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Diversity in proteinase specificity of thermophilic lactobacilli as revealed by hydrolysis of dairy and vegetable proteins

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Abstract Ability of industrially relevant species of thermophilic lactobacilli strains to hydrolyze proteins from animal (caseins and β-lactoglobulin) and vegetable (soybean and wheat) sources, as well as influence of peptide content of growth medium on cell envelope-associated proteinase (CEP) activity, was evaluated. *Lactobacillus delbrueckii* subsp. *lactis* (CRL 581 and 654), *L. delbrueckii* subsp. *bulgaricus* (CRL 454 and 656), *Lactobacillus acidophilus* (CRL 636 and 1063), and *Lactobacillus helveticus* (CRL 1062 and 1177) were grown in a chemically defined medium supplemented or not with 1 % Casitone. All strains hydrolyzed mainly β-casein, while degradation of α_s-caseins was strain dependent. Contrariwise, κ-Casein was poorly degraded by the studied lactobacilli. β-Lactoglobulin was mainly hydrolyzed by CRL 656, CRL 636, and CRL 1062 strains. The *L. delbrueckii* subsp. *lactis* strains, *L. delbrueckii* subsp. *bulgaricus* CRL 656, and *L. helveticus* CRL 1177 degraded gliadins in high extent, while the *L. acidophilus* and *L. helveticus* strains highly hydrolyzed soy proteins. Proteinase production was inhibited by Casitone, the most affected being the *L. delbrueckii* subsp. *lactis* species. This study highlights the importance of proteolytic diversity of lactobacilli for rational strain selection when formulating hydrolyzed dairy or vegetable food products.

Keywords Lactic acid bacteria · Proteinase activity · Casein · Gliadin · Soy protein · β-Lactoglobulin

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Introduction

Lactic acid bacteria (LAB) are nutritionally exigent microorganisms that require exogenous amino acids and peptide sources to grow (Hebert et al. 2000a, 2004, 2008). In rich protein culture media with low amino acid availability, LAB growth depends on production of proteolytic and peptidolytic enzymes. These enzymes, together with peptide and amino acid transport systems, act coordinately to use proteins as an external amino acid source (Savijoki et al. 2006). This proteolytic system is essential for bacterial growth as formation of peptides and amino acids also plays a key role in development of organoleptic properties of fermented foods (Savijoki et al. 2006). In addition, certain LAB strains contribute to release of bioactive peptides that promote health beyond the basic nutrition (Hebert et al. 2008, 2010; Meisel 2004).

The specialized proteolytic system of LAB consists of a cell envelope-associated proteinase (CEP), transport systems to allow uptake of the resulting peptides, and several intracellular peptidases, which degrade peptides into amino acids (Liu et al. 2010; Savijoki et al. 2006). The CEP is the key enzyme of this system as it is involved in the first step of protein degradation.

Biochemical and genetic aspects of LAB proteinase activity have been mainly studied in *Lactococcus lactis* (Kunji et al. 1996). Hydrolysis specificity of lactococci proteinases, determined on caseins or α_{s1}-casein fragment comprising

residues 1 to 23, varies among strains, and different classifications have been proposed (Exterkate 1990; Visser et al. 1986). *L. lactis* proteinases were originally classified into two specificity groups, P_I or P_{III}. P_I-type cleaves β-casein preferentially and, to a lesser extent, κ-casein and α_{s1}-casein. In contrast, P_{III}-type cleaves β-, κ-, and α_{s1}-caseins equally.

Compared to *L. lactis*, thermophilic lactobacilli are largely deficient in amino acid biosynthesis that is compensated by large number of peptidases, amino acid permeases, and multiple oligopeptide transport systems they encode (Klaenhammer et al. 2005; Liu et al. 2010). The proteolytic system of lactobacilli displays a proteinase activity with substrate specificity differing from that of lactococci (Hebert et al. 2002; Kunji et al. 1996; Savijoki et al. 2006). Most of the CEPs found in mesophilic lactobacilli belong to the P_I type, whereas those of thermophilic lactobacilli to P_{III} or a mixed type between P_I and P_{III} (Hebert et al. 2002; Martín-Hernández et al. 1994). However, P_I-type CEPs were also described in the thermophilic strains *Lactobacillus delbrueckii* subsp. *lactis* BGPFI and *Lactobacillus acidophilus* CH2 and V74 (Fira et al. 2001; Tsakalidou et al. 1999). Some strains of *Lactobacillus helveticus* (Genay et al. 2009; Smeianov et al. 2007) and *L. delbrueckii* subsp. *bulgaricus* (Stefanitsi et al. 1995) have been reported to contain two distinct CEPs that differ in their substrate specificity. Six different types of CEPs were cloned and characterized from LAB, including PrtP from *L. lactis* and *Lactobacillus paracasei*, PrtH and PrtH2 from *L. helveticus*, PrtR from *Lactobacillus rhamnosus*, PrtS from *Streptococcus thermophilus*, and PrtB from *L. delbrueckii* subsp. *bulgaricus* (Fernandez-Espla et al. 2000; Gilbert et al. 1996; Holck and Naes 1992; Kok et al. 1988; Pastar et al. 2003; Pederson et al. 1999; Sadat-Mekmene et al. 2011). In *L. helveticus* CNRZ 32, two different proteinases (PrtH and PrtH2) have been described (Genay et al. 2009). The amino acidic sequences of different PrtP in lactococci showed an identity higher than 98 %, while in *L. helveticus* PrtH and PrtH2, they displayed low identity, indicating broad specificity diversity dependent on genera, species, and strain level.

Degradation of proteins from different sources other than caseins from milk by LAB has become of interest for the development of novel products or functional foods (Pescuma et al. 2007). Moreover, breakdown of proteins such as those present in whey and soybean have shown to improve protein digestibility and solubility, to decrease their allergenic content, and to release nutritionally important amino acids such as branched-chain amino acids (Aguirre et al. 2008; Pescuma et al. 2010, 2011). Therefore, the aim of this study was to compare ability of industrially relevant species of thermophilic lactobacilli strains to hydrolyze proteins from animal (caseins and whey proteins) and vegetable (soybean and gliadins) sources. In addition, the influence of peptide supply on CEP activity was evaluated.

Materials and methods

Microorganisms, media, and growth conditions

L. delbrueckii subsp. *lactis* CRL 581 and CRL 654, *L. delbrueckii* subsp. *bulgaricus* CRL 454 and CRL 656, *L. acidophilus* CRL 636 and CRL 1063, and *L. helveticus* CRL 1062 and CRL 1177, belonging to the culture collection of the Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina), were used in this study. The *L. delbruecki* subsp. *lactis* strains were isolated from Argentinean artisanal hard cheeses, while *L. helveticus* CRL 1062 and *L. helveticus* CRL 1177 were isolated from French Gruyère de Comtè cheese and Italian Grana cheese, respectively. *L. delbrueckii* subsp. *bulgaricus* CRL 454 was isolated from an Argentinean commercial cheese, while *L. delbrueckii* subsp. *bulgaricus* CRL 656 was from commercial yogurt. *L. acidophilus* CRL 636 was isolated from commercial fermented milk, whereas *L. acidophilus* CRL 1063 was from human intestine.

