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ORIGINAL ARTICLE

Functionality of lactic acid bacteria peptidase activities in the hydrolysis of gliadin-like fragments

C.L. Gerez[1], G. Font de Valdez[1,2] and G.C. Rollán[1]

1 Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco, Tucumán, Argentina
2 Cátedra Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

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Correspondence
Dr Graciela Rollán, Chacabuco 145 (4000)-Tucumán, Argentina.
E-mail: rollan@cerela.org.ar

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Abstract

Aims: To evaluate the role of the peptidase activities from sourdough lactic acid bacteria (LAB) in the degradation of α-gliadin fragments.
Methods and Results: Different proline-containing substrates were hydrolysed by LAB indicating pro-specific peptidase activities. *Lactobacillus plantarum* CRL 775 and *Pediococcus pentosaceus* CRL 792 displayed the highest tri- and di-peptidase activities, respectively. *Lactobacillus plantarum* strains hydrolysed more than 60% α-gliadin fragments corresponding to the 31–43 and 62–75 amino acids in the protein after 2 h. None of the LAB strains alone could hydrolyse 57–89 α-gliadin peptide; however, the combination of *L. plantarum* CRL 775 and *P. pentosaceus* CRL 792 led to hydrolysis (57%) of this peptide in 8 h.
Conclusions: The capacity of LAB strains to degrade α-gliadin fragments was not correlated to individual peptidase activities. Several strains separately degraded the 31–43 and 62–75 α-gliadin fragments, while the 57–89 peptide degradation was associated with the combination of peptidase profiles from pooled LAB strains. This is the first report on the peptide hydrolase system of sourdough pediococci and its ability to reduce α-gliadin fragments.
Significance and Impact of the Study: This study contributes to a better knowledge of sourdough LAB proteolytic system and its role in the degradation of proline-rich α-gliadin peptides involved in celiac disease.

Introduction

Gluten, the main wheat flour protein is composed of two fractions: gliadins (α-, β-, γ- and ω-gliadin sub-roups) and glutenins. Both fractions are important contributors to the rheological properties of wheat dough. However, several residues of the N-terminus of α-gliadin are immunologically active, e.g. amino acids at position 31–43, 62–75 and 57–89 in the protein (Fasano and Catassi 2001; Maki *et al.* 2003). These fragments are difficult to hydrolyse owing to their high content of proline (P) residues within the sequence. Proline has a cyclic structure, which imposes many restrictions in the structural aspects of peptides and proteins, giving particular biological properties; hence, a group of P-specific peptidases are necessary to hydrolyse this type of peptides (Hausch *et al.* 2002). Certain bacterial and fungal enzymes are able to degrade various proline-rich gluten peptides; however, they have a limiting practical application (Stepniak *et al.* 2005; Bethune *et al.* 2006; Siegel *et al.* 2006; Mitea *et al.* 2008).

The proteolytic system of lactic acid bacteria (LAB) species commonly isolated from traditional sourdoughs, such as *Lactobacillus sanfranciscensis, Lactobacillus brevis* and *Lactobacillus plantarum*, has been subjected to several studies (Gobbetti *et al.* 1996; Gallo *et al.* 2005; Rollán *et al.* 2005; Vermeulen *et al.* 2005). The gluten proteolysis by sourdough LAB releases small peptides and free amino acids, which are important for the nutritional and technological points of view (Rollán and Font de Valdez 2001). Moreover, LAB might be used as a tool to hydrolyse α-gliadin-derived fragments in wheat-baked goods (Di Cagno *et al.* 2002, 2004). Recently, the combination of LAB strains with fungal proteases was considered an efficient approach to eliminate gluten in baked products (Rizzello *et al.* 2007).

In previous research, 13 sourdough LAB strains (nine lactobacilli and four pediococci) were selected for their ability to hydrolyse gluten (Gerez *et al.* 2006). The aim of the present work was to evaluate the peptidase activities of sourdough LAB (lactobacilli and pediococci strains) and to determine their potential relationship between these enzymatic activities and the reduction of the α-gliadin-fragments.

Materials and methods

Micro-organisms and growth conditions

Eleven strains isolated from homemade wheat sourdough and belonging to the culture collection of the Centro de Referencia para Lactobacilos (CERELA- CONICET, Tucumán, Argentina) were used in this study: *L. brevis* CRL 781, *Lactobacillus curvatus* CRL 760, *L. plantarum* CRL 769 and 775, *Lactobacillus reuteri* CRL 1098, 1099 and 1100, and *Pediococcus pentosaceus* CRL 761, 792, 793 and 797. The strains were grown in de Man Rogosa sharpe (MRS) broth (de Man *et al.* 1960) at 37°C for 16 h; subsequently, cells 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), suspended (20% w/v) in the same buffer, and disrupted with French press (French Pressure Cells; Thermo-Spectronic, Rochester, NY, USA). Cell-free extracts (CFE) were obtained by centrifugation (9000 ***g*** for 15 min at 4°C) and used for enzymatic assays. Protein concentration of CFE was determined according to Bradford (1976) using bovine serum albumin as standard.

