol	98 ± 1·4	94 ± 1·9	87 ± 1·3	71 ± 2·1	80 ± 2·0	73 ± 2·1
L-cysteine	90 ± 2·0	125 ± 3·4	73 ± 2·4	57 ± 1·5	39 ± 1·1	41 ± 1·2
PMSF	87 ± 1·6	93 ± 2·5	80 ± 2·5	41 ± 1·3	91 ± 2·4	68 ± 2·1
DTT	98 ± 2·4	103 ± 2·9	127 ± 3·0	62 ± 2·0	39 ± 1·3	39 ± 1·5
p-CMB	68 ± 2·0	39 ± 1·1	89 ± 2·0	48 ± 1·3	87 ± 1·5	77 ± 2·1

DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; *p*-CMB, *p*-chloromercuribenzoate, PMSF, phenylmethylsulfonyl fluoride.
*Aminopeptidase, dipeptidase and tripeptidase activities were determined against Leu-*p*-NA, Leu–Leu and Leu–Leu–Leu as substrates, respectively, as described in Materials and methods. The enzyme was pre-incubated with the chemical reagents for 30 min at 37°C before substrate addition.
†Mean ± SD of three independent measurements.

Table 4 Effect of metal ions on the enzyme activities of *Lactobacillus plantarum* CRL 759 and CRL 778

Cations (10 mmol l^{-1})	Relative activity (%)					
	Aminopeptidase (AP)*		Dipeptidase (DP)*		Tripeptidase (TP)*	
	CRL 759	CRL 778	CRL 759	CRL 778	CRL 759	CRL 778
Control	100	100	100	100	100	100
Co^{2+}	150 ± 3·0	143 ± 2·0	258 ± 2·6	192 ± 3·5	243 ± 3·7	149 ± 2·6
Mn^{2+}	11 ± 1·1	13 ± 0·9	20 ± 1·3	18 ± 0·5	57 ± 2·3	67 ± 2·1
Fe^{2+}	6 ± 0·4	6 ± 0·2	4 ± 0·1	4 ± 0·3	15 ± 0·8	13 ± 0·9
Cu^{2+}	17 ± 1·0	7 ± 0·4	0 ± 0·0	2 ± 0·2	1 ± 0·1	0 ± 0·0
Ca^{2+}	66 ± 2·9	52 ± 2·0	13 ± 1·8	10 ± 0·7	14 ± 1·0	27 ± 1·3
Hg^{2+}	13 ± 1·0	4 ± 0·3	11 ± 0·8	3 ± 0·3	5 ± 0·2	0 ± 0·0
Mg^{2+}	47 ± 2·2	30 ± 2·0	142 ± 3·5	85 ± 3·5	143 ± 2·7	171 ± 3·0
Zn^{2+}	11 ± 0·6	7 ± 0·5	34 ± 1·9	26 ± 1·0	21 ± 1·2	18 ± 1·7
Cd^{2+}	21 ± 1·1	10 ± 0·9	9 ± 0·3	5 ± 0·2	27 ± 1·2	4 ± 0·1

Values are expressed as mean ± SD of three independent measurements.
*AP, DP and TP activities were determined as described in Materials and methods. The enzyme was pre-incubated for 10 min at 37°C with divalent cations in 10 mmol l^{-1} potasium phosphate buffer, pH 6·0 for AP and pH 7·0 for DP and TP activities, prior to the addition of the substrates.

unstarted dough (Fig. 1). The concentration of sulfur- and hydroxyl-containing amino acids decreased 46% and 10% in dough fermented by *Lact. plantarum* CRL 759 and CRL 778 respectively.

Hydrolysis of 31–43 α-gliadin fragment

The capacity of *Lact. plantarum* strains to hydrolyse synthetic peptides, homologous to the α-gliadin 31–43 amino sequence, was evaluated. The degradation of this peptide, a toxic factor related to the coeliac disease, was assayed by using cell wall and intracellular extracts, which showed the major part of peptidase activities. After 4 h of incubation, hydrolysis of the 31–43 peptide of α-gliadin by enzyme preparations of *Lact. plantarum* CRL 759 and CRL 778 was 73% and 36% (expressed as percentage reduction of the peak area of the untreated substrate) respectively (Fig. 2).

DISCUSSION

The intracellular peptide hydrolase system of *Lact. plantarum* CRL 759 and CRL 778 involves the activity of AP, DP, TP, PDA and EP enzymes and it is close to that of *Lactobacillus helveticus* (Miyakawa *et al.* 1992), *Lactococcus*