Working cultures of the lactobacilli strains were propagated twice in MRS broth (Biokar Diagnostics, France) at 40 °C for 16 h. To eliminate carryover nutrients, cells were harvested by centrifugation at 8,000×*g* for 15 min, washed twice in sterile 0.85 % (w/v) saline solution, and resuspended in this solution to the original volume. This cell suspension was used to inoculate a chemically defined medium (CDM) previously described by Hebert et al. (2000a) supplemented or not with 0.1 % (w/v) of Casitone at an initial optimal density at 560 nm (OD_{560}) of 0.08. After reaching exponential growth phase of incubation, cells grown in CDM were harvested by centrifugation (10,000×*g*; 10 min, 4 °C), washed twice with saline solution and resuspended to a final OD_{560} of approximately 10 in 100 mM sodium phosphate (pH 7.0). This suspension was used for enzymatic assays.

Proteinase activity

Proteinase activity was measured against succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (S-Ala; Sigma-Aldrich, St. Louis, MO, USA) by monitoring release of *p*-nitroaniline at 410 nm at 40 °C (Espeche Turbayet al. 2009) in a VersaMax microplate reader (Molecular Devices Corp.; Sunnyvale, CA, USA). One unit of proteinase was defined as the amount required to release 1 nanomole of nitroanilide per minute; specific activity was expressed as units per unit of OD_{560}.

Protein hydrolysis

Caseins

Whole cells, obtained as mentioned above, were mixed with 3 mg/ml of α-, β-, or κ-caseins (Sigma-Aldrich) previously dissolved in 100 mM sodium phosphate (pH 7.0) at 1:1 cell:protein ratio. The resulting mixtures were incubated at 40 °C for 4 h and centrifuged (10,000×*g*; 10 min, 4 °C). The supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Espeche Turbay et al. (2009). Either Coomassie Brilliant Blue R-250 or silver staining (Bio-Rad) was used to visualize the protein bands. Protein hydrolysis was evaluated by densitometric analysis of gels by using the QuantiScan software (Biosoft International, Ferguson, MO, USA). Casein hydrolysis was expressed as a percentage of disappearance of the protein fraction bands with respect to the control (noninoculated casein).

Protein hydrolysis and peptide released were also analyzed in supernatants by RP-HPLC. RP-HPLC was performed using a KNAUER Smartline System HPLC (Berlin, Germany) (Manager 5000 gradient programmer, pump 1000) with a KNAUER Smartline 2600 UV detector (Berlin, Germany) fitted with a Pursuit column (4.6×250 mm, 300 Å, 5 μm; Varian (Lexington, USA)). The operating conditions were the following: flow rate, (0.2 ml/min); solvent A, 0.1 % (v/v) trifluoroacetic acid (TFA) (sequential grade, Sigma-Aldrich) in ultrapure water; and solvent B, acetonitrile (sequential grade, Sigma-Aldrich) in TFA (0.1 %, v/v). The column was equilibrated with 5 % solvent B. Separation of peptides was effected with a gradient of 5 to 60 % solvent B over 90 min. The absorbance of the column effluent was recorded at 220 nm using an HPLC software KNAUER EuroChrom 305P4 (Berlin, Germany).

β-Lactoglobulin

Cell suspensions were incubated at a cell–protein ratio of 2:1 (v/v) using β-lactoglobulin (BLG, ICN, 3 mg/ml) as protein source. This compound was previously dissolved in 100 mM sodium phosphate (pH 7.0) and heated at 80 °C for 30 min. The cell–protein mixtures were incubated for 4 h at 40 °C and then centrifuged (10,000×*g*, 10 min 4 °C). β-Lactoglobulin hydrolysis was analyzed in supernatants by 16 % (w/v) Tricine SDS-PAGE (Pescuma et al. 2007). Either Coomassie Brilliant Blue R-250 or silver staining was used to visualize the proteins. RP-HPLC was performed with the same solvents and flow rate as specified above. The gradient applied was 100 % solvent A up to 10 min and 10 to 60 % solvent B in a linear fashion between 10 and 60 min.

Gliadins

Gliadins (Sigma-Aldrich) dissolved in 70 % ethanol were mixed with cell suspensions at a 1:1 (v/v) cell–protein ratio. The mixtures were incubated at 40 °C for 4 h. Hydrolysis of proteins was analyzed in supernatants by 10 % Tricine SDS-PAGE. Either Coomassie Brilliant Blue R-250 or silver staining was used to visualize the proteins. RP-HPLC was performed with the same solvents and flow rate as described previously for casein chromatography using the following lineal gradient from 25 to 55 % of B during 100 min.

Soybean proteins

Soybean proteins were extracted according to the method of Aguirre et al. (2008). Briefly, defatted commercial soybean flour (20 g) was extracted once with 400 ml of 30 mM tris–HCl buffer (pH 8.0) at room temperature with agitation for 1 h. The soybean sample was then centrifuged at 15,000×*g* for 20 min at 4 °C and the resulting supernatant (soybean protein extract, SPI), a glycinin and β-conglycinin-rich fraction, was sterilized through 0.22-μm filters (Millipore, Bedford, MA, USA). The SPI was immediately kept at −20 °C until use. SPI was heat treated at 80 °C during 30 min before the assay for protein denaturation, since no hydrolysis of these proteins in their native form was detected in previous work (Aguirre et al. 2008). Cell suspensions were incubated with SPI at a 2:1 (v/v) cell:protein ratio at 40 °C for 4 h and then centrifuged (10,000×*g*, 10 min 4 °C). Protein hydrolysis was analyzed in supernatants by RP-HPLC with the same solvents and flow rate as described previously. The gradient applied was from 0 % solvent B at time 0 min to 40 % of solvent B at 30 min and held under this condition for 15 min.

Results

Proteinase activity

The proteinase activity levels of eight industrially relevant strains of thermophilic lactobacilli on the chromophoric substrate S-Ala were investigated (Table 1). To analyze the effect of the peptide supply on CEPs, lactobacilli cells were grown in CDM and in CDM supplemented with Casitone (a pancreatic digest of casein consisting of small peptides and amino acids in a ratio of about 4:1, Hebert et al. 2008). Different degrees of proteinase activity among lactobacilli were observed; *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *L. delbrueckii* subsp. *lactis* CRL 581 showed the highest activity values, while the *L. acidophilus* strains degraded this substrate scarcely (Table 1). Proteinase activity levels of cells grown in CDM were higher than those obtained in CDM plus

Table 1 Proteinase activities of thermophilic lactobacilli after their growth in a chemically defined medium (CDM) and CDM supplemented with Casitone (CDMCas)