Aminopeptidase (AP), X-prolyl-dipeptidyl aminopeptidase (PDA), endopeptidase (EP), dipeptidase (DP) and tripeptidase (TP) activities were determined as described by Rollán *et al.* (2005).

Hydrolysis of α-gliadin-derived fragments

The degradation of the α-gliadin fragments was determined in the reaction mixture containing 160 μl of 20 mmol l^{-1} phosphate buffer (pH 7·0); 75 μl of 1 mmol l^{-1} of each α-gliadin fragment; 4 μl of 0·05% (w/v) NaN_3, and 100 μl of CFE (5 mg protein per ml). This mixture was incubated at 37°C for 24 h, and the reaction was stopped by adding 0·1% (v/v) trifluoracetic acid (TFA) at regular intervals (2 h). The concentration of the α-gliadin fragment was determined by RP-HPLC (ISCO system, Microsorb 100A column, UV detector; Lincoln, NE, USA) operating at 210 nm. A linear gradient (20–100%) of acetonitrile in 0·1% (v/v) TFA was used as mobile phase at a flow rate of 0·5 ml min^{-1}. Results were expressed as percentage (%) of the peak area reduction corresponding to the substrate without treatment with CFE.

The α-gliadin fragments corresponding to amino acids 31–43 (L-G-Q-Q-Q-P-F-P-P-Q-Q-P-Y); 62–75 (P-Q-P-Q-L-P-Y-P-Q-P-Q-S-F-P) and 57–89 (L-Q-L-Q-P-F-P-Q-P-Q-L-P-Y-P-Q-P-Q-L-P-Y-P-Q-P-Q-L-P-Y-P-Q-P-Q-P-F) in the protein, were chemically synthesized at Bio-Synthesis Lab (Lewisville, TX, USA).

Statistical analysis

Assays were determined in three independent experiments and mean values ± standard deviation (SD) are given. Data were compared by analisis of variance (ANOVA) and Dunnett *t*-test. The statistical significance ($P < 0·05$) was determined by using Minitab-12 software. The principal-component analysis (PCA) was applied to the LAB peptidase activity data.

Results and discussion

The LAB strains used in this study, previously selected by their ability to hydrolyse gluten (Gerez *et al.* 2006), showed wide spectrum of peptidase activities (Table 1). The AP activities were, in general, higher when leucine (L-*p*NA) occupied the N-terminal position with respect to glutamic acid (Q-*p*NA) or proline (P-*p*NA). All LAB strains, except *P. pentosaceus* CRL 793, showed prolyl iminopeptidase activity (P-*p*NA) with values between 0·9 and 6·1 μmol min^{-1} mg^{-1} protein. Among the LAB tested, 36% showed DP activity on L-P dipeptide (prolidase activity). LAB strains showed X-prolyl dipeptidyl aminopeptidase activity (PDA) between 10·4 and 37·6 μmol min^{-1} mg^{-1} protein. The prolyl iminopeptidase, PDA, and prolidase activities indicated the presence of P-specific peptidases, which are considered key enzymes for degradation of P-rich α-gliadin polypeptides in LAB strains. These polypeptides are involved in the immune response of celiac patients (Gallo *et al.* 2005).

Tripeptidase enzymes showed a broad range of activity values; *L. plantarum* CRL 775 showed the highest (476 μmol min^{-1} mg^{-1} protein), while the lowest (28 μmol min^{-1} mg^{-1} protein) corresponded to *L. reuteri* CRL 1099. Only five strains (CRL 769, CRL 775, CRL 1098, CRL 781 and CRL 792) displayed EP activities on *N*-succinyl L-phenyl-alanine-*p*-NA (SF) and *N*-glutaryl L-phenyl-alanine-*p*-NA (QF).

To compare the peptidase profile of assayed LAB strains, data obtained were analysed by the PCA). In Fig. 1a,b, the LAB strains, represented as points in the plane, are disposed in different quadrants according to their peptidase activity profiles. Distances between the points indicate differences in their proteolytic profiles.