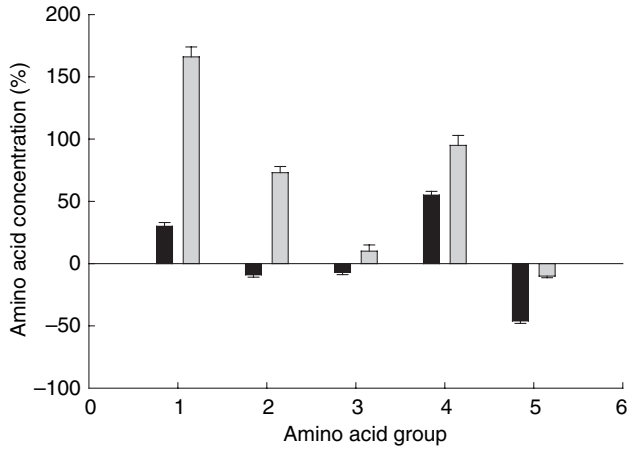

Fig. 1 Amino acids content of dough bread started with *Lactobacillus plantarum* with respect to the control (0%, dough fermented without lactic acid bacteria added); ■, *Lact. plantarum* CRL 759; ▩, *Lact. plantarum* CRL 778

lactis, *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (El Soda and Desmazeaud 1982). Similarities in the enzymes characteristics were found for both *Lact. plantarum* CRL 759 and CRL 778, nevertheless, significant differences concerning the level of specific activities were detected; *Lact. plantarum* CRL 778 displayed the highest AP activities. As wheat flour contains variable but considerable amounts of free amino acids and low molecular weight peptides, the DP and TP may be more useful than other proteolytic activities for the growth of lactobacilli in sourdough (Gobbetti *et al.* 1996b).

All *Lact. plantarum* CRL 759 and CRL 778 peptidase enzymes were located in the intracellular fraction, however, DP activity of *Lact. plantarum* CRL 759 also showed low activity associated to the cell membrane fraction. DP and TP activities associated mainly to the cell membrane-wall fraction were found in *Lactobacillus reuteri* CRL 1098, other sourdough LAB (Rollán and Font de Valdez 2001). Evidence for the membrane localization of DP and TP in LAB was also reported for *L. lactis* ssp. *lactis* (Tan *et al.* 1991) and sourdough *Lactobacillus* (Gobbetti *et al.* 1996a).

Lactobacillus plantarum CRL 759 and 778 have a wide spectrum of peptidase activity (AP, DP, TP, PDA and EP) profiles, which were active against all the substrates used in this study. AP activities using *p*-NA derivatives, as substrates were higher when a hydrophobic amino acid (Leu, Ala or Phe) or diaminomonocarboxylic acid (Lys) occupied the N-terminal position. When leucyl di- or tripeptides were used as substrates, the DP or TP activities were relatively high. The higher DP activity on Leu–Leu rather than on Leu–Gly, and the preferential hydrolysis of dipeptides containing Leu, Ala or Phe, showed a considerable similarity between the DP substrate specificity from

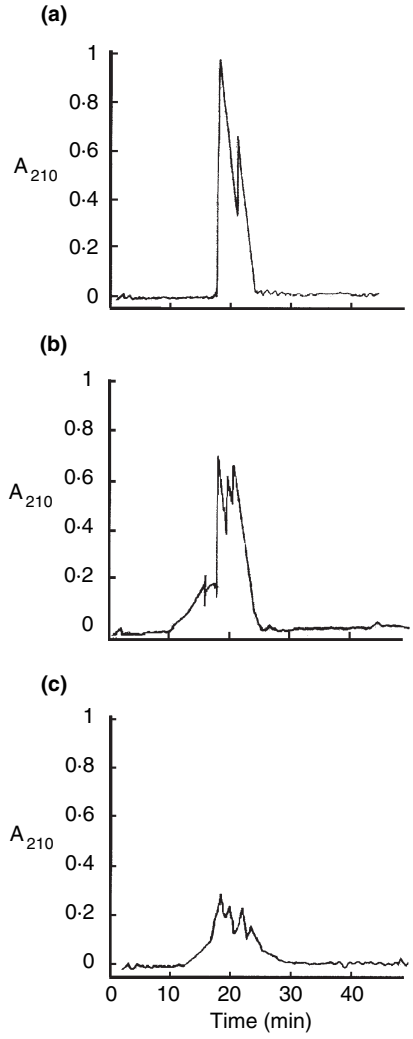

Fig. 2 Reverse-phase fast protein liquid chromatograms of the 31–43 α-gliadin fragment (a), 31–43 α-gliadin fragment treated with a mixture of cell wall and cytoplasmic enzyme preparations of *Lactobacillus plantarum* CRL 778 (b), *Lact. plantarum* CRL 759 (c)

Lact. plantarum CRL 759 and CRL 778 and that isolated from *Lact. bulgaricus* B14 (Wohlrab and Bockelmann 1992).