Strains	Specific proteinase activity[a,b]		
	CDM	CDMCas	Inhibition factor[c]
L. delbrueckii subsp. *lactis* CRL 581	192.79±22.99	1.61±0.62	119.18
L. delbrueckii subsp. *lactis* CRL 654	31.70±0.53	0.51±0.31	61.58
L. delbrueckii subsp. *bulgaricus* CRL 454	101.95±8.99	13.23±0.41	7.70
L. delbrueckii subsp. *bulgaricus* CRL 656	218.09±58.34	69.04±0.93	3.15
L. acidophilus CRL 636	14.45±2.75	7.28±0.93	1.98
L. acidophilus CRL 1063	9.89±1.51	6.40±0.31	1.54
L. helveticus CRL 1062	49.96±10.03	6.73±0.15	7.43
L. helveticus CRL 1177	34.08±0.26	3.53±0.21	9.66

[a] Values are the means from three independent experiments+SD

[b] Specific activity is expressed as nanomoles min^{-1} OD_{560}

[c] Ratio between the values obtained in CDM and CDMCas

Casitone, with this effect being strain dependent. The highest inhibition degree was observed in the *L. delbrueckii* subsp. *lactis* strains, this inhibition factor being about 119- and 62-fold (Table 1). On the contrary, a slight reduction in enzyme activity was observed in whole cells of *L. acidophilus* grown in a peptide-rich medium (Table 1).

Protein hydrolysis

Caseins

Hydrolysis of α_s-caseins was strain dependent (Fig. 1); these protein fractions were highly degraded by the *L. helveticus* strains, which showed similar hydrolysis percentage (92.0 %) values but different peptide profiles as revealed by SDS-PAGE (lanes 8 and 9) and RP-HPLC. *L. helveticus* CRL 1062 (Fig. 1a, lane 8) released several peptides showing three main bands of 10, 14, and 23 kDa (marked with arrows), while *L. helveticus* CRL 1177 (Fig. 1a, lane 9) released peptides of different moleular weight (MW); a more intense 23-kDa band and two bands of 18 and 20 kDa in lower amounts were observed in the gels. *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *L. acidophilus* CRL 636 also degraded α_s-caseins to a high extent (79.5 and 89.8 %, respectively), while *L. delbrueckii* subsp. *lactis* CRL 654, *L. delbrueckii* subsp. *bulgaricus* CRL 454, and *L. acidophilus* CRL 1063 scarcely hydrolyzed this protein. On the other hand, a complete inhibition of α_s-casein hydrolysis was observed when the LAB strains were grown in CDM plus Casitone (Fig. 1b).

In the RP-HPLC chromatograms, two peaks corresponding to α_{s1} and α_{s2}-caseins were observed in the nondegraded α-casein sample (Fig. 1c, 1). All strains were able to degrade α_{s2}-casein almost completely, except for *L. acidophilus* CRL 636 and *L. helveticus* CRL 1062, for which this protein was still detected (Fig. 1c, 6–8, indicated with arrows). α_{s1}-Casein was hydrolyzed by the studied strains albeit in much lesser extent (Fig. 1c, 6, 7, and 9) than α_{s2}.

Concerning β-casein, this fraction was almost completely (78–100 %) hydrolyzed by the assayed strains, except for *L. acidophilus* CRL 1063 (12 %) (Fig. 2a). In general, the strains released low MW peptides with highly hydrophilic characteristics (Fig. 2a, c). *L. delbrueckii* subsp. *bulgaricus* CRL 454 degraded this protein into several peptides showing two main bands of approximately 22 and 19 kDa and several peaks eluting along the chromatogram (Fig. 2c, 4). When the LAB strains were previously grown in CDM plus Casitone, β-casein hydrolysis was drastically (88–100 %) reduced, except for the samples hydrolyzed by *L. delbrueckii* subsp. *bulgaricus* CRL 454 and CRL 656, for which inhibition percentages were 6 and 10 %, respectively (Fig. 2b).

κ-Casein was the least degraded casein fraction as slight changes in the band intensity of this protein were observed in the SDS-PAGE (Fig. 3a). Interestingly, *L. delbrueckii* subsp. *bulgaricus* CRL 454 displayed two additional low MW peptides originating from the κ-casein hydrolysis. The RP-HPLC profiles revealed a poor hydrolysis of this protein by all strains releasing mainly hydrophilic peptides; some hydrophobic peaks (30–55 min) were detected in the hydrolysates from *L. helveticus* CRL 1062 (Fig. 3c, 8). Complete inhibition of κ-casein degradation was observed when the assayed strains were grown in the presence of Casitone (Fig. 3b).

β-Lactoglobulin

All analyzed strains were able to degrade BLG although in different extent (8.4–98.1 %, Fig. 4), the strains of *L. acidophilus* CRL 636 and *L. delbrueckii* subsp. *bulgaricus* CRL 656 being the ones that hydrolyzed this protein the most (98.1 and 82.5 %, respectively). The peptide patterns for each of these BLG hydrolysates were different; four bands corresponding to peptides of approximate MW between 11.6 and 5.5 kDa were released by *L. acidophilus* CRL 636, while three bands of approximate MW between 14.4 and 9.4 kDa were obtained for *L. delbrueckii* subsp. *bulgaricus* CRL 656 (Fig. 4a). *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* CRL 1062 showed similar peptide profiles, except for the