Table 1 Peptidase activities of sourdough lactic acid bacteria (LAB) strains

	Aminopeptidases			Dipeptidases		PDA		Tripeptidases		Endopeptidases	
LAB	L-*p*-NA	P-*p*-NA	Q-*p*-NA	L-L	L-P	GP	L-L-L	L-G-G,	L-G-F	SF	QF
L. brevis											
CRL 781	47* ± 2	3·6 ± 0	3·0 ± 0	133 ± 10	0·0 ± 0	23·3 ± 1	142 ± 9	150 ± 11	33 ± 8	3·1 ± 0	3·6 ± 0
L. curvatus											
CRL 760	14 ± 3	6·1 ± 1	2·2 ± 0	71 ± 6	0·0 ± 0	10·4 ± 1	345 ± 9	326 ± 9	101 ± 5	0·0 ± 0	4·8 ± 0
L. plantarum											
CRL 769	28 ± 0	0·9 ± 0	5·9 ± 0	44·4 ± 7	0·0 ± 0	35·2 ± 1	130 ± 11	245 ± 12	52 ± 15	1·7 ± 0	0·8 ± 0
CRL 775	39 ± 0	1·7 ± 0	8·6 ± 0	174 ± 16	0 ± 0	30·3 ± 3	459 ± 10	476 ± 29	214 ± 5	4·7 ± 0	7·0 ± 0
P. pentosaceus											
CRL 761	89 ± 3	3·7 ± 1	0·6 ± 0	77 ± 6	0·0 ± 0	27·4 ± 1	217 ± 14	290 ± 0	79 ± 0	0·0 ± 0	0·0 ± 0
CRL 792	64 ± 2	4·1 ± 1	20·6 ± 0	183 ± 6	7·0 ± 3	33·9 ± 7	298 ± 8	438 ± 10	126 ± 2	1·5 ± 0	10·6 ± 1
CRL 793	25 ± 1	0·0 ± 0	1·5 ± 0	64 ± 2	0·0 ± 0	30·2 ± 1	149 ± 7	200 ± 15	67 ± 1	0·0 ± 0	3·7 ± 0
CRL 797	10 ± 2	1·1 ± 0	1·2 ± 0	58 ± 4	0·0 ± 0	25·6 ± 3	168 ± 10	120 ± 7	36 ± 6	0·4 ± 0	0·0 ± 0
L. reuteri											
CRL 1098	32 ± 0	4·5 ± 1	4·4 ± 0	10 ± 0	5·5 ± 2	25·6 ± 0	33 ± 4	127 ± 8	33 ± 3	2·5 ± 0	1·2 ± 0
CRL 1099	27 ± 5	2·9 ± 1	0·0 ± 0	21 ± 6	3·1 ± 1	37·6 ± 2	30 ± 6	53 ± 10	28 ± 10	0·0 ± 0	0·0 ± 0
CRL 1100	15 ± 0	1·2 ± 0	0·0 ± 0	27 ± 0	6·6 ± 3	23·3 ± 1	47 ± 3	171 ± 8	36 ± 1	1·2 ± 0	0·0 ± 0

L., Lactobacillus; PDA, X-prolyl-dipeptidyl aminopeptidase; L-L, Leu-Leu; L-P, Leu-Pro; GP, Gly-Pro-*p*-NA; L-L-L, Leu-Leu-Leu; L-G-G, Leu-Gly-Gly; L-G-F, Leu-Gly-Phe; SF, *N*-succinyl-L-phenyl-alanine-*p*-NA; QF, *N*-glutaryl-l-phenyl-alanine-*p*-NA.
*In μmol min^{-1} mg^{-1} protein.

Lactobacillus reuteri CRL 1098, CRL 1099 and CRL 1100 showed similar enzymes behaviour, e.g. high activity on L-P and low TP activity (Fig. 1a: quadrant A). *Lactobacillus plantarum* CRL 775, which displayed opposite characteristics to these strains, was placed in Fig. 1a (quadrant D). *Pediococcus pentosaceus* strains (CRL 761, CRL 793 and CRL 797) showed similar EP activities but had no activity on L-P (Fig. 1a: quadrant C), while *P. pentosaceus* CRL 792 (Fig. 1a: quadrant B) exhibited high activity on L-P, L-*p*NA, Q-*p*Na and QF. *Lactobacillus curvatus* CRL 760 showed the highest (6·1 μmol min^{-1} mg^{-1} protein) P-*p*NA activity (Fig. 1b: quadrant B), which was higher than that reported by other authors for combinations of different LAB strains (Di Cagno *et al.* 2002; de Angelis *et al.* 2006).