EDTA and 1,10-phenanthroline, metal-complexing reagents, inhibited AP activities from *Lact. plantarum*, indicating that these peptidases are metalloenzymes, and thus, making these enzymes comparable with those isolated from several other LAB (Tan *et al.* 1991; Miyakawa *et al.* 1992; Rollán and Font de Valdez 2001). Metal-dependent enzymes with Lys-*p*-NA or Leu-*p*-NA as the preferential substrate, and with an optimum pH in the range of 6·0–8·0, have also been reported in *Lactobacillus casei* ssp. *rhamnosus* (Arora and Lee 1994), *Lactobacillus delbrueckii* ssp. *bulgaricus* (Wohlrab and Bockelmann 1994). TP activity of *Lact. plantarum* strains showed different sensitivities for *o*-phen-

anthroline (metal-complexing reagent), TP from CRL 778 was strongly inhibited (83%) while the enzyme from CRL 759 was slightly inhibited (28%) by this reagent. AP of *Lact. plantarum* CRL 759 and CRL 778 and DP of *Lact. plantarum* CRL 759 were not inhibited by specific S–S reducing agents, such as DTT or β-mercaptoethanol, possibly indicating that intact disulfide groups were not essential for these enzymes mechanism of action. AP (CRL 759 and CRL 778), DP (CRL 759) and TP (CRL 759) were not serine enzymes, as the specific-serine protease inhibitor PMSF did not inhibit them.

The effects of divalent cations on peptidase activities were also studied. Fe^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+} and Cd^{2+} strongly inhibited all peptidase activities. AP, DP and TP from *Lact. plantarum* CRL 759 and CRL 778 showed different sensitivities for Mn^{2+}, Ca^{2+}, and Mg^{2+}. The AP enzymes of both *Lact. plantarum* strains were activated by Co^{2+}, inhibited by chelating reagents such as EDTA and *o*-phenanthroline, and partially inhibited by the sulfhydryl group modifying reagent (*p*-CMB). These observations suggest that the AP from *Lact. plantarum* CRL 759 and CRL 778 are metalloenzymes possessing a thiol group at their active sites.

The amino acids evolution during sourdough fermentation is complex and, it is related to metabolic requirements and the proteolytic activities of LAB. The peptidase activities may contribute to proteolysis during sourdough fermentation by degrading smaller peptides into amino acids, which would result in either desirable texture or aroma changes in bread making. In this study, biochemical changes during dough fermentation in free amino acid content clearly differentiated doughs with and without microbial starters. Dicarboxylic and aliphatic amino acids were predominant amino acids in dough bread. Basic and aromatic amino acids increased in dough bread started with LAB in contrast to aliphatic (9%), dicarboxylic (7%) and sulfur- and hydroxyl-containing amino acids (46%), which decreased in dough fermented by *Lact. plantarum* CRL 759. The net decrease in some individual amino acids suggests that they are utilized as metabolites in a greater extent rather than they are being replaced by proteolytic activity. Degree of proteolysis was higher for dough started with *Lact. plantarum* CRL 778, producing significant levels of basic, aliphatic, and aromatic amino acids. The qualitative and quantifiable composition of amino acids of fermented doughs are of interest as they affect positively bread flavour mainly through the formation of Maillard compounds where some amino acids react with sugars forming typical flavours and aromas described as toasty and bread like (Collar *et al.* 1991). Amino acids represent precursors of aromatic compounds produced during heating. Thus, selection and utilization of proteolytic LAB have important effects on overall quality of bread (Gobbetti *et al.* 1994).

Investigations carried out with fragments of α-gliadin or with homologous synthetic peptides to this α-gliadin amino acid sequence and the sequence 44–55, confirmed the toxicity of these proteins and peptides for coeliac patients (Maiuri *et al.* 1996; Silano and De Vincenzi 1999b). *Lactobacillus plantarum* CRL 778 and CRL 759 were both able to hydrolyse the chemically synthesized 31–43 fragment of α-gliadin. Results reported by Di Cagno *et al.* (2002) showed that this fragment was also hydrolysed by *Lactobacillus alimentarius* 15M and *Lact. brevis* 14G in a range of 54–50%, and by *Lactobacillus sanfransisco* 7A (43%) and *Lactobacillus hilgardii* 51B (35%).

The results of this study show that *Lact. plantarum* CRL 778 was more proteolytic towards synthetic substrates (*p*-NA, di- and tri-peptides) than *Lact. plantarum* CRL 759; however, the former strain was less active than *Lact. plantarum* CRL 759 to hydrolyse the toxic 31–43 α-gliadin fragment. This result could be explained by differences in the peptidases substrates specificities. Thus content of biologically active peptides, which usually contain a large proportion of Pro residues within the sequence, make them very resistant to hydrolysis by nonspecific Pro-bonds peptidases (Cunningham and O'Connor 1997; Shan *et al.* 2002). Proline is unique among the 20 amino acids because of its cyclic structure. This specific conformation imposes many restrictions on the structural aspects of peptides and proteins and confers particular biological properties, because of this; a group of specific peptidases are necessary to hydrolyse peptide bonds in which a proline residue occurs as a potential substrate (Hausch *et al.* 2003).

In this study, it has been shown that the use of *Lact. plantarum* in sourdough fermentation allowed reducing a gliadin-like fraction. Further *in vitro* and *in vivo* studies, involving sourdough LAB on the hydrolysis of toxic fragments of gliadin, are necessary to understand the role of these bacteria in reducing the gluten allergenic compounds responsible for coeliac disease.

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