Detection and Localization of a Peptidoglycan Hydrolase in *Lactobacillus delbrueckii* subsp. *bulgaricus*

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ABSTRACT

Peptidoglycan hydrolase activities in *Lactobacillus delbrueckii* subsp. *bulgaricus* were detected by analysis of bacterial extracts on denaturing polyacrylamide gel electrophoresis containing lyophilized *Micrococcus lysodeikticus* cells as substrate. A hydrolase with an estimated molecular mass of 80 kDa was found to cross-react on Western blot with monoclonal antibodies raised against muramidase-2 of *Enterococcus hirae*. These antibodies were also used to demonstrate that the method of cell sample preparation affected protein detection. Slot and Western blots indicate that the peptidoglycan hydrolase from *L. bulgaricus* is bound to the cell wall. Immuno-labeling followed by optical and electron microscopic observations suggest that this hydrolase is intracellular and restricted mainly to the space between the membrane and the cell wall.

(**Key words:** peptidoglycan hydrolase, electron and fluorescence microscopy, *Lactobacillus*)

Abbreviation key: DIC = differential interference contrast, FITC = fluorescein isothiocyanate, FLM = fluorescence microscopy, GAM = goat anti-mouse, LRW = London resin white, OD = optical density, TEM = transmission electron microscopy.

INTRODUCTION

Lactobacillus has a major role in the dairy industry as a producer of lactic acid for casein coagulation and as a contributor to flavor development and ripening of dairy products. *Lactobacillus delbrueckii* subsp. *bulgaricus* (henceforth described as *L. bulgaricus*), used mainly for making yogurt, is often involved in cheese production, particularly Swiss and Mozzarella (Ezzat, 1990; Valence and Lortal, 1995).

Various enzymes are involved in cheese maturation, particularly proteases (Martin-Hernandez et al., 1993; Yamamoto et al., 1993; Kunji et al., 1996), peptidases (Christensen et al., 1999) and esterases (Hikey et al., 1983; Ezzat et al., 1986). Peptidoglycan hydrolases are endogenous cell wall bacterial enzymes that hydrolyse bonds in the peptidoglycan structure (Shockman and Høltje, 1994). Several enzymes with different specificities may be produced by a single bacterial species (Lortal et al., 1997; Smith et al., 2000). These include *N*-acetylmuramidases (lysozymes), *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases and endopeptidases (Ghuysen et al., 1966). A hydrolase that degrades the peptidoglycan of the producing strain is referred to as an autolysin (Ghuysen et al., 1966). Autolytic activity has been demonstrated during cheese production and may contribute to flavor development (Valence and Lortal, 1995).

The characterization of individual peptidoglycan hydrolases was complicated due to many bacterial species possessing more than one enzyme with the same lytic activity. Many of these have now been sequenced and cloned (Shockman and Høltje, 1994; Buist et al., 1995; Husson-Kao et al., 2000; Cibik et al., 2001), which has allowed the construction of specific hydrolase inactivated mutants. The analysis of such mutants and of their peptidoglycan structure has revealed that autolysins play a role in a number of important cellular processes: peptidoglycan maturation, cell separation, motility, competence, cell expansion, cell-wall turnover, protein secretion, germination, vegetative growth, and differentiation (Shockman and Høltje, 1994; Navarre and Schneewind, 1999; Smith et al., 2000).

Factors such as pH, temperature, salt concentration, and carbon source appear to influence the autolytic process (Valence and Lortal, 1995). The degree of autolysis is strain dependent, and the process normally starts after the exponential growth phase. Peptidases may, thus, be released into the cheese matrix after cell lysis, allowing them to participate in cheese ripening and

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flavor development. Autolysis has been described in cheese (Bottazzi, 1993; Wilkinson et al., 1994; Govindasamy-Lucey et al., 2000) and in buffered solutions (Lortal et al., 1989; Cibik and Chapot-Chartier, 2000). However, few studies of autolytic enzymes in *Lactobacillus* species are available. We have recently reported ultrastructural observations of *L. bulgaricus* cell wall degradation and evaluated factors (temperature, pH, NaCl concentration, growth phase, strain dependence) influencing the autolytic process (Kang et al., 1998a). We have also studied the specificity of *L. bulgaricus* autolysins by identification of the newly exposed reducing and free amino acid groups produced by *L. bulgaricus* cell wall hydrolysis. We concluded that at least two autolysins were present in *L. bulgaricus* cell walls, although it was not possible to identify their type and localization (Kang et al., 1998b).