The LAB strains used in this work were further evaluated for their ability to hydrolyse the chemically synthesized α-gliadin fragments: 31–43, 62–75 and 57–89, considered as allergenic for celiac patients (Fasano and Catassi 2001; Maki *et al.* 2003). *Lactobacillus plantarum* CRL 769 and CRL 775 hydrolysed more than 85% of the α-gliadin fragments 31–43 and 62–75 after 2 h, whereas *L. reuteri* CRL 1099 showed no activity on these fragments (Fig. 2a,b). *Pediococcus pentosaceus* CRL 793 and CRL 797 degraded 90% of the 62–75 α-gliadin fragment after 6 h, while they were not able to hydrolyse the 31–43 fragment. Previous studies reported that a pool of LAB strains was able to reduce 50% (4 h) and 100% (30 min) the α-gliadin fragments 31–43 and 62–75, respectively (Di Cagno *et al.* 2002). Our results show that *L. plantarum* strains hydrolysed the 31–43 α-gliadin fragment with higher efficiency (greater than 85% in 2 h), while the 62–75 fragment was degraded by all the evaluated LAB strains with lower efficiency (less 80% in 2 h) with respect to that reported by Di Cagno *et al.* (2002).

None of the LAB strains assayed was capable to hydrolyse the 57–89 α-gliadin fragment, even after 24 h. This fact led us to assay seven different mixtures of CFE of LAB strains (in a 1 : 1 ratio) to evaluate the degradation of this fragment (Table 2). These combinations were able to hydrolyse, in varying degrees, the 57–89 fragment. The greatest degradation (56·6%) was obtained with the mixture *P. pentosaceus* CRL 792/*L. plantarum* CRL 775 after 8 h. These strains showed high AP activities on Q-*p*NA, EP on QF, DP on L-L and TP on the tripeptides evaluated (Table 1). The smallest degradation (21·3%) of the 57–89 fragment was obtained with the combination of *L. reuteri* CRL 1100/CRL 1099, which showed low TP activity and absence of activity on Q-*p*NA and on QF.

Peptidase activities of each LAB strain included in the mixture *P. pentosaceus* CRL 792/*L. plantarum* CRL 775 and *L. reuteri* CRL 1100/CRL 1099 were compared. All strains showed activity on P-*p*NA and on L-P dipeptide, both substrates containing proline. On the other hand, the combinations: *L. plantarum* CRL 775/*P. pentosaceus* CRL 761 and *L. curvatus* CRL 760/*L. plantarum* CRL 775 hydrolysed up to 50% the 57–89 fragment in 24 h, but they showed no prolidase activity, indicating that this activity was not essential to hydrolyse the 57–89 fragment.

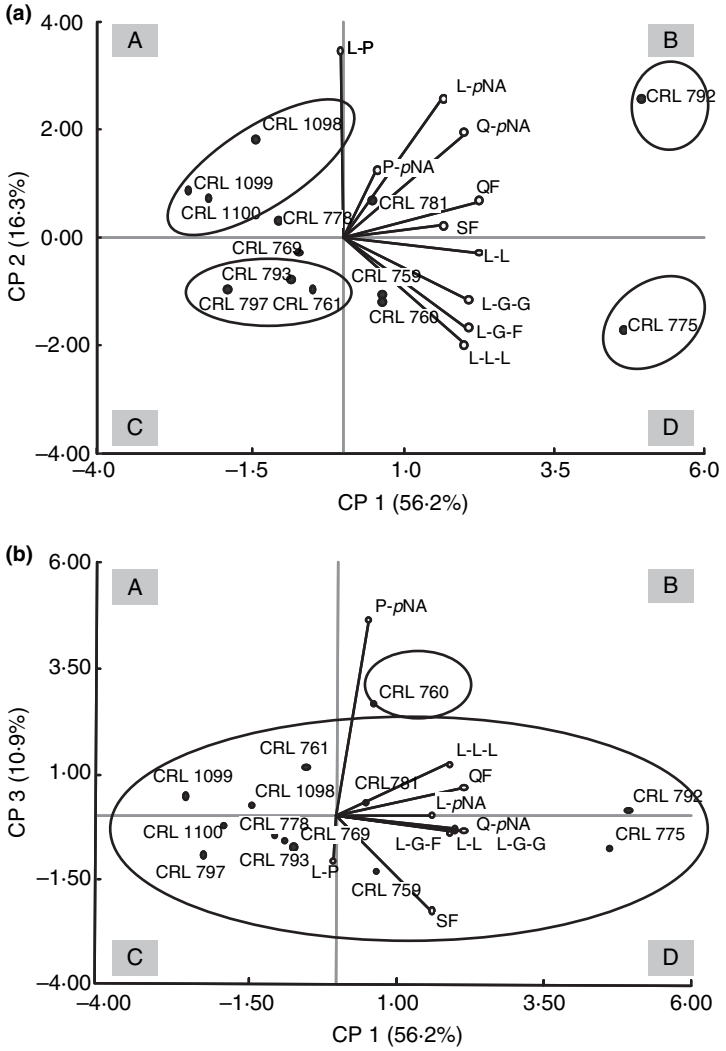

Figure 1 Principal component analyses of lactic acid bacteria (LAB) peptidase activities: (a) PC1 *vs.* PC2 and (b) PC1 *vs.* PC3.