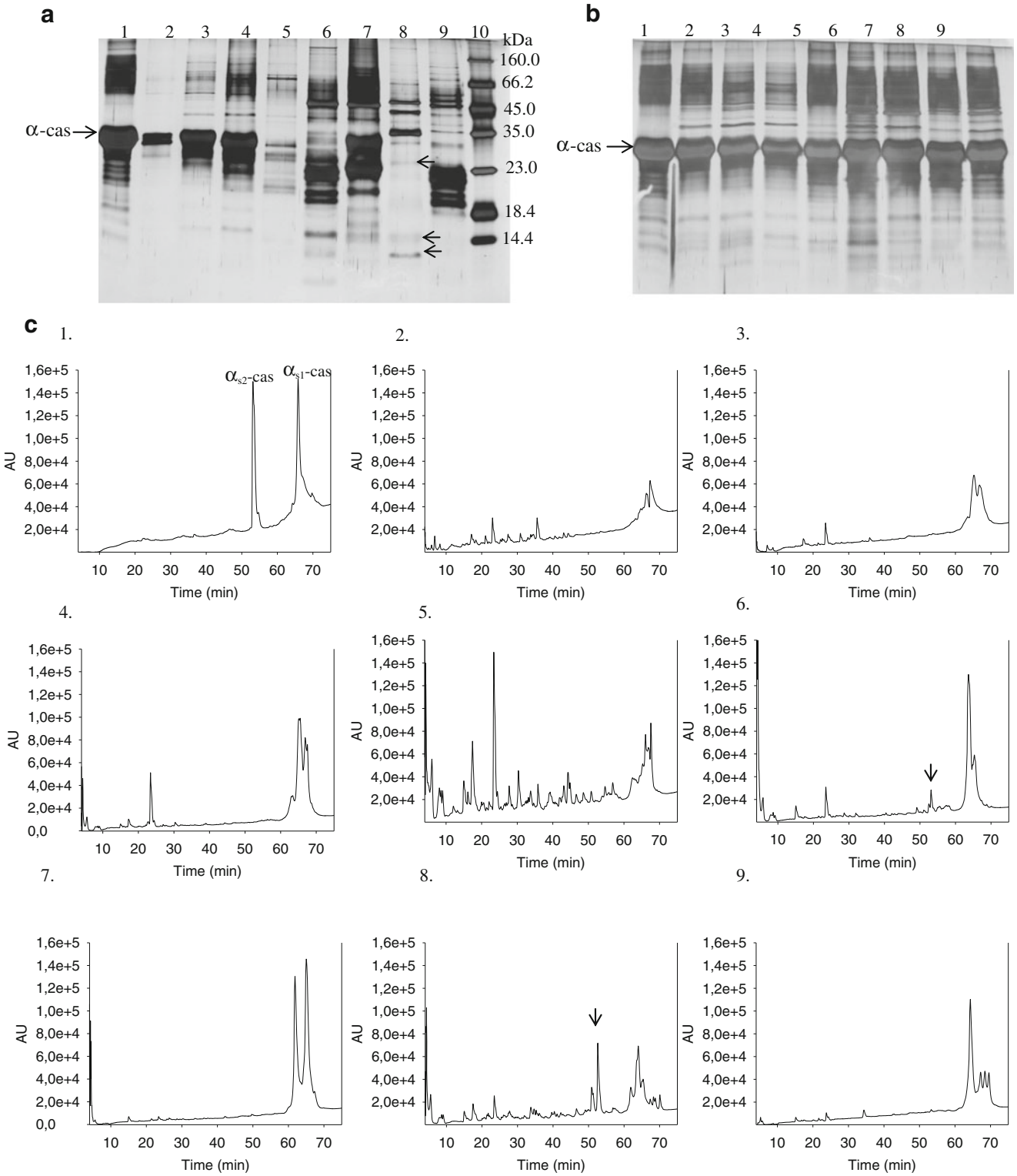

Fig. 1 α-Casein hydrolysis by LAB strains grown in CDM (**a**) and CDM supplemented with 1 % (w/v) Casitone (**b**) observed in SDS-PAGE and RP-HPLC profiles of α-casein hydrolysates (**c**) by noninoculated protein used as control (*1*), *L. delbrueckii* subsp. *lactis* CRL 581 (*2*), and CRL 654 (*3*); *L. delbrueckii* subsp. *bulgaricus* CRL 454 (*4*) and CRL 656 (*5*); *L. acidophilus* CRL 636 (6) and CRL 1063 (*7*); and *L. helveticus* CRL 1062 (*8*) and CRL 1177 (*9*). Molecular weight marker (*10*)

peaks indicated with a cross in Fig. 4b (4–5). In general, *L. acidophilus* strains released large amounts of highly hydrophilic peptides eluting during the first 10 min; a similar result was observed for *L. helveticus* CRL 1062 (Fig. 4b, 8).

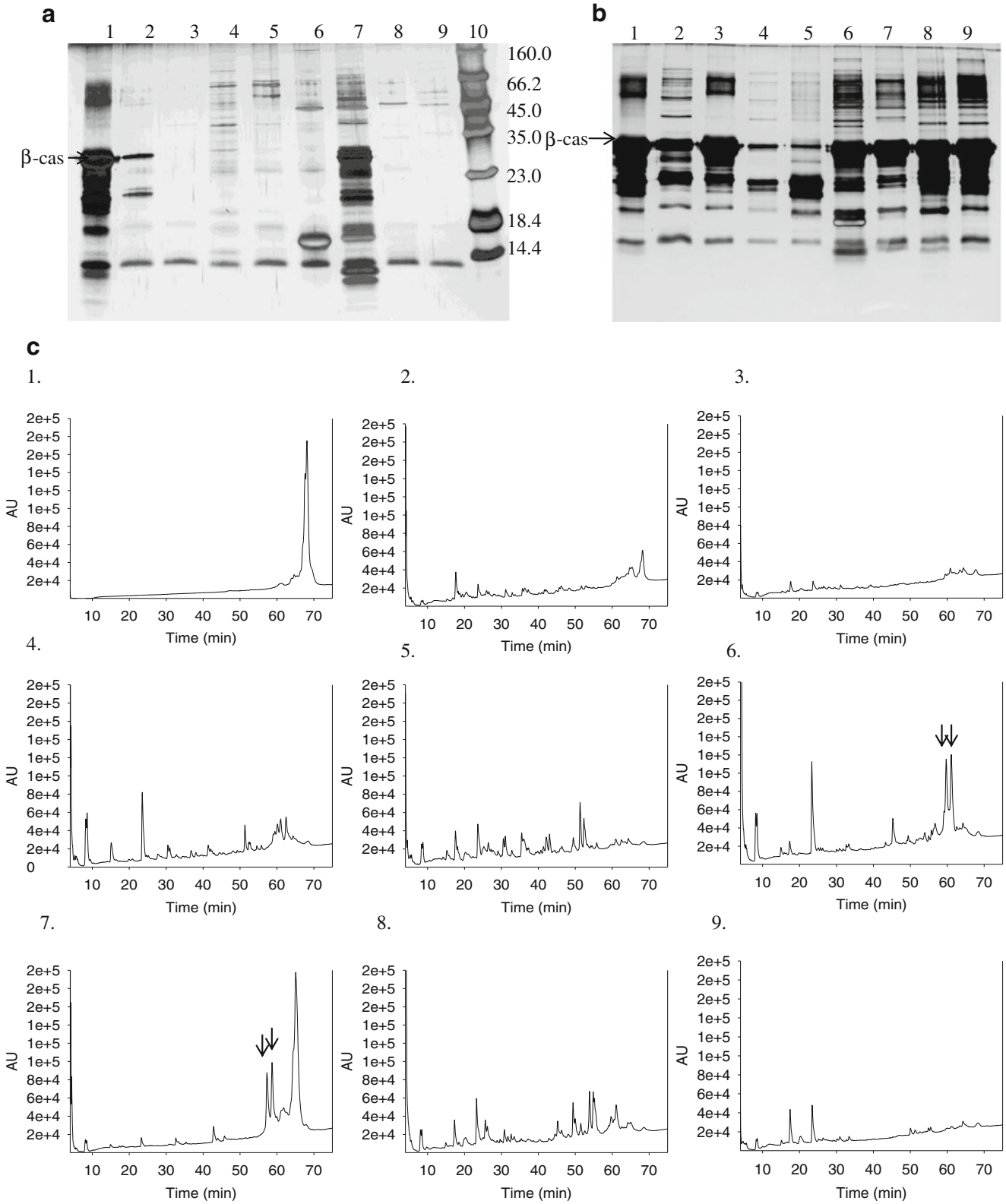

Fig. 2 β-Casein hydrolysis by LAB strains grown in CDM (**a**) and CDM supplemented with 1 % (w/v) Casitone (**b**) observed in SDS-PAGE and RP-HPLC profiles of β-casein hydrolysates (**c**) by noninoculated protein used as control (*1*); *L. delbrueckii* subsp. *lactis* CRL 581 (*2*) and CRL 654 (*3*); *L. delbrueckii* subsp. *bulgaricus* CRL 454 (*4*) and CRL 656 (*5*); *L. acidophilus* CRL 636 (*6*) and CRL 1063 (*7*); and *L. helveticus* CRL 1062 (*8*) and CRL 1177 (*9*). Molecular weight marker (*10*)