In the present work, we report the detection of an 80 kDa peptidoglycan hydrolase from *Lactobacillus delbrueckii* subsp. *bulgaricus* able to degrade lyophilized *Micrococcus lysodeikticus* cells. This enzyme was found to cross-react with monoclonal antibodies raised against *Enterococcus hirae* N-acetyl-muramidase-2 (Kariyama and Shockman, 1992). These antibodies were also used to evaluate the influence of cell sample preparation method on measured hydrolase level and to determine its cellular localization.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Lactobacillus delbrueckii subsp. *bulgaricus* UL12 was obtained from a local culture collection (STELA, Université Laval). Bacterial growth was performed at 37°C in MRS broth (Difco, Montréal, Québec, Canada) and was monitored by optical density measurements at 650 nm (OD₆₅₀) using a 1001 Plus spectrophotometer (Milton Roy, Rochester, NY) and by dry biomass determination. Biomass recovered by centrifuging 20 ml of culture at 5500 × g for 15 min, washing the pellet three times with distilled water and filtering on 0.45 µm nitrocellulose membrane was oven dried at 104°C for 24 h according to Bibal et al. (1988).

Slot and Western Blotting

Lactobacillus bulgaricus cells from cultures at 4, 6, 8, 10, or 12 h following inoculation were collected by centrifugation at 5500 × g for 15 min at 4°C and washed three times in distilled water. Samples of each washed cells were incubated 3 h in 0.2 M NaCl at 40°C for autolysis (Kang et al., 1998a) and centrifuged as above. The pellets thus obtained and their supernatants were denatured separately at 100°C in 125 mM Tris-HCl (pH

6.7), 4% (wt/vol) SDS plus 2% (vol/vol) β-mercaptoethanol, and total protein in the extracts was quantified using the Bradford reagent (Bio-Rad, Mississauga, Canada). Samples containing ~50 µg of protein were blotted under vacuum (Prioult et al., 2000) on polyvinylidene difluoride (Immobilon-P) membranes (Millipore, Mississauga, Canada) using a Bio-Dot SF apparatus (Bio-Rad, Mississauga, Canada). Immunodetection was done using a Chemiluminescence Blotting Reagent Kit, (Boehringer Mannheim, Laval, Canada). The membrane was blocked for 1 h at 22°C in 25 ml TBS buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 0.05% (vol/vol) Tween 20 and 10% (wt/vol) nonfat dry milk (Kariyama and Shockman, 1992) and then held for 2 h at 22°C with gentle shaking with *Enterococcus hirae* (1/500 in TBS) muramidase-1 (1B6) or muramidase-2 (4B2) mouse monoclonal antibody (Kariyama and Shockman, 1992). After washing three times in TBS-Tween buffer, the membrane was exposed for 2 h at 22°C to a second antibody (1/500 in TBS), which consisted of an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G and M (heavy and light chains) from Jackson Immunoresearch Laboratories (West Grove, PA). This step was then followed by a final washing in TBS-Tween. Antibody reacting spots were visualized using a BCIP/NBT color development kit from Fisher Scientific (Montréal, Canada) according to the manufacturer's instructions.

For Western blotting, the proteins were transferred to Immobilon-P membranes at 100 mA for 1 h in 25 mM Tris/200 mM glycine (pH 8.3) buffer containing 20% (vol/vol) methanol at 4°C (Towbin et al., 1979) with a Mini Protean-II electroblotting apparatus (Bio-Rad, Mississauga, Canada). Immunodetection was done as described above.

SDS-PAGE

Exponential (OD₆₅₀ = 0.8) and stationary (OD₆₅₀ = 3.2) cultures (4 and 12 h, respectively) of *L. bulgaricus* were centrifuged at 5500 × g for 15 min at 4°C and the pellets washed three times in distilled water before freeze-drying at -52°C in a LYPH Lock 4.5 system (Labconco, Kansas City, USA). Cell samples (2.2 mg dry weight each) were then treated separately as follows: a) resuspended in 125 mM Tris-HCl (pH 6.7); b) sonicated six times for 10 s (Ultrason, Fisher, Model 300, small probe, 4000-intensity setting); c) autolysed 3 h at 40°C in 0.2 M NaCl (Kang et al., 1998a); or d) treated by mechanical disruption for 10 min in a refrigerated French Pressure cell (SLM Instruments, Urbana, IL) at 138 MPa (40K Manual-Fill Cell). Following French Press treatment, unbroken cells were removed by centrifugation at 5500 × g for 15 min at 4°C. Cell walls

were recovered from the supernatant by centrifugation at $30,000 \times g$ for 30 min at 4°C , washed five times in distilled water and then lyophilized. Cell wall samples (20 mg dry weight) were resuspended in 1 ml of 1% (vol/vol) Triton X-100, held for 5 min at room temperature, and pelleted again by centrifugation. This last step was repeated six times, and the cell wall pellet was finally washed three times with distilled water. The soluble proteins in the pooled Triton X-100 supernatants were precipitated with acetone for 1 h at -20°C and recovered by centrifugation at $10,000 \times g$ for 30 min.

Protein samples for hydrolytic detection were prepared as follows: *Lactobacillus bulgaricus* cultures were centrifuged at $5500 \times g$ for 15 min at 4°C and resuspended in 5 M guanidine-HCl, centrifuged again and resuspended in Tris-HCl 0.1 M pH 8. Soluble proteins from the guanidine-HCl supernatant were precipitated with acetone for 1 h at -20°C and recovered by centrifugation at $10,000 \times g$ for 30 min, washed with cold (-20°C) acetone, air-dried and resuspended in Tris-HCl 0.1 M buffer (pH 8). Proteins from these extracts were separated under reducing conditions in 10% (wt/vol) polyacrylamide denaturing gels (Laemmli, 1974) and stained with Coomassie Blue R-250. Prestained low range standard proteins (161-0305) were purchased from Bio-Rad (Mississauga, Canada). Detection of peptidoglycan hydrolase activities was performed in identical gels containing 0.2% (wt/vol) lyophilized *Micrococcus lysodeikticus* cells (Sigma, St. Louis, MO) as substrate (Potvin et al., 1988). Following electrophoretic separation, the slab gel was incubated overnight at 37°C with gentle shaking in 50 mM Tris-HCl buffer (pH 8) containing 1% (vol/vol) Triton X-100 for protein renaturation (Potvin et al., 1988). Lytic zones in gels appeared as clear bands within the opaque greyish substrate when examined with light from the back. Gels were photographed (Kodak black-and-white Polaroid Type 667 film) against a dark background, making lytic zones appear as dark bands on photos (Potvin et al., 1988).

DIC and Fluorescence Microscopy (FLM)

Exponential phase *L. bulgaricus* cells were washed three times in distilled water and resuspended. One aliquot of this suspension was kept as a control, while another was autolysed for 1 h at 40°C in 0.2 M NaCl and then held at room temperature for 1 h with Triton X-100 (1% vol/vol). Both samples were rinsed with 0.5% (vol/vol) blotto blocking buffer for 1 h at room temperature in order to decrease the number of nonspecific labeling bond sites (Prioult et al., 2000). Untreated and autolysed cells were incubated for 2 h at room temperature in 0.1 M Pipes (1,4-piperazinediethanesulfonic

acid) buffer (pH 6.8). They were then washed and centrifuged three times at $5500 \times g$ for 15 min in the same buffer and incubated overnight at 4°C with anti-muramidase-2 (1/10 dilution) in TBS buffer (pH 7.2). They were washed in TBS buffer and then held for 2 h at 37°C in darkness in the same buffer with the second antibody (1/10 dilution), anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM) FITC conjugated (Sigma Chemical, St. Louis, MO). After washing again in TBS buffer, cells were mounted in antifade (DABCO: 1,4-diazabicyclo (2.2.2) octane, 25 mg/ml in 80% glycerol, pH 8.6) on glass slides (Johnson et al., 1982). Observations were made at 1250 X on a Polyvar fluorescence microscope (Reichert, Munich, Germany) equipped for Nomarski DIC (Padawer, 1968) and for fluorescence with an HBO 200W/4 mercury lamp configured for blue irradiation with a BP 450-490 nm excitation filter, a DS 510 separator mirror, and an LP 515 barrier-filter. Color photomicrographs were taken on Kodak Ektachrome 160 ASA films.

Immunogold Localization and Transmission Electron Microscopy (TEM)

Lactobacillus bulgaricus intact and autolysed cells as previously prepared for FLM were centrifuged and resuspended in 2% (wt/vol) molten agar. Following solidification, 2-mm-thick agar slices were fixed in 0.5% (vol/vol) glutaraldehyde, 4% (vol/vol) freshly prepared paraformaldehyde (in 0.1 M sodium cacodylate, 1 mM CaCl_2 , adjusted to pH 7.4 with HCl) and held for 2 h at room temperature followed by 18 h at 4°C (Newman and Hobot, 1989). After rinsing in 0.1 M sodium cacodylate (pH 7.4), the slices were treated 20 min in 0.1% (wt/vol) NaBH_4 to neutralize free aldehyde groups, which may otherwise interfere with antibody probing (Willingham, 1983), then rinsed with 0.1 M (wt/vol) sodium cacodylate and dehydrated in a graded series of ethanol solutions. Half of the samples were embedded in Epon 812 resin (J. B. EM Services) and heat-cured at 60°C for 48 h, while the other half were embedded in London Resin White (LRW) from J. B. EM Services (Montréal, Québec, Canada) and polymerized at 4°C under UV light for 18 h.

Ultra-thin sections (90 nm) of both resin-embedded cells were obtained with an ultramicrotome (Reichert-Jung, Vienna, Austria) and collected on Formvar-coated nickel grids. Sections on grids were floated on a drop of 0.1% (wt/vol) BSA in 0.1 M TBS for 5 min, treated with blotto for 15 min, and held for 15 h at 4°C followed by 1 h at room temperature with the primary anti-muramidase-2 (undiluted) monoclonal antibody. After two gentle washes in TBS buffer, the grids were exposed to the second antibody (1/10 dilution) E. M. GAF (IgG

Figure 1. Growth curves of *Lactobacillus bulgaricus*. Bacterial growth was performed in MRS medium at 37°C, and monitored by OD measurements at 650 nm (○) and by cell-biomass (▲) as described in materials and methods.

+ IgM) and finally to 10 nm gold-conjugated GAM immunoglobulin from Cedarlane Laboratories (Hornby, Ontario, Canada) in TBS-BSA buffer. After rinsing with TBS buffer and distilled water, sections were stained with 2% uranyl acetate and lead citrate according to Reynolds (1963). Observations were made with a JEOL 1200 EX electron microscope (Jeol, Tokyo, Japan) operated at 80 kV.

RESULTS AND DISCUSSION

Evaluation of Muramidase Antibodies

Dry biomass and OD₆₅₀ measurements of *L. bulgaricus* cultures are shown in Figure 1. *L. bulgaricus* reached the exponential and stationary growth phases after 4 h and 12 h, respectively. Similar results were obtained using the Bio-Rad protein quantification assay (data not shown), indicating that the different methods used are equally reliable for *L. bulgaricus* biomass estimation.

Slot blot assays were used to evaluate the putative interaction between proteins from *L. bulgaricus* and two monoclonal mouse antibodies raised against mura-

Figure 2. Slot blot detection of proteins from *L. bulgaricus* extracts cross-reacting with *E. hirae* muramidase-2 antibody. Washed cell pellets (lane a) and culture broth supernatants (lane c) from cells grown in MRS medium at 37°C for different times (h). Pellets (lane b) and supernatants (lane d) from cells autolysed for 3 h at 40°C in 0.2 M NaCl.

midase-1 and muramidase-2 from *Enterococcus hirae*. Muramidase-1 is a cell-bound, latent (protease activatable) glycoenzyme (Kariyama and Shockman, 1992), whereas muramidase-2 is produced and secreted as an active enzyme in the culture medium and possesses long repeating amino acid sequences which may be involved in substrate binding (Kariyama and Shockman, 1992