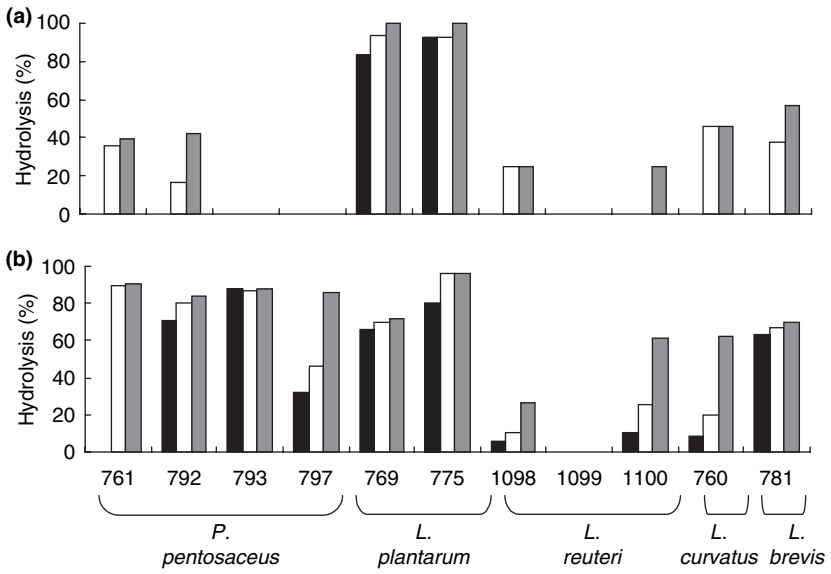

Figure 2 Hydrolysis of the 31–43 (a) and 62–75 (b) α-gliadin-derived fragments by LAB strains. 2 h 4 h 6 h.

Table 2 Hydrolysis of the 57–89 α-gliadin fragment using different lactic acid bacteria (LAB) combinations

LAB	Time (h) 8	24
L. plantarum CRL 759 and *L. curvatus* CRL 760	2·4* ± 4	35·5 ± 1
L. plantarum CRL 775 and *L. curvatus* CRL 760	29·0 ± 2	44·1 ± 3
L. plantarum CRL 775 and *P. pentosaceus* CRL 761	10·6 ± 1	51·5 ± 6
L. plantarum CRL 775 and *P. pentosaceus* CRL 792	56·6 ± 0	60·5 ± 4
P. pentosaceus CRL 792 and *L. curvatus* CRL 760	19·3 ± 5	25·1 ± 6
P. pentosaceus CRL 761 and CRL 792	41·2 ± 2	47·7 ± 1
L. reuteri CRL 1100 and CRL 1099	21·5 ± 0	21·3 ± 2

L., Lactobacillus.
*Percentage of reduction (%) of the peak area corresponding to the 57–89 fragment without treatment with cell-free extracts.

The enzymatic activities of the mixtures *P. pentosaceus* CRL 761/CRL 792 and *L. plantarum* CRL 775/*P. pentosaceus* CRL 761 were also compared. The strains of both combinations had high activity on L-*p*NA y Q-*p*NA, but only the first mixture showed prolidase activity and degraded (41·2%) the 57–89 fragment with high efficiency, in 8 h. This result gave evidence that the combination of prolidase activity with high AP activities increased the degradation rate. Gallo *et al.* (2005) and Gobbetti *et al.* (2007) showed that the combination of P-specific peptidases from *L. sanfranciscensis* CB1 with AP activities was more efficient to hydrolyse the 57–89 fragment.

No correlation between the individual peptidase activities and the capacity of the LAB strains to degrade α-gliadin-derived fragment was found. Among the LAB studied, several strains degraded, separately, the α-gliadin fragments 31–43 and 62–75, while the degradation of the 57–89 fragment was associated with a synergic or complementary effect between different peptidase profiles from two combined LAB strains.

Pediococci represent 40% of the total LAB microbiota isolated from sourdoughs of north of Argentina (unpublished data). To our knowledge, the present study is the first report on the peptide hydrolase system of pediococci strains isolated from sourdoughs and its ability to reduce α-gliadin fragments.

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