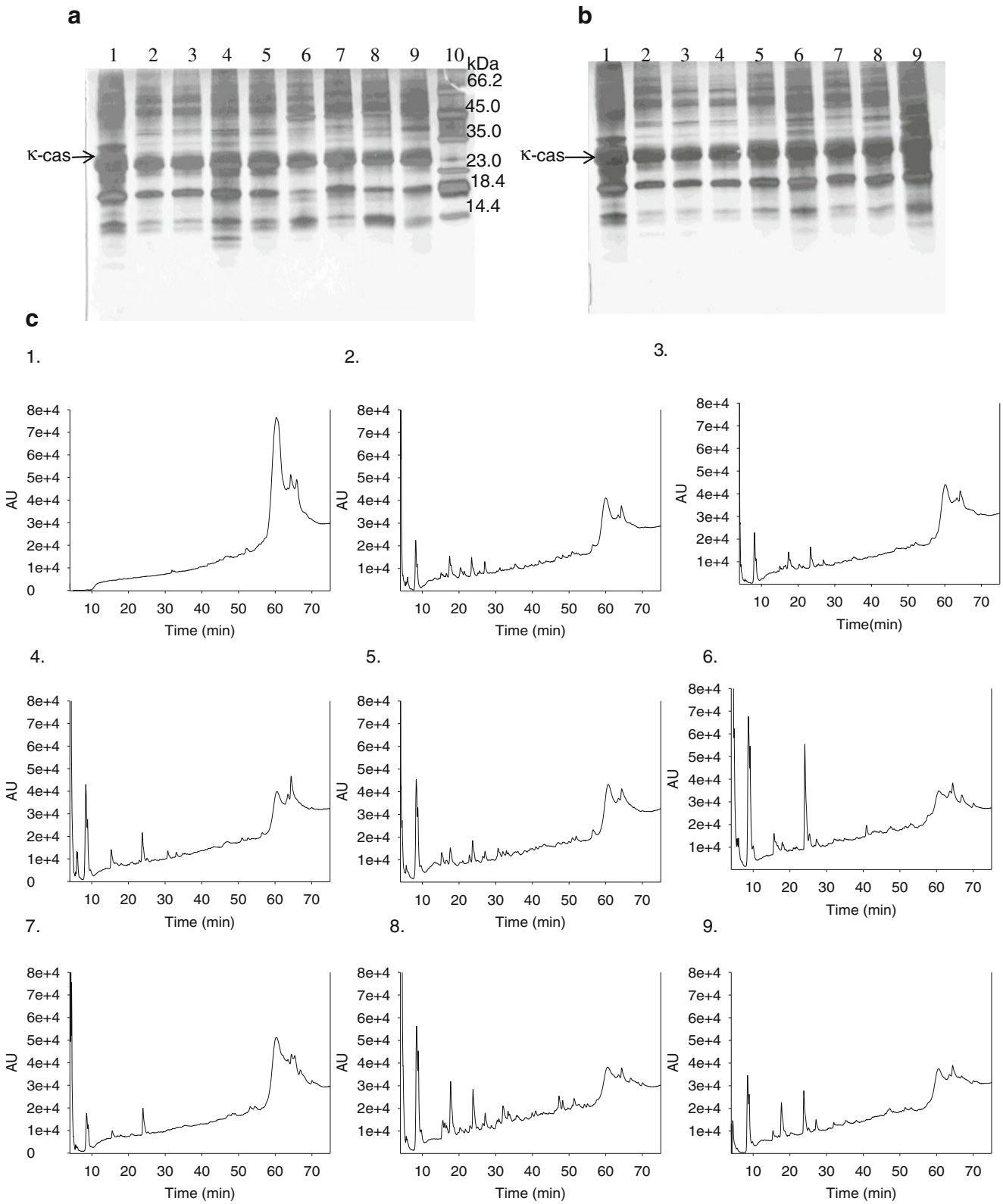

Fig. 3 κ-Casein hydrolysis by LAB strains grown in CDM (**a**) and CDM supplemented with 1 % (w/v) Casitone (**b**) observed in SDS-PAGE and RP-HPLC profiles of κ-casein hydrolysates (**c**) by noninoculated protein used as control (*1*); *L. delbrueckii* subsp. *lactis* CRL 581 (*2*) and CRL 654 (*3*); *L. delbrueckii* subsp. *bulgaricus* CRL 454 (*4*) and CRL 656 (*5*); *L. acidophilus* CRL 636 (*6*) and CRL 1063 (*7*); and *L. helveticus* CRL 1062 (*8*) and CRL1177 (*9*). Molecular weight marker (*10*)

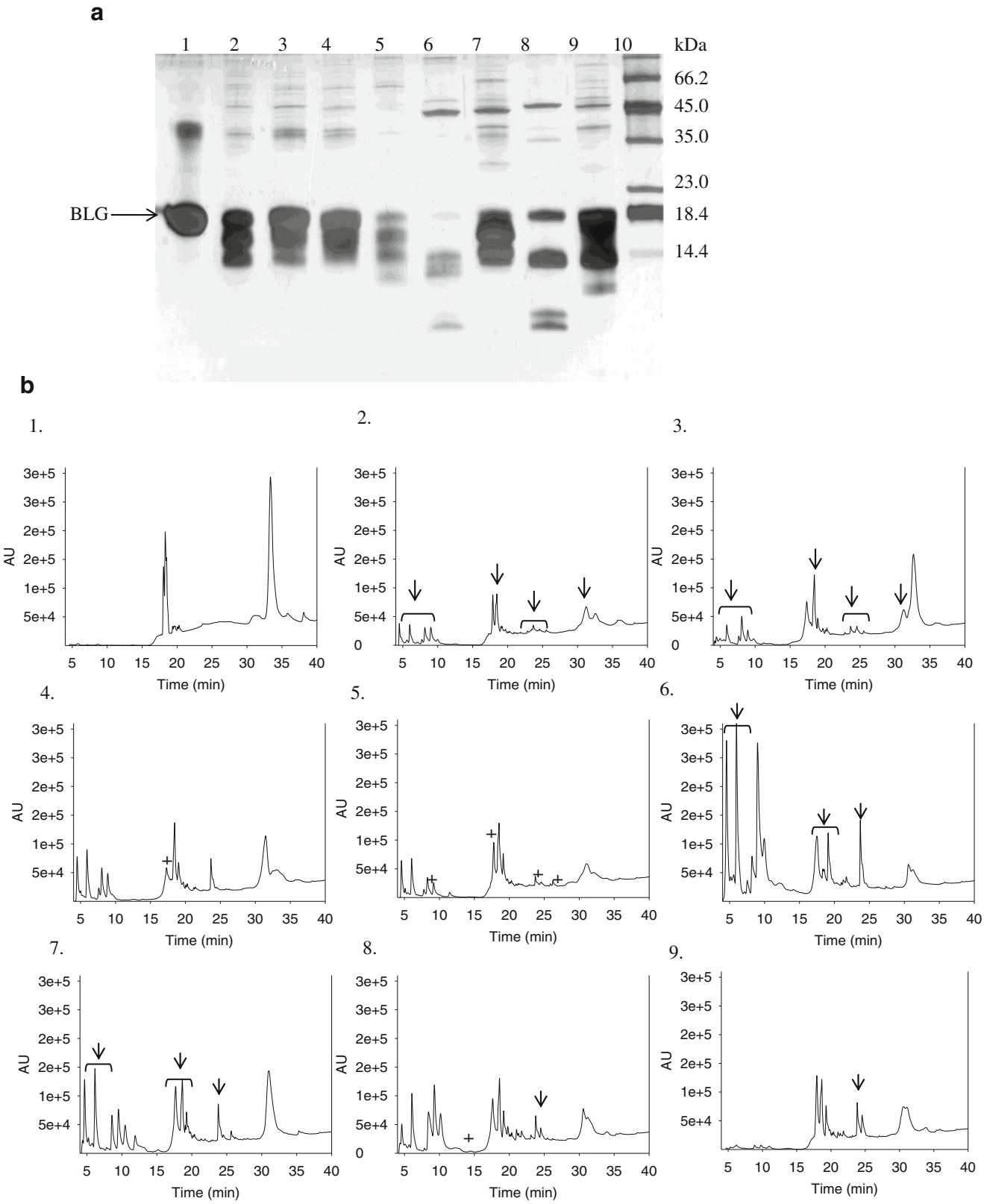

Fig. 4 β-Lactoglobulin hydrolysis by LAB strains grown in CDM observed in SDS-PAGE (**a**) and RP-HPLC profiles of β-lactoglobulin hydrolysates obtained (**b**) by noninoculated protein used as control (1); *L. delbrueckii* subsp. *lactis* CRL 581 (*2*) and CRL 654 (*3*); *L. delbrueckii* subsp. *bulgaricus* CRL 454 (*4*) and CRL 656 (*5*); *L. acidophilus* CRL 636 (*6*) and CRL 1063 (*7*); and *L. helveticus* CRL 1062 (*8*) and CRL 1177 (*9*). Molecular weight marker (*10*)

Gliadins

All evaluated LAB degraded gliadins, although their proteinase specificity towards the different fractions varied among strains and species (Fig. 5). *L. delbrueckii* subsp. *lactis* strains degraded the 51-, 45-, 38-, and 13-kDa fractions the most; similar results were obtained with the *L. delbrueckii* subsp. *bulgaricus* strains, although the presence of new hydrophilic peptides were observed for the hydrolysate from *L. delbrueckii* subsp. *bulgaricus* CRL 454 (Fig. 5b, 4) as compared with those from the other strains. In contrast, *L. acidophilus* strains and *L. helveticus* CRL1062 poorly degraded the 45- and 38-kDa protein bands (Fig. 5a). The RP-HPLC results showed that the studied strains released mainly hydrophilic peptides eluting during the first 20 min (Fig. 5b).

Soy proteins

Overall, all strains degraded the low MW fractions of soy proteins (16.7, 12.7, 10.3, and 8.3 kDa) the most, while the 30.7-kDa fraction was scarcely hydrolyzed (Fig. 6a). All CEPs released hydrophilic peptides (RP-HPLC, Fig. 6b), some of them showing different profiles. RP-HPLC profiles showed that almost all strains degraded fractions 1, 3, and 9, except for *L. helveticus* CRL 1177, which was unable to hydrolyze fraction 1. Interestingly, the *L. acidophilus* strains degraded fraction 7 almost completely. A new peak eluting at 18 min, although with different intensity, was detected in all the hydrolysates.

Discussion

LAB are extensively used in the food industry due to their capacity to produce a broad range of compounds that can improve sensorial quality, storage time, and functional characteristics of fermented foods. LAB are used in fermentation of animal and vegetable products such as milk, meat, fish, wheat, soy, and different vegetables to obtain added value foods (Champagne et al. 2009; Galle et al. 2011; Settanni and Corsetti 2008). Up to date, the most outstanding industrial application of LAB is as starter cultures for the production of fermented foods justifying the huge research efforts destined to the study of these microorganisms.

The ability of eight thermophilic strains, used in the fermented food industry, to hydrolyze milk and vegetable proteins was analyzed. All the strains were able to degrade the studied proteins (caseins, β-lactoglobulin, soy proteins, and gliadins), although in a different extent, suggesting different specificities of their proteinases. *L. delbrueckii* subsp. *lactis* CRL 581, *L. delbrueckii* subsp. *bulgaricus* CRL 656, *L. acidophilus* CRL 636, and the *L. helveticus* strains could be classified as harboring a mixed type P_I–P_{III} proteinase as they were able to degrade α_s and β-caseins to a high extent; the remaining strains degraded mainly β-casein being classified as belonging to the P_I type group. Several reports (Hebert et al. 2000a, 2002,2008; Miladinov et al. 2001; Pastar et al. 2003) have demonstrated that the presence of peptides in the growth medium inhibits the proteinase activity of some LAB such as *L. helveticus*, *L. delbrueckii* subsp. *lactis*, *L. lactis*, and *L. rhamnosus*. In this work, the CEP activity levels of lactobacilli cells grown in CDM supplemented with Casitone were decreased compared with those found in a CDM, suggesting that the nitrogen source modulates the proteinase biosynthesis in lactobacilli. However, the range of inhibition was strain dependent. The less affected strains were those belonging to the *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* species. Interestingly, a complete inhibition of α-casein hydrolysis was observed for the *L. delbrueckii* subsp. *bulgaricus* strains, while almost no inhibition was achieved for β-casein degradation, suggesting the presence of two proteases with different specificities and regulation. In this respect, it has been reported that *L. delbrueckii* subsp. *bulgaricus* ACA DC235 bears two CEPs, a zinc-dependent metalloprotease and a serine protease (Stefanitsi et al. 1995). Moreover, these types of proteases were detected in the genome of *L. delbrueckii* subsp. *bulgaricus* ND02 (GenBank CP002341). On the other hand, the low inhibition values obtained for the *L. acidophilus* strains could be explained by the fact that these microorganisms degraded the chromogenic substrate S-Ala poorly. It has been already established that LAB proteinases have different affinities towards several substrates (Bruinenberg and Limsowtin 1995). Fernandez-Espla et al. (2000) studied the ability of a *S. thermophilus* strain to degrade seven different substrates commonly used for classifying the *L. lactis* proteinases, demonstrating that this strain could hydrolyze peptides bearing aromatic amino acids in position P1 and failed to degrade those having a negative-charged amino acid in position P3. Hebert et al. (2008) have already reported that *L. delbrueckii* subsp. *lactis* CRL 581 CEP prefers a glutamine or glutamic acid in position P1; phenylalanine was also found at this position although in lower frequency. The differences observed in the peptide profiles of casein hydrolysates with the studied lactobacilli suggest that they may degrade other milk and vegetable proteins in a distinct way.

β-Lactoglobulin is the major whey protein in ruminants; this protein is highly resistant to hydrolysis by digestive and nondigestive enzymes and is one of the major cause of milk allergy together with α_{s1}-casein (Picariello et al. 2011). Several studies demonstrated that some LAB strains are capable of growing and degrading whey proteins (Pescuma et al. 2007, 2008,2010, 2012). In this respect, whey, the main by-product of the cheese industry could be used as substrate for lactic fermentation to elaborate novel fermented foods with specific (i.e., hypoallergenic) properties. Recently, Pescuma et al. (2008, 2012) showed that the strains *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *L. acidophilus* CRL 636 were able to degrade

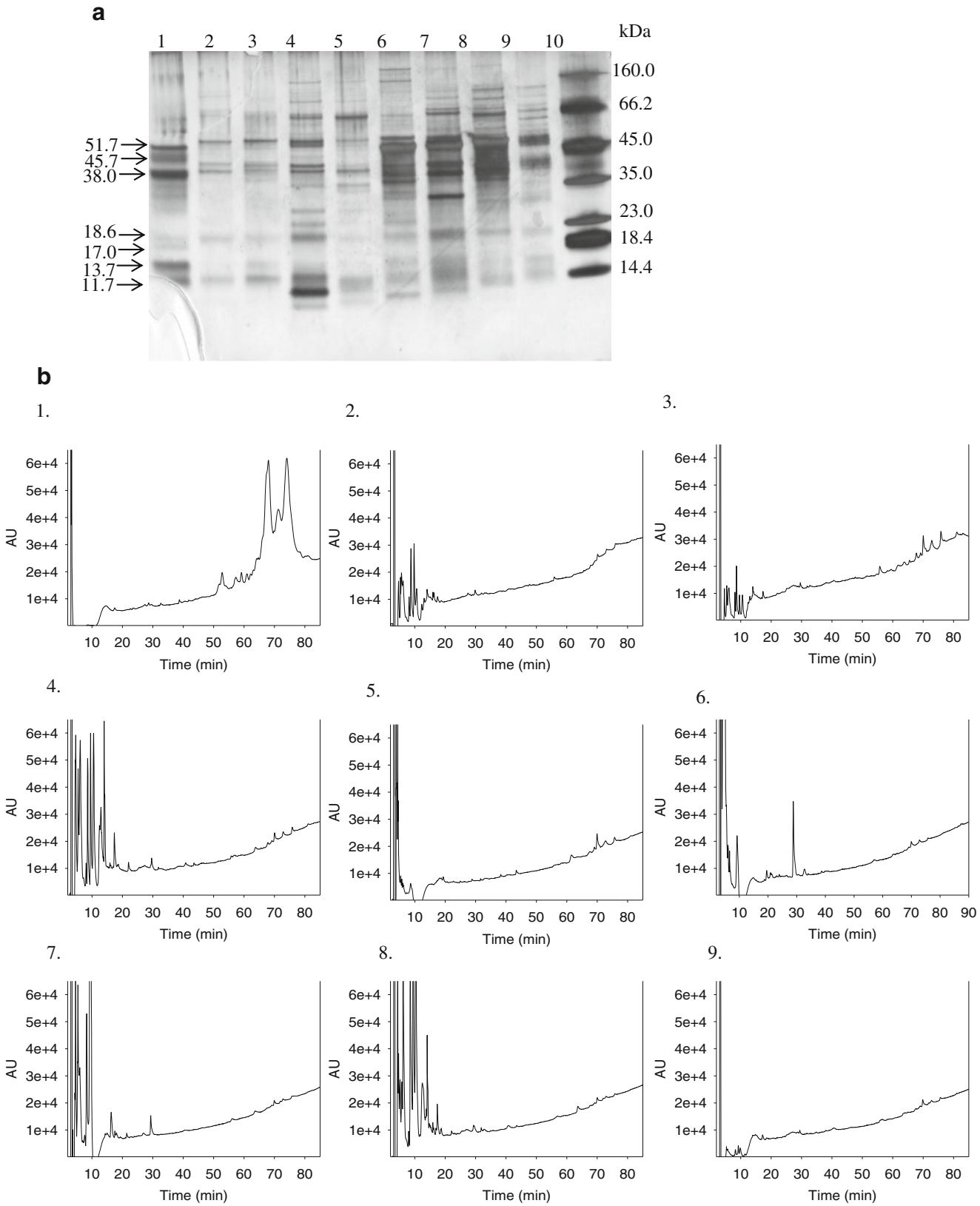

Fig. 5 Gliadin hydrolysis by LAB grown in CDM observed in SDS-PAGE (**a**), and RP-HPLC profiles of gliadin hydrolysates (**b**) by noninoculated gliadin protein used as control (*1*); *L. delbrueckii* subsp. *lactis* CRL 581 (*2*) and CRL 654 (*3*); *L. delbrueckii* subsp. *bulgaricus* CRL 454 (*4*) and CRL 656 (*5*); *L. acidophilus* CRL 636 (*6*) and CRL 1063 (*7*); and *L. helveticus* CRL 1062 (*8*) and CRL 1177 (*9*). Molecular weight marker (*10*). Main gliadin protein fractions are indicated with *arrows*

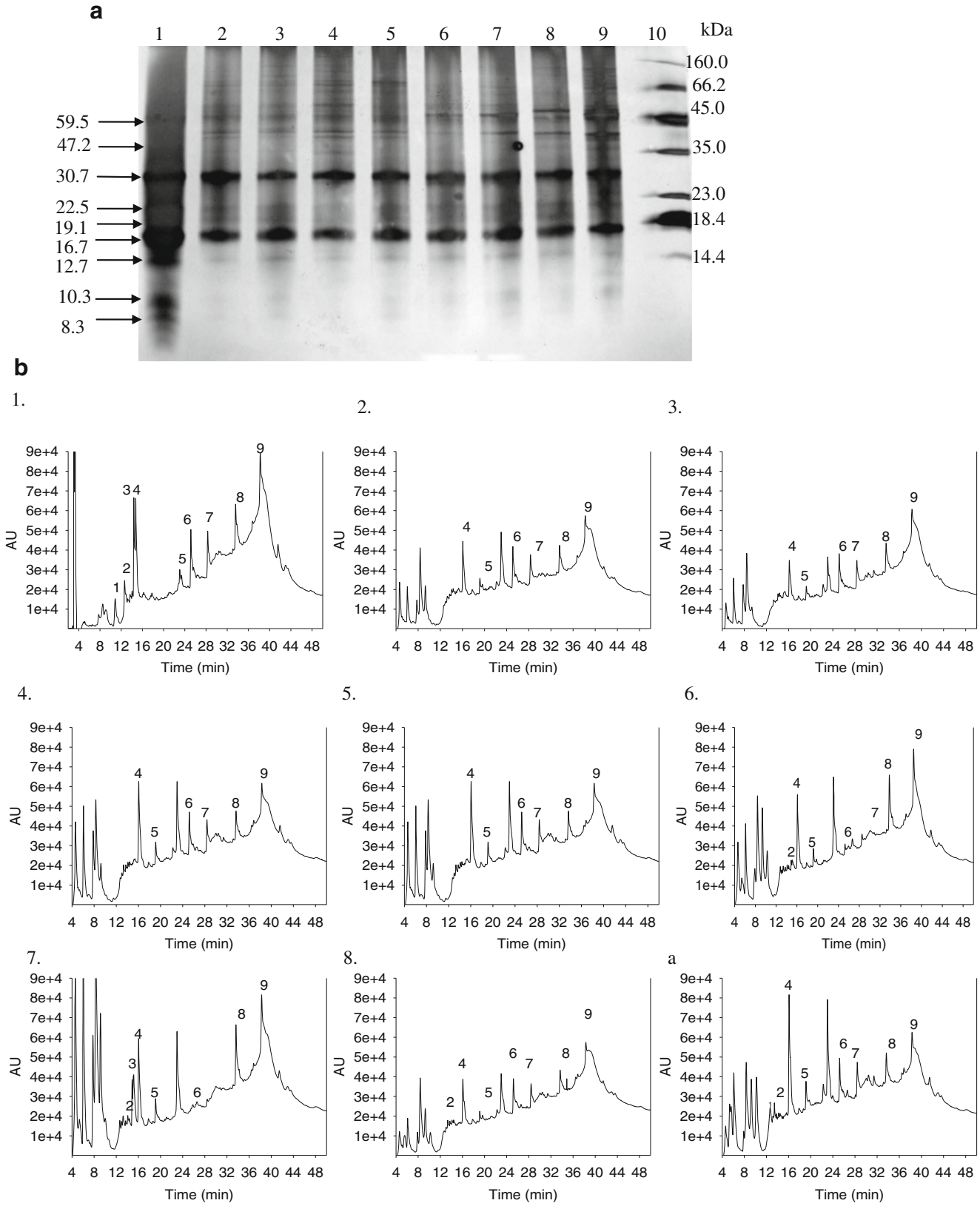

Fig. 6 Soy protein hydrolysis by LAB strains grown in CDM observed in SDS-PAGE (**a**) and RP-HPLC profiles of soy protein hydrolysates (**b**) by noninoculated soy protein used as control (*1*); *L. delbrueckii* subsp. *lactis* CRL 581 (*2*) and CRL 654 (*3*); *L. delbrueckii* subsp. *bulgaricus* CRL 454 (*4*) and CRL 656 (*5*); *L. acidophilus* CRL 636 (*6*) and CRL 1063 (*7*); and *L. helveticus* CRL 1062 (*8*) and CRL 1177 (*9*). Molecular weight marker (*10*). Main soy protein fractions are indicated with *arrows*

BLG when growing both in whey and in whey protein concentrate as substrates. In our work, the assayed thermophilic lactobacilli were able to hydrolyze BLG when using the pure protein. Among the studied strains, *L. delbrueckii* subsp. *bulgaricus* CRL 656, *L. helveticus* CRL 1062, and *L. acidophilus* CRL 636 were the strains that showed the highest activity against BLG. *L. helveticus* CRL 1062, currently used as a starter culture in the manufacture of Argentinian hard cheeses (Hebert et al. 2000b), could, therefore, contribute to the hydrolysis of the remaining BLG present in the coagulum, since allergenic reactions can be elicited even with very low concentrations of the antigen. Interestingly, this strain was also able to degrade almost completely the allergenic protein α_{s1}-casein. Recently, other authors have shown that some LAB strains are able to decrease casein immunoreactivity through proteolysis (El-Ghaish et al. 2010; Hadji-Sfaxi et al. 2012).

Gliadins from wheat flour are the cause of celiac disease in susceptible people (Picariello et al. 2011). Degradation of gliadins during sourdough fermentation has already been published (Di Cagno et al. 2007; Gerez et al. 2012; Zotta et al. 2006); however, hydrolysis of wheat proteins is carried out by wheat endopeptidases as well as by the action of proteases coming from several LAB and yeasts present in the dough. In this work, the CEPs of eight strains belonging to different species were able to degrade gliadins contributing to the primary hydrolysis of these proteins, usually attributed to wheat endopeptidases (Thiele et al. 2002).

Soybeans are an abundant and relatively inexpensive source of proteins that are widely recognized for their high nutritional value and excellent functional properties. The major components of soy proteins are storage proteins known as β-conglycinin (low MW) and glycinin (high MW), which account for 65 to 80 % of total seed proteins (Wang and Gonzalez de Mejia 2005). Acidic hydrolysis and enzymatic hydrolysis have been extensively applied to improve the solubility and other functional properties of soy protein products (Wang and Gonzalez de Mejia 2005). Enzymatic methods are easier to control and do not cause amino acid damage. Therefore, enzymatic hydrolysis, involving commercial or microbial enzymes, is the most commonly used method to produce food-grade protein hydrolysates and to release bioactive peptides from their protein precursors (Wang and Gonzalez de Mejia 2005). Aguirre et al. (2008) reported on the ability of *L. helveticus* and *L. delbruekii* subsp. *lactis* strains to breakdown soybean proteins. Here, we observed that *L. delbruekii* subsp. *bulgaricus* CRL 454 and CRL 656, and *L. acidophilus* CRL636 and CRL 1063 were also able to hydrolyze soy proteins.

The majority of the studied LAB strains were isolated from fermented milk products, except for *L. acidophilus* CRL 1063. This strain, isolated from human intestine, was the least proteolytic one. In this respect, it has been reported that human isolates of *L. acidophilus* grow poorly in milk, a behavior that may be associated to low proteinase activities (Banina et al. 1998).

Results showed that majority of the strains degraded α_s-caseins, β-casein, and BLG the most. In addition, some strains effectively degrade gliadins, while slight differences on their ability to degrade soy proteins were detected. The *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* strains degraded effectively α_{s1}-casein, while *L. delbrueckii* subsp. *bulgaricus* CRL 656 was the only strain capable of degrading BLG to a high extent. As this latter species is commonly used in yogurt manufacture, the ability of this strain to decrease the immunoreactivity of milk proteins should be investigated for the elaboration of hypoallergenic yogurts. Moreover, the studied *L. delbrueckii* strains could be used for reducing the immunoreactivity of gliadins.

This is the first work showing differences on the ability of several strains belonging to different species to degrade vegetable and milk proteins. This information becomes crucial when formulating hydrolyzed food products of different (dairy or vegetable) origin by selected LAB.

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