Effect of High Pressure on the Viability and Enzymatic Activity of Mesophilic Lactic Acid Bacteria Isolated from Caprine Cheese

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ABSTRACT

The effects of high pressure on the viability and acidifying and peptidolytic activities of lactococci and mesophilic lactobacilli strains were studied. We inoculated 10% reconstituted bovine skim milk with cells in the stationary phase of growth (~8 $\log_{10}$ cfu/ml) without incubation. Cells were treated at high pressure (100 to 400 MPa) for 20 min at 20°C. The lactococci were more sensitive than the lactobacilli to pressures of 100 to 350 MPa. The pressure-treated cells exhibited lower acidification rates, even with treatments that did not affect cell viability. High pressures increased the hydrolytic activity of lactococci and lactobacilli on the carboxyl-terminal fragment from β-casein (C-peptide), which contributes to bitterness in cheese. Activity levels were highest after treatment of lactococci and lactobacilli at 300 and ≥350 MPa, respectively. At these pressures, there was no reduction of aminopeptidase or dipeptidase activity of *Lactococcus lactis* ssp. *lactis* IFPL 359, but these activities were partially inhibited in *Lactobacillus casei* ssp. *casei* IFPL 731 after pressure treatment at 400 MPa. This paper also discusses the possibility of lactic bacteria being subjected to high pressure to create an extra supply of enzymes with debittering properties.

(**Key words**: high pressure, lactic acid bacteria, enzymatic activity)

Abbreviation key: **RBSM** = reconstituted bovine skim milk.

INTRODUCTION

Many studies have been conducted to decrease ripening time or to improve the flavor of cheeses (11) by modification of manufacturing conditions, the use of exogenous enzymes, or the addition of larger amounts of starter culture. However, the last procedure can cause excessive acidity or the release of intermediate-sized peptides, resulting in a bitter flavor (17), although not all researchers (30) agree that bitterness is related purely to starter cell numbers. Microbial proteinases and peptidases must be at least partially responsible for the production of bitter peptides from larger peptides produced by action of rennet, and specific peptidases are important for the removal of bitter peptides once formed (19). There appears to be general agreement that the incorporation of appropriate proteolytic and peptidolytic activities at an optimum ratio in the cheese is important for balanced and increased flavor development (5).

Overproduction of acid in cheese can be prevented by using modified strains of lactococci with reduced proteolytic activity (prt^-) and capacity to metabolize lactose (lac^-) (15) or with cells treated with cold or heat as starter adjuncts (3, 7, 9, 12, 16, 20, 24, 34). The use of these adjuncts has also led to a marked decrease in bitterness in cheese (1, 2, 4, 5). The removal of bitterness could be connected with the release of intracellular peptidases that undergo cellular lysis, as proposed by Feirtag and McKay (10) for nonbitter strains of *Lactococcus lactis* ssp. *cremoris* AM2 or SK11. Thermolytic response was observed in broth shortly after heating to 38 to 40°C during manufacture of Cheddar cheese. Ardo et al. (1), Tan et al. (31), and Smit et al. (29) also suggested the possible involvement of early lysis of strains in the degradation of bitter peptides.

One possible alternative to the methods used to accelerate cheese ripening is to use lactic acid bacteria cells attenuated by high pressure. Effects of pressure on acid-producing and peptidolytic activities of thermophilic lactic bacteria have been studied by Tanaka and Hatanaka (32) for yogurt cultures and Miyakawa et al. (18) for *Lactobacillus helveticus*, but the enzymatic activity of lactococci and mesophilic lactobacilli has not yet been studied. The aim of the present work was to find a sublethal technique to lower the lactic-acid producing activity of these microorganisms without causing damage to the pep-

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tidolytic enzymes with a view to using pressurized cells as a cheese-ripening agent.

MATERIALS AND METHODS

Bacterial Cultures and Preparation of Inocula

The microorganisms used in this study were three strains of *Lactococcus lactis* ssp. *lactis* IFPL 22, IFPL 60, and IFPL 359; *Lactobacillus casei* ssp. *casei* IFPL 731 and IFPL 99; and *Lactobacillus plantarum* IFPL 935. The strains had been isolated from semi-hard farmhouse cheese made with caprine raw milk and were from the collection of the Instituto del Frío (Madrid, Spain). Microorganisms had been selected on the basis of their aminopeptidase, X-prolyl-dipeptidyl aminopeptidase, and dipeptidase activities reported by Requena et al. (26) and Asensio et al. (3). Two of these strains, *Lc. lactis* ssp. *lactis* IFPL 359 and *Lb. casei* ssp. *casei* IFPL 731, had already been successfully used as starters in the production of semi-hard caprine cheese (25). The lactococci and lactobacilli employed in this study were stored at –80°C in the respective broths, M17 and MRS (Oxoid, Hampshire, United Kingdom), both containing 15% glycerol as a cryoprotective agent.

The strains were subcultured twice overnight at 30°C before being used to inoculate the batch culture. Cells were harvested during the early stationary growth period by centrifugation (8160 × *g* for 15 min at 4°C), washed, and resuspended in 50 m*M* Tris·HCl, pH 7.0, to a final volume equivalent to 10% of the initial volume.

High Hydrostatic Treatment

We inoculated 10% reconstituted bovine skim milk (**RBSM**) with the bacterial suspensions to a final concentration of ~8 $\log_{10}$ cfu/ml and then divided the mixture into two parts. In each experiment, one part served as an untreated control. The other part was dispensed in approximately 50-ml volumes into polyethylene bottles, avoiding air bubbles, placed inside another vacuum pouch, and vacuum-sealed. The samples were then placed in a pressure unit filled with water as a pressure medium.

Pressurization of samples was carried out using a hydrostatic pressure unit with 2350-ml capacity, maximum pressure of 450 MPa, and potential maximum temperature of 95°C (ACB Gec Alsthom; Nantes, France). Each experiment involved inoculating the RBSM without incubation and exposing them to pressures up to 400 MPa for 20 min at 20°C. All experiments were replicated three times using inocula obtained from different cultures. The rate of increase in pressure with time was linear (150 MPa/min). The time to lower the pressure to atmospheric pressure was 2.5 min. The controls and pressure-treated samples were immediately analyzed for bacterial counts and acidifying activity. Sodium citrate was added to samples (1% final concentration), which were stirred for 30 min and stored at –80°C until used for enzymatic analysis.

Bacterial Counts

Sampling and dilutions were performed in accordance with the International Dairy Federation (13). Lactobacilli and lactococci viable counts were made on MRS and M17 agar (Oxoid, Ltd., London, United Kingdom). The results of pressure inactivation are expressed as $\log_{10} N_o/N$, where N_o = number of viable cells per milliliter before pressure treatment, and N = number of survivors after treatment.

Acidification Activity on Milk

Lactic acid-producing activity of cells was evaluated at 30°C in 10% reconstituted skim milk by measuring the decrease of pH after 6 h of incubation. The milk was inoculated (2%, vol/vol) with either pressure-treated or untreated cells suspensions in RBSM.

Enzyme Extraction

The frozen and thawed suspensions of pressure-treated and control cells of *Lc. lactis* ssp. *lactis* IFPL 359 and *Lb. casei* ssp. *casei* IFPL 731 in sodium citrate were concentrated by 15 min of centrifugation (8600 × *g* at 4°C), washed three times in 50 m*M* Tris·HCl, pH 7.0, and resuspended in the same buffer to a final volume equivalent to 10% of the initial volume.

The bacterial suspensions, submerged in a bath of ice, NaCl, and ethanol, were disrupted with an ultrasonic homogenizer (Labsonic U; B. Braun Biotech, Melsungen, Germany) (8 times for 1 min each with pauses of 30 s). Between 95 and 99% of the cells were destroyed as estimated by viable counts before and after disruption. Unbroken cells and cell fragments were removed by centrifugation (10,000 × *g* for 30 min at 4 °C). The supernatant (enzyme extract) was then passed through 0.22-μm filters (Millipore Corp., Bedford, MA).

Peptidolytic Activities

The hydrolytic activity of the cells on the carboxyl-terminal fragments from the β-CN (f 193–209) was determined; aminopeptidase, x-prolyl-dipeptidyl aminopeptidase, and dipeptidase activities of the enzyme extracts of the most active peptidolytic strains, *Lc. lactis* ssp. *lactis* IFPL 359 and *Lb. casei* ssp. *casei* IFPL 731, were also analyzed. The preparation and isolation of C-peptide was a modified version of the method described by Vreeman et al. (36). One milliliter of chymosin (Chymogen 190; Chr. Hansen, Copenhagen, Denmark) (190 International Milk Clotting Units, method Remcat) was added to 100 ml of a β-CN solution (20 g/L) containing 100 m*M* KCl and 10 m*M* imidazole, pH 6.7. The mixture was incubated for 40 min at 20°C. To stop the reaction, the pH was raised to 9.1 by addition of 500 m*M* NaOH. The C-peptide was isolated and purified from the reaction mixture as described by Vreeman et al. (36).

The assays were performed at 30°C in 50 m*M* sodium acetate buffer, pH 5.4, containing 3% NaCl with 100 μg of purified C-peptide using 1 ml of pressure-treated or untreated cell suspensions obtained as follows. After bacterial suspensions were clarified with sodium citrate, the cells were harvested by centrifugation (8600 × *g* at 4°C for 15 min) and washed twice by resuspension in 200 m*M* sodium acetate buffer containing 10 m*M* $CaCl_2$, pH 5.4, at 4°C. The pellets were resuspended in the incubation buffer to an absorbance of 2.5 at 578 nm. The decrease of C-peptide concentration (micrograms per milliliter) was measured during the incubation by reverse-phase HPLC as described by Smit et al. (29) but using a HiPore RP-318 column (Bio-Rad, Hertsfordshire, United Kingdom) and the following elution conditions: buffer A, 0.1% (vol/vol) trifluoroacetic acid in acetonitrile-MilliQ water (1:9) (Millipore, Saint-Quentin-Yvelines, France); and buffer B, 0.08% (vol/vol) trifluoroacetic acid in acetonitrile-water (9:1). The eluent flow rate was 1 ml/min, and the injection volume was 20 μl. Column temperature was 30°C throughout the experiment. Elution with 80% buffer A and 20% buffer B for 3 min was followed by a linear gradient over 27 min to 50% buffer B and then to 100% buffer B over the next 3 min. The column was washed with 100% buffer B for 2 min, followed by equilibration with 80% buffer A and 20% buffer B for 5 min before the next injection. Peak detection was at 220 nm.

Aminopeptidase (EC 3.4.11) and x-prolyl-dipeptidyl aminopeptidase (EC 3.4.14.5) activities were studied using the substrates L-leucine-*p*-nitroanilide (Leu-*p*-NA) and glycyl-proline-*p*-nitroanilide (Gly-Pro-*p*-NA) (Sigma Chemical Co., St. Louis, MO). The assays were performed at 37°C in 800 μl of 50 m*M* Tris·HCl buffer, pH 7.0, with 100 μl of enzyme extract and 100 μl of substrate (10 m*M*). Initial rates were measured by spectrophotometry at 410 nm (1). One unit of aminopeptidase activity was defined as a variation of 0.01 U of absorbance during 1 min.

Dipeptidase (EC 3.4.13) activity was determined on glycyl-L-leucine (Gly-Leu) (Sigma Chemical Co.). The reaction mixture consisted of 900 μl of dipeptide (1 m*M*) in 50 m*M* Tris·HCl buffer (pH 7.0) and 100 μl of enzyme extract. Assays were carried out at 37°C. After 2 h of incubation, an aliquot (500 μl) was taken, to which 100 μl water and 600 μl 24% of TCA were added. A pellet was separated by 15 min of centrifugation (5000 × *g*) at 4°C. Amino acid content was determined in 50 μl of supernatant by spectrophotometer reading at 340 nm after 2 min of incubation at room temperature with 1000 μl of *o*-phthaldialdehyde reagent (8). The unit of dipeptidase activity was defined as a variation of 0.01 U of absorbance at 15 min.

The results of enzymatic activities were expressed as a percentage of specific activity of untreated cells, determined as the number of units of activity per milligram of extract proteins, and measured by the method of Lowry (Bio-Rad DC Protein Assay, Bio-Rad, Redmond, CA).

RESULTS AND DISCUSSION

Viability and Acidifying Activity of Strains

Pressure treatment at 100 (data not shown) to 200 MPa did not affect the viability of *Lc. lactis* ssp. *lactis* IFPL 60 or *Lc. lactis* ssp. *lactis* IFPL 22, and 250 MPa did not affect viability of *Lc. lactis* ssp. *lactis* IFPL 359 (Table 1). At higher pressures, viability was reduced in all strains, and especially *Lc. lactis* ssp. *lactis* IFPL 22. The lactococci proved more sensitive than did the lactobacilli to pressures within the range of 100 to 350 MPa; these pressures had practically no effect on the viability of the lactobacilli. After treatment at 400 MPa, *Lb. casei* ssp. *casei* IFPL 99 and *Lb. plantarum* 935, the most resistant strains, showed reductions of 0.17 and 1.31 $\log_{10}$ for the initial number of viable cells per number of survivors, respectively, as compared with reductions of 2.96 log for the initial number of viable cells per number of survivors for *Lb. casei* ssp. *casei* IFPL 731 in the same conditions. These results demonstrate major variation in pressure sensitivity within strains of the same species. Carlez et al. (6) also reported high resistance of

TABLE 1. Effects of high pressure on viability of lactococci and lactobacilli strains (n = 3).[1]

	Lactococcus lactis ssp. *lactis*						*Lactobacillus casei* ssp. *casei*				*Lactobacillus plantarum*	
Pressure	IFPL22		IFPL 60		IFPL 359		IFPL 99		IFPL 731		IFPL935	
(MPa)	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
200	0.06	0.04	0.01	0.01	0.0	. . .	0.0	. . .	0.0	. . .	0.0	. . .
250	0.71	0.03	0.35	0.03	0.05	0.01	0.0	. . .	0.0	. . .	0.0	. . .
300	1.56	0.04	0.81	0.04	1.14	0.01	0.06	0.03	0.03	0.03	0.09	0.03
350	3.00	0.07	2.80	0.04	1.70	0.05	0.11	0.01	0.39	0.06	0.13	0.04
400	3.18	0.09	2.98	0.02	1.87	0.10	0.17	0.05	2.96	0.02	1.31	0.06

[1]Values are log (number of cells before pressure/number of cells after pressure).

Lactobacillus spp. in pressure-treated minced meat, which were only slightly affected at 100 and 200 MPa with reductions of 1.50 log for the initial number of viable cells per number of survivors after treatment at 300 MPa and extensive inactivation (>4 $\log_{10}$ cfu/g) at 400 and 450 MPa.

High pressure seemed to affect the acidifying activity of the lactococci and lactobacilli more than cell viability. *Lactococcus lactis* ssp. *lactis* strains IFPL 60, IFPL 22, and IFPL 359 treated at 200 MPa lost, respectively, 48.4 (±5.3), 57.5 (±5.3), and 65.8% (±7.4) of acid-producing ability with respect to the untreated control. Reduction of the acidifying activity was more marked as the pressure increased. *Lactobacillus casei* ssp. *casei* strains IFPL 731 and IFPL 99 and *Lb. plantarum* 935 showed less acidifying activity than lactococci with almost no change in the pH of milk after treatments at 300 MPa, in which conditions there was no apparent loss of cell viability. We can offer no clear explanation for these results, which could be related to the effects of high pressure on the enzymes specific to lactose phosphorylation and transportation or to events connected with the acid-base physiology of the cell (37). High pressure-induced deactivation and subsequent reactivation have been observed for other enzymes such as glyceraldehyde-3-phosphate dehydrogenase from baker's yeast (27), chymotrypsin, trypsin, and collagenase (28). Such deactivation and reactivation could account for the effect of the treatment having an effect at 6 h (acidifying test) but not at 48 h (viability test). A transitory effect of high pressure on microbial injury has also been reported by Carlez et al. (6) and Patterson et al. (23), who found that the growth rate of the recovered organisms after the initial lag was similar to that of the untreated controls, which has important implications for the storage of pressure-treated food. On the other hand, Wouters et al. (37) reported that the activity of F_oF_1 ATPase activity of *Lb. plantarum*, which is closely related to the ability of cells to generate a proton gradient, was reduced at 250 MPa. They concluded that under these conditions the proton translocation step is inhibited and that the acid efflux from cells was impaired. According to those researchers (37), the ATP pool increased after mild pressure treatment, which suggests that glycolysis is less affected than F_oF_1 ATPase by high pressure.

The results in the present study are consistent in qualitative terms with the findings of others for thermophilic microorganisms, yogurt cultures (32), and *Lb. helveticus* (18). Pressure treatment of yogurt at 200 to 300 MPa at room temperature (10 min) prevented acidification after treatment but maintained the initial number of lactic acid bacteria, and higher pressure prevented acidification after pressure treatment but also reduced the number of viable bacteria. According to those authors (32), a lack of further acidification by the yogurt cultures with no drop in their viable cell counts suggests that the bacteria may have been sufficiently injured by the pressure exposure to prevent replication in the low pH environment. Miyakawa et al. (18) also found inhibition of acid production for cells of *Lb. helveticus* resuspended in phosphate buffer and treated at 400 MPa at 30°C for 10 min.

Peptidolytic Activities of Strains

Studies were made of the effect of high pressures (up to 400 MPa) on the hydrolytic activity of *Lc. lactis* ssp. *lactis* strains IFPL 359, IFPL 22, and IFPL 60; *Lb. casei* ssp. *casei* strains IFPL 731 and IFPL 99; and *Lb. plantarum* IFPL 935 on the carboxyl-terminal fragment 193–209 of β-CN (the C-peptide, relative molecular weight of 1880). This peptide is of interest because it contributes to bitterness of cheese (35).

As a general rule, the cells subjected to high pressure exhibited greater hydrolytic activity on the C-peptide when compared with fresh cells. The maxi-

TABLE 2. Hydrolysis of the β-CN (f 193–209) during the incubation with untreated cells and pressure treated cells of lactococci and lactobacilli strains (n = 3).

Microorganism	Pressure treatment	Decrease of β-CN (f 193–209) after incubation (%)			
		1.5 h		3.0 h	
		$\bar{X}$	SD	$\bar{X}$	SD
Lactococcus lactis ssp. *lactis*					
IFPL 359	None	17.1	1.6	20.6	3.1
	300 MPa	34.2	2.5	50.4	2.9
IFPL 22	None	5.0	0.5	9.1	1.6
	300 MPa	13.5	1.3	23.8	2.9
IFPL 60	None	2.0	0.2	2.8	0.7
	300 MPa	4.5	0.8	9.4	1.8
Lactobacillus casei ssp. *casei*					
IFPL 731	None	31.0	3.6	63.2	3.9
	350 MPa	72.4	4.3	100.0	0.1
IFPL 99	None	1.9	0.2	3.4	0.4
	400 MPa	17.5	1.8	28.6	2.5
Lactobacillus plantarum					
IFPL 935	None	3.4	0.5	4.6	0.8
	400 MPa	18.6	2.0	29.0	2.0

mum increase of activity was found after treatment at 300 and ≥350 MPa in lactococci and lactobacilli, respectively. Table 2 compares hydrolysis of the C-peptide by the control cells and cells treated at the designated pressures of all strains. Control and pressurized cells of *Lb. casei* ssp. *casei* IFPL 731 and *Lc. lactis* ssp. *lactis* IFPL 359 showed the highest rate of hydrolysis of C-peptide followed by other strains; by the end of incubation, the pressurized cells of *Lb. casei* IFPL 731 and *Lc. lactis* IFPL 359 had hydrolyzed 100.0 and 50.4% of the C-peptide as compared with 63.2 and 20.6% in the case of the untreated controls. The rate of hydrolysis of the C-peptide could be associated with debittering ability of strains as previously reported by Smit et al. (29), although organoleptic analysis of hydrolyzed samples would be needed to verify it. Those researchers (29) found that cheeses made with strains selected for their high hydrolytic activity on the C-peptide were less bitter than those made with starter alone, but there was no reduction in bitterness of the cheese made with some other strains. In such cases, bitter-tasting peptides other than the C-peptide might have been released in the cheese, or degradation products of the C-peptide might still be large enough to result in a bitter taste.

The high peptidolytic activity observed in pressurized cells may be due to increased cell lysis or to enhancement of membrane permeability, because the C-peptide is too large to cross the cell membrane of the lactic acid bacteria (29). The effect of pressure treatment at over 200 MPA on membrane permeability and the release of intracellular substances from cells has been reported by Osumi et al. (22) and Isaacs et al. (14) for *Candida tropicalis*, *Escherichia coli*, and *Listeria monocytogenes*. We know of no similar studies of lactic acid bacteria that were pressure treated.

The effect of the high pressures would be similar to that found by Tsuchido et al. (33), who reported destruction of the permeability barrier, leakage of intracellular material from the cells, and sensitization to hydrophobic dyes and some hydrophobic antibiotics in heat-shocked cells of *E. coli*. Feirtag and McKay (10) also observed a release of intracellular peptidases of *Lc. lactis* ssp. *cremoris* after heating to 38 to 40°C during the manufacture of Cheddar cheese.

The effects of high pressure on the intracellular peptidase activities of *Lc. lactis* ssp. *lactis* IFPL 359 and *Lb. casei* ssp. *casei* IFPL 731 were studied to determine the enzymatic potential of the pressurized cells for degradation of other bitter peptides too. As Figure 1 shows, the aminopeptidase, X-prolyl-dipeptidyl aminopeptidase, and dipeptidase activities of cell-free extracts of both strains were not negatively affected by pressures of 100, 200, or 300 MPa. Nevertheless, 400 MPa (the most severe condition) decreased the X-prolyl-dipeptidyl-aminopeptidase (48%), aminopeptidase (65%), and dipeptidase (75%) activities of *Lb. casei* ssp. *casei* IFPL 731, but *Lc. lactis* ssp. *lactis* IFPL 359 was unaffected. Miyakawa et al. (18), using sonicated cell suspensions, reported an increase of aminopeptidase and X-prolyl dipeptidyl aminopeptidase activities of *Lb. hel-*

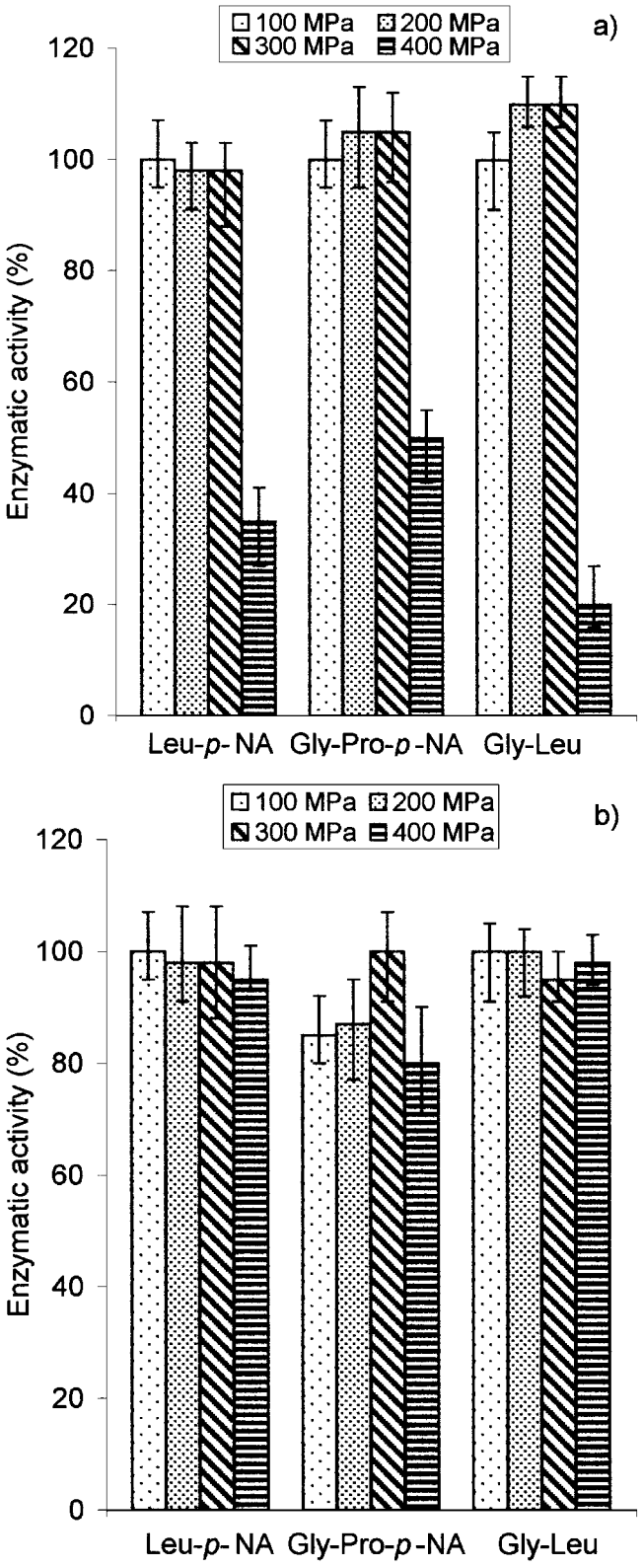

Figure 1. Effects of high pressure treatment on the activity of *Lactobacillus casei* ssp. *casei* IFPL 731 (a) and *Lactococcus lactis* ssp. *lactis* IFPL 359 (b) on L-leucine-*p*-nitroanilide (Leu-*p*-NA), glycyl-proline-*p*-nitroanilide (Gly-Pro-*p*-NA), and glycyl-L-leucine (Gly-Leu).

veticus after cells were subjected to 400 MPa at 30°C for 10 min in phosphate buffer. Unlike those results or the results in the present study, Ohmori et al. (21) reported lower resistance of beef peptidases to high pressure. In that study, the extracts from pressure-treated beef retained 80% of aminopeptidase activity and 90% of carboxypeptidase activity after treatment at 200 MPa, but these peptidases were completely inactivated at 400 or 500 MPa.

CONCLUSIONS

The lactic acid-producing capacity of lactococci and lactobacilli cells was reduced after treatment at 200 MPa or higher. If cells of these strains treated at those pressures were used as starter adjunct in cheese making, the normal process of acidification would not be disturbed. At the same time, high pressure augmented the hydrolytic activity of all strains tested on the β-CN (f 193–209), which contributes to bitterness in cheese; the highest activities were found in *Lc. lactis* ssp. *lactis* IFPL 359 and *Lb. casei* ssp. *casei* IFPL 731 treated at 300 and 350 MPa. At these pressures, the lactococci and, quite possibly, the lactobacilli retained high aminopeptidase and dipeptidase activities because these enzymes in strain IFPL 731 were unaffected at 300 MPa and were only partially inactivated at 400 MPa. The results of this study suggest that cells of *Lc. lactis* ssp. *lactis* IFPL 359 and *Lb. casei* ssp. *casei* IFPL 731 treated at 300 and 350 MPa, respectively, may be added during cheese making to give the cheese an extra supply of enzymes with potential debittering properties. Further research is needed to determine whether high pressures induce increased permeability of cell membranes and whether the effect is due to induction of cell lysis with consequent early release of enzymes. It would also be necessary to determine whether these systems are effective in a dynamic biological system such as cheese. This work is now proceeding at our laboratory.

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