

## High Pressure Effects on Proteolytic and Glycolytic Enzymes Involved in Cheese Manufacturing

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### ABSTRACT

The activity of chymosin, plasmin, and *Lactococcus lactis* enzymes (cell envelope proteinase, intracellular peptidases, and glycolytic enzymes) were determined after 5-min exposures to pressures up to 800 MPa. Plasmin was unaffected by any pressure treatment. Chymosin activity was unaffected up to 400 MPa and decreased at 500 to 800 MPa. Fifty percent of control chymosin activity remained after the 800 MPa treatment. The lactococcal cell envelope proteinase (CEP) and intracellular peptidase activities were monitored in cell extracts of pressure-treated cells. A pressure of 100 MPa increased the CEP activity, whereas 200 MPa had no effect. At 300 MPa, CEP activity was reduced, and 400 to 800 MPa inactivated the enzyme. X-Prolyl-dipeptidyl aminopeptidase was insensitive to 5-min pressure treatments of 100 to 300 MPa, but was inactivated at 400 to 800 MPa. Aminopeptidase N was unaffected by 100 and 200 MPa. However, 300 MPa significantly reduced its activity, and 400 to 800 MPa inactivated it. Aminopeptidase C activity increased with increasing pressures up to 700 MPa. High pressure did not affect aminopeptidase A activity at any level. Hydrolysis of Lys-Ala- $\rho$ -NA doubled after 300-MPa exposure, and was eliminated at 400 to 800 MPa. Glycolytic enzyme activities of pressure-treated cells were evaluated collectively by determining the titratable acidity as lactic acid produced by cell extracts in the presence of glucose. The titratable acidities produced by the 100 and 200 MPa samples were slightly increased compared to the control. At 300 to 800 MPa, no significant acid production was observed. These data demonstrate that high pressure causes no effect, activation, or inactivation of proteolytic and glycolytic enzymes depending on the pressure level and enzyme. Pressure treatment of cheese

may alter enzymes involved in ripening, and pressure-treating *L. lactis* may provide a means to generate attenuated starters with altered enzyme profiles.

(**Key words:** cheese ripening, *Lactococcus*, peptidase, protease)

**Abbreviation key:** CEP = cell envelope protease, HPP = high pressure processing, PepX = X-prolyl-dipeptidyl aminopeptidase, PepN = aminopeptidase N, PepC = aminopeptidase C, PepA = aminopeptidase A,  $\rho$ -NA =  $\rho$ -nitroanilide.

### INTRODUCTION

Cheddar cheese maturation involves complex microbiological and biochemical changes. Proteolytic, glycolytic, and lipolytic enzymes can play roles. The appearance of peptides and amino acids as a result of casein proteolysis is essential for achieving a characteristic Cheddar cheese flavor and texture (Fox et al., 1996; Kunji et al., 1996; Law, 2001). The sources of proteolytic enzymes in Cheddar cheese are indigenous milk enzymes, the coagulant, and starter and non-starter microorganisms.

Chymosin is responsible for the initial hydrolysis of casein during maturation (Fox et al., 1996). Plasmin, a native milk enzyme, also participates. These enzymes produce large and intermediate-sized peptides. The starter culture, *Lactococcus lactis*, has a cell envelope-associated proteinase (CEP) and a battery of specific intracellular peptidases. The CEP hydrolyzes the intermediate molecular weight peptides into low molecular weight peptides that are then degraded to free amino acids by intracellular peptidases after importation into the cell or cell lysis (Kunji et al., 1996; Mierau et al., 1996; Christensen et al., 1999). Peptidolysis in cheese does not readily occur until the starter bacteria lyse, which results in the release of intracellular peptidases in the cheese matrix where they have direct access to their peptide substrates (Boutrou et al., 1998). These peptidases hydrolyze low molecular weight peptides to form free amino acids that can participate in further chemical reactions.

Although the majority of lactose in milk is fermented during curd production or removed with the

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whey, the fresh cheese contains 0.7 to 1.5% lactose that is fermented by the starter organisms during the early stages of cheese ripening (Fox et al., 1996). Therefore, assessment of the lactic acid-producing capabilities of pressure-treated cells is relevant to cheese maturation studies. Lactic acid production by pressure-treated cells is also of interest as a potential means to generate attenuated starter cultures. Attenuated starter cultures are disabled in their ability to ferment lactose to L-lactic acid and can be added along with the normal starter culture to provide an additional source of proteolytic enzymes without affecting acid production rate during cheese making (Fox et al., 1996).

High pressure processing (**HPP**) is a novel technology that may have many applications in the food industry. Much of the published work involving pressure processing has been aimed at inactivating food pathogens and degradative enzymes (Cheftel, 1992). Yet, substantial evidence exists that low to moderate pressures (100 to 400 MPa) can activate enzyme activity (Anese et al., 1995; Cano et al., 1997), whereas higher pressures inactivate the same enzymes (Gomes and Ledward, 1996; Goodner et al., 1998). In the case of cheese, HPP has minimal effect on texture and consistency, but if applied early in ripening, may affect cheese-ripening parameters through modulation of enzymatic reactions or viable bacterial populations (Miyakawa et al., 1994; Messens et al., 1999; O'Reilly et al., 2000).

In this study, the effects of high pressure on acid-producing and protein-degrading enzymes of *L. lactis* subsp. *cremoris* MG1363, and on chymosin and plasmin activities were investigated. Results will help evaluate the biochemical basis for the proteolytic changes and flavor formation during ripening of high-pressure treated Cheddar cheese.

## MATERIALS AND METHODS

### Sample Preparation and Pressure Treatment

Bovine plasmin (5 U/ml, Roche Applied Science, Indianapolis, IN) was diluted to 0.3 U/ml in 1% Tween-20, 0.83 mM PEG 6000, 50 mM glycine (pH 2.5). Aliquots (0.24 ml) of the diluted plasmin were heat-sealed in sterile plastic bags (Nasco, Fort Atkinson, WI) before pressure treatment. Likewise, a commercial chymosin product (Chy-Max Extra, Chr. Hansen, Milwaukee, WI) was dispensed (0.5 ml) without dilution into sterile plastic bags that were then sealed.

*Lactococcus lactis* subsp. *cremoris* MG1363 (Gasson, 1983) was grown to late exponential phase at 30°C in M17 broth (Difco, Detroit, MI) with 0.5% glu-

cose (Terzaghi and Sandine, 1975). The cell pellet was collected from 15 ml of culture and washed once in cold, sterile PBS at pH 7.0, then resuspended in 15 ml of PBS. The cell suspension was aseptically transferred to a sterile plastic bag that was sealed.

Pressures tested were in 100-MPa intervals from 100 to 800 MPa for 5 min at 25°C in a Quintus High Pressure Food Processor (Flow Pressure Systems, Kent, WA). The pressure transmitting fluid was one part distilled water:one part Houghto-Safe 620-TY (Houghton International, Valley Forge, PA). The initial temperature of the pressure transmitting fluid was controlled to account for adiabatic heating. Setting the temperature of the water-jacketed pressure chamber to the desired process temperature ensured constant process temperatures. Pressure, product temperature, and water-jacket temperature were monitored and recorded in 3-s intervals using a 21X Micrologger (Campbell Scientific Inc., Logan, UT) connected to a computer running PC208W datalogger support software (Campbell Scientific Inc., Logan, UT). An untreated control was left at 25°C at atmospheric pressure (0.1 MPa) for 5 min while each pressure treated sample was being pressurized. Enzyme activities were determined immediately after pressure treatment.

### Plasmin Activity Assay

Plasmin activity was determined using Chromozym PL (tosyl-Gly-Pro-Lys- $\rho$ -nitroanilide) as the substrate following the manufacturer's instructions (Roche Applied Science). Release of  $\rho$ -nitroaniline ( $\rho$ -NA) was measured spectrophotometrically at 405 nm for 30 s. Enzyme activity was calculated as  $\mu\text{mol min}^{-1} \text{ml}^{-1}$  using the molar extinction coefficient at 405 nm ( $\epsilon_{405}$ ) at  $10.4 \text{ mM}^{-1}\text{cm}^{-1}$ .

### Chymosin Activity Assay

The pressure-treated chymosin samples were diluted 1:10 in 0.1 M NaCl, 0.01 M acetate buffer and were assayed by the method of Salesse and Garnier (1976) using a 0.4 mM solution of the substrate H-Leu-Ser- $\rho$ -nitro-Phe-Nle-Ala-Leu-OMe-TFA (Bachem, Bubendorf, Switzerland). The change in molar extinction coefficient at 310 nm ( $\epsilon_{310}$ ) of  $0.990 \text{ mM}^{-1}\text{cm}^{-1}$  was used to calculate the enzyme activity as  $\text{mmol min}^{-1}\text{ml}^{-1}$  (Martin et al., 1981). Activities reported are for the undiluted sample.

### CEP Activity Assay

The CEP assay was performed by the method of Mierau et al. (1996) with some modifications. After

the pressure treatment, the cell pellet from 1 ml of cell suspension was washed in 80 mM Tris-HCl, pH 7.0 containing 5 mM CaCl<sub>2</sub>, then suspended in the same buffer containing 2 mM methoxy-Suc-Arg-Pro-Tyr- $\rho$ -nitroanilide (DiaPharma Group Inc., West Chester, OH). The suspension was incubated at 30°C and aliquots of the supernatant were monitored for release of  $\rho$ -NA spectrophotometrically at 410 nm. Enzyme activity was calculated as  $\mu\text{mol } \rho\text{-NA min}^{-1}$  using  $\epsilon_{410} = 8.8 \text{ mM}^{-1}\text{cm}^{-1}$ . Specific activities were calculated as  $(\mu\text{mol } \rho\text{-NA}) (\text{min})^{-1} (\text{mg of protein})^{-1}$ .

### Peptidase Activity Assays

Peptidase activities were determined by the methods of Mierau et al. (1996) and Vesanto et al. (1995) with some modifications. The cell pellet from 1.5 ml of pressure- or nonpressure-treated samples ( $\sim 1 \times 10^8$  cfu/ml) was washed and resuspended in 300  $\mu\text{l}$  of 20 mM Tris-HCl (pH 7.0). Approximately 0.1 g of glass beads (0.1 mm) was added to the cell suspension. The cells were broken using a Mini-BeadBeater (Bio-Spec Products, Inc., Bartlesville, OK) by shaking for three 30-s intervals at maximum speed. Between each interval, the samples were placed on ice for 5 min. Cell extracts were recovered after centrifugation.

The enzyme activities were determined at 30°C using 0.2 ml of cell extract (0.3 mg ml<sup>-1</sup> protein) added to 0.8 ml of 20 mM Tris-HCl (pH 7.0) containing 0.2 mM substrate. Each reaction mixture was monitored for release of  $\rho$ -NA spectrophotometrically at 410 nm over time. Enzyme specific activities were calculated as described above for CEP activity. X-Prolyl-dipeptidyl aminopeptidase (**PepX**) activity was evaluated using two substrates, Gly-Pro- $\rho$ -NA and Ala-Pro- $\rho$ -NA (Christensen et al., 1999). Aminopeptidase N (**PepN**) and aminopeptidase A (**PepA**) were evaluated using Lys- $\rho$ -NA and Glu- $\rho$ -NA, respectively. Aminopeptidase C (**PepC**) is also active on these substrates (Christensen et al., 1999), thus PepN and PepA activities were distinguished from PepC by the addition of 5 mM EDTA. PepN and PepA are metalloproteins that are inhibited by EDTA (Niven, 1991; Exterkate and DeVeer, 1987; Tan and Konings, 1990), whereas PepC is not (Neviani et al., 1989). PepN and PepA activities were calculated as the EDTA-sensitive activity on the appropriate substrate by subtracting the activity in the presence of EDTA from the activity without EDTA. PepC activity was reported by hydrolysis of Lys- $\rho$ -NA in the presence of 5 mM EDTA. Hydrolysis of Lys-Ala- $\rho$ -NA was also evaluated. All  $\rho$ -nitroanilide substrates were purchased from Bachem (Bubendorf, Switzerland).

### Protein Concentration

The protein concentration of each cell extract was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) based on the Bradford method (Bradford, 1976). Bovine serum albumin was the standard. Cell extracts were standardized to 0.3 mg ml<sup>-1</sup> protein with 20 mM Tris-HCl before peptidase assays. Protein concentration was also determined in selected cell extracts by analysis in a Perkin Elmer Series II Nitrogen Analyzer 2410 (Perkin Elmer, Inc., Wellesley, MA).

### Lactic Acid Production by Cell Extracts

Lactic acid production by cell extracts was determined by the method of Miyakawa et al. (1994) with some modifications. Cells were grown to late exponential phase ( $\sim 1 \times 10^8$  cfu/ml) in 250 ml of G-M17. The cells were washed and concentrated in 20 ml of PBS (pH 7.0). The cell suspension was pressure treated in a sterile plastic bag. After the pressure treatment, the cell suspension was sonicated for 10 min at 4°C (Ultrasonic Processor 36800, Cole Parmer, Vernon Hills, IL). Following centrifugation, 5 ml of each cell extract were added to 45 ml of a 1.1% (wt/vol) glucose solution. The samples were incubated for 1 h at 30°C. The percent lactic acid (g/100 ml) was estimated by titration with 0.1 N NaOH using phenolphthalein as the pH indicator.

### Statistical Analysis

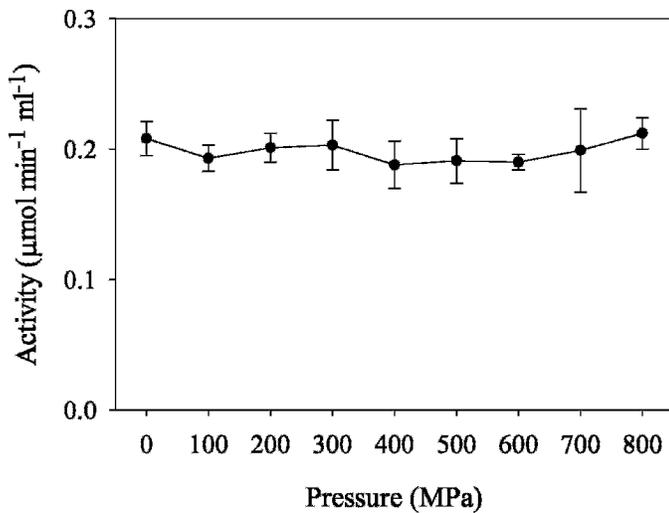
Data are shown as mean  $\pm$  SD and are the results of at least three independent replications of each experiment. Mean comparisons between different pressures for each enzyme evaluated were performed with a one-way ANOVA followed by Tukey's test with  $\alpha = 0.05$  (JMPin, SAS Institute Inc., Cary, NC and SigmaStat 5.0, SPSS Inc., Chicago, IL).

## RESULTS AND DISCUSSION

### Chymosin and Plasmin Activities

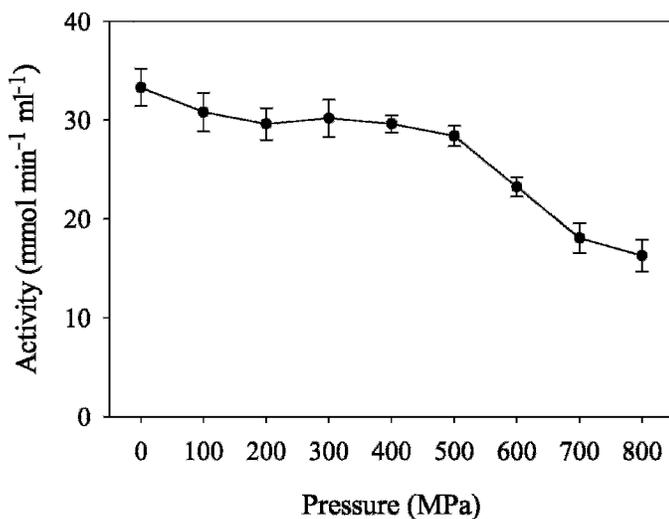
Plasmin activity was not affected significantly by any pressure treatment ( $P > 0.05$ ) (Figure 1). Chymosin treated with 100 to 400 MPa did not differ significantly in activity from the control ( $P > 0.05$ ) (Figure 2). At 500 to 800 MPa, chymosin activity was reduced with increasing pressure ( $P < 0.05$ ). Approximately 50% of the control chymosin activity remained after treatment at 800 MPa.

Based on SDS-PAGE analysis of pH 4.6-insoluble nitrogen, Messens et al. (1999) concluded that plas-

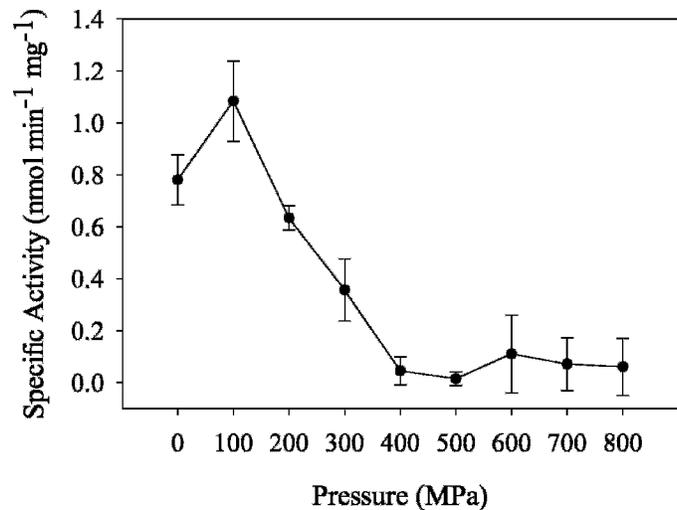


**Figure 1.** Plasmin activity ( $\mu\text{mol min}^{-1} \text{ml}^{-1}$ ) after pressure exposure for 5 min at 25°C. Error bars represent  $\pm 1$  SD,  $n \geq 3$ .

min and chymosin activities in Gouda cheese were not affected by pressure treatments of 50 to 400 MPa for 20 to 100 min. Scollard et al. (2000) found that plasmin is pressure stable in most systems and is able to hydrolyze  $\beta$ -casein after pressure treatments up to 700 MPa. A treatment of 400 MPa at 25°C for 30 min did not inactivate plasmin in milk (García-Risco et al., 1998). Rennet proteolysis in milk was not affected by 127 MPa for 90 min (Ohmiya et al., 1987). The data presented here concur with those in the literature, though the medium in which the enzymes were treated, the enzyme assay method, and the duration of pressure treatment differed.



**Figure 2.** Chymosin activity ( $\text{mmol min}^{-1} \text{ml}^{-1}$ ) after pressure exposure for 5 min at 25°C. Error bars represent  $\pm 1$  SD,  $n \geq 3$ .



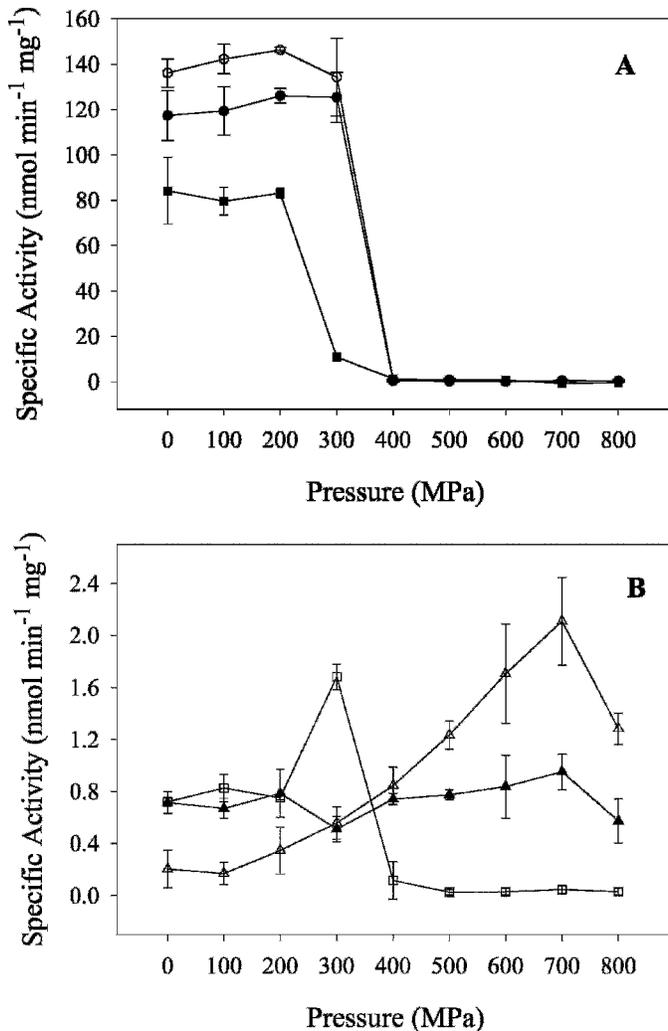
**Figure 3.** Specific activity ( $\text{nmol min}^{-1} \text{mg}^{-1}$ ) of the *Lactococcus lactis* MG1363 cell envelope proteinase (CEP) after pressure exposure for 5 min at 25°C. Error bars represent  $\pm 1$  SD,  $n \geq 3$ .

### CEP Activity

After exposure to 100 MPa, CEP activity was significantly higher than at all other pressures, including the atmospheric pressure control ( $P < 0.05$ ) (Figure 3). The 0.1- and 200-MPa samples were not statistically different. The CEP activity was significantly reduced at 300 MPa and was essentially inactivated at 400 to 800 MPa. No previous reports of lactococcal CEP activity post-pressurization are available.

### Peptidase Activities

Activities of two broad specificity aminopeptidases (PepN and PepC), a glutamyl aminopeptidase (PepA), and PepX were evaluated in cell extracts after pressure treatment of intact cells. In addition, hydrolysis of Lys-Ala- $\rho$ -NA was evaluated. PepN was unaffected by 100- and 200-MPa treatments compared with the control (Figure 4A). However, at 300 MPa, PepN activity was dramatically reduced; and at 400 to 800 MPa, PepN was inactivated. High pressure had a positive effect on PepC activity (Figure 4B). PepC activity increased with increasing pressure from 0.1 MPa up to 700 MPa and declined at 800 MPa. At 400 to 800 MPa, PepC activity was significantly increased ( $P < 0.05$ ) compared with the control (0.1 MPa). At 700 MPa, a maximum activity of  $2.11 \mu\text{mol min}^{-1} \text{mg}^{-1}$  was observed, whereas the 0.1- and 800-MPa samples had activities of 0.20 and  $1.28 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , respectively. PepA activity was not significantly affected by HPP at any level (Figure 4B).



**Figure 4.** Specific activities (nmol min<sup>-1</sup> mg<sup>-1</sup>) of intracellular peptidases after pressure exposure of *Lactococcus lactis* MG1363 for 5 min at 25°C. Error bars represent  $\pm 1$  SD,  $n \geq 3$ . Panel A: PepX hydrolysis of Gly-Pro- $\rho$ NA (●) and Ala-Pro- $\rho$ NA (○) and PepN hydrolysis (EDTA-sensitive) of Lys- $\rho$ NA (■). Panel B: PepA hydrolysis (EDTA-sensitive) of Glu- $\rho$ NA (▲), PepC hydrolysis (EDTA-resistant) of Lys- $\rho$ NA (△), and hydrolysis of Lys-Ala- $\rho$ NA (□).

PepX was unaffected by 100-, 200-, and 300-MPa pressure treatments compared with the control ( $P < 0.05$ ) (Figure 4A). PepX was inactivated at pressures of 400 MPa and greater. PepX activity was examined on two substrates, Gly-Pro- $\rho$ -NA and Ala-Pro- $\rho$ -NA. Both substrates yielded the same enzyme inactivation profile indicating that activities on two different substrates are affected similarly by pressure treatment.

Hydrolysis of Lys-Ala- $\rho$ -NA was unaffected by 100 and 200 MPa and inactivated at pressures of 400 to 800 MPa (Figure 4B). At 300 MPa, Lys-Ala- $\rho$ -NA hydrolysis was significantly higher ( $P < 0.05$ ) than at all other pressures with double the activity of the

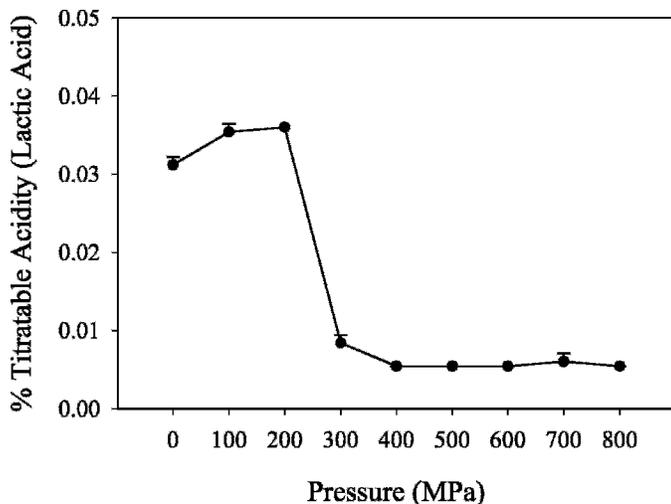
nonpressure-treated control. To our knowledge, Lys-Ala- $\rho$ -NA hydrolysis by *L. lactis* cell extracts has not been described previously. Release of the chromogenic  $\rho$ -NA from this substrate could result from dipeptidyl peptidase activity or from two consecutive aminopeptidase reactions, with the second releasing  $\rho$ -NA from Ala- $\rho$ -NA. We cannot distinguish between these possibilities with the present data, although the pressure inactivation profiles suggest that hydrolysis of Lys-Ala- $\rho$ -NA is not solely due to the general aminopeptidases, PepC and PepN.

Other researchers have reported inactivation and activation of bacterial proteolytic enzymes by high pressure. Pressurizing *Lb. helveticus* cells to 400 MPa at 30°C for 10 min increased aminopeptidase and PepX activities (Miyakawa et al., 1994). In contrast, Casal and Gómez (1999) found reduced aminopeptidase, PepX and dipeptidase activity after a 20-min, 400-MPa treatment of *Lb. casei*, whereas no reduction was observed in *L. lactis*. These studies suggest interspecific variation in pressure sensitivity of aminopeptidases. Differences in pressure application time, pressurization medium, bacterial species, and peptidase assay substrates make direct comparison to the present data difficult. Nonetheless, it is apparent that pressure has different effects on different peptidolytic enzymes within a bacterial strain. Additional studies are required to fully understand any inter- and intraspecific variations in pressure sensitivity of enzymes.

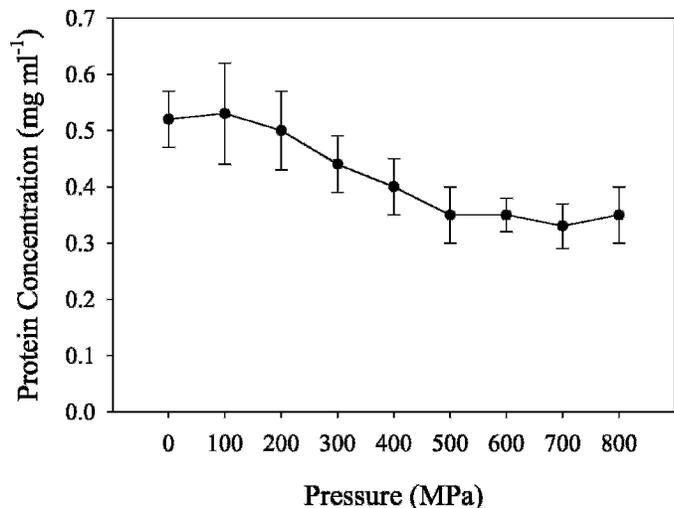
#### Lactic Acid Production by Cell Extracts

The glycolytic capacity of pressure-treated cells was assessed by incubating cell extracts with glucose for 1 h and measuring titratable acidity as percent lactic acid (g/100 ml). The titratable acidities of the 100- and 200-MPa samples were slightly increased compared to the control level of 0.031% ( $P < 0.05$ ) (Figure 5). In samples treated at 300 to 800 MPa, titratable acidities were significantly decreased to 0.006 to 0.008%, indicating a loss in activity of one or more enzymes in the glycolytic pathway. Cell extracts incubated without glucose yielded 0.006% titratable acidity. We previously showed that *L. lactis* MG1363 is not viable after pressure treatments of 300 to 800 MPa (Malone et al., 2002), corresponding to pressures at which cellular glycolytic capability is inactivated.

Pressurizing *Lb. helveticus* at 400 MPa for 10 min at 30°C completely inhibited its acid producing ability (Miyakawa et al., 1994). Pressurizing *L. lactis* at 200 MPa for 20 min at 20°C resulted in lower acidification rates when the pressure-treated cells were subsequently incubated in milk (Casal and Gómez, 1999), though the methodology used makes it unclear



**Figure 5.** Percent titratable acidity (as lactic acid) produced by cell extracts incubated with glucose. Cell extracts were prepared from *Lactococcus lactis* MG1363 cells after pressure exposure for 5 min at 25°C. Error bars represent plus 1 SD,  $n \geq 3$ .



**Figure 6.** Protein concentration (mg ml<sup>-1</sup>) in cell extracts from pressure-treated cells determined using the Bio-Rad protein assay. Values are mean  $\pm$  1 SD ( $n = 12$ ).

whether the decreased acidification rate is due to damage to the glycolytic enzymes specifically or due to a reduced growth rate caused by pressure-induced general cell damage.

#### Protein Concentrations of Cell Extracts Prepared from Pressure-Treated Cells

The protein concentrations in lactococcal cell extracts were determined with a Bradford dye-binding assay to standardize the peptidase and CEP enzyme activity assays across pressure treatments. During the course of these experiments, we observed a decrease in protein concentration in the cell extracts with an increase in pressure (Figure 6) though all samples contained approximately the same number of cells. This phenomenon was also reported in tomato extracts (Shook et al., 2001). Possible explanations include: 1) an apparent decrease in protein concentration due to a change in the dye binding capacity of proteins postpressure treatment or 2) an actual decrease in protein concentration caused by aggregation or insolubility of pressure-denatured proteins that are subsequently removed by centrifugation during cell extract preparation. To ensure proper standardization of enzyme activities, we confirmed the cell extract protein concentrations by total nitrogen analysis on cell extracts from 0.1-, 400-, and 800-MPa treated cells. The nitrogen analysis revealed a similar decrease in protein concentration with increasing pressure as observed with the dye-binding assay (data not shown). The protein concentration in cell extracts was

dependent on pressure, and the pressure dependency was not statistically different between the Bradford and total nitrogen assays. Thus, the variations in protein concentration we observed were not an artifact of the protein quantification method used and are likely due to removal of denatured, aggregated proteins during cell extract preparation.

#### CONCLUSIONS

Applications of HPP to cheese may include acceleration, arrest, or modulation of cheese ripening and production of attenuated starters with modified peptidolytic capabilities. The present study investigated the effect of pressure on several enzymes involved in Cheddar cheese ripening. Plasmin is recalcitrant to inactivation by any pressure. Chymosin activity is unaffected up to 400 MPa and decreased by 50% after an 800 MPa treatment. Depending on the pressure treatment, *L. lactis* proteolytic and glycolytic enzymes could be activated, inactivated, or unaffected. A summary of the effect of different pressures on the enzymes studied is presented in Table 1.

Pressure treatment at 300 MPa or higher may provide a new method for attenuation of starter cultures. Acid production by cell extracts was abolished by treatments of 300 to 800 MPa, yet most peptidases studied retained activity after a 300 MPa exposure, and two aminopeptidases retained activity at 400 to 800 MPa (Table 1). Using attenuated starters with selectively activated or inactivated proteolytic en-

**Table 1.** Summary of changes in enzyme activity due to high pressure exposure.

Pressure (MPa)	Increased activity <sup>1</sup>	Unchanged activity <sup>1</sup>	Reduced activity*	Inactive <sup>1</sup>
100	Acid production, CEP	Plasmin, chymosin, PepA, PepC, PepN, PepX, Lys-Ala- $\rho$ NA hydrolysis		
200	Acid production	Plasmin, chymosin, CEP, PepA, PepC, PepN, PepX, Lys-Ala- $\rho$ NA hydrolysis		
300	Lys-Ala- $\rho$ NA hydrolysis	Plasmin, chymosin, PepA, PepC, PepX	CEP, PepN	Acid production
400	PepC	Plasmin, chymosin, PepA		Acid production, CEP, PepN, PepX, Lys-Ala- $\rho$ NA hydrolysis
500–800	PepC	Plasmin, PepA	Chymosin	Acid production, CEP, PepN, PepX, Lys-Ala- $\rho$ NA hydrolysis

<sup>1</sup>Relative activity are in comparison to the non-pressure-treated control.

zymes may provide new variations in the flavor profiles generated by these additives.

The present study has implications for pressure-treated cheese. We demonstrated that pressure has different effects on different enzymes. Thus, pressure treatment of young cheeses will likely alter the active enzyme profiles, potentially changing the flavor profile of the resulting ripened cheese. HPP may provide a novel way by which the flavor of cheeses can be manipulated. Additional research is required to determine if Cheddar cheese ripening can be manipulated positively by pressure treatment of young cheeses.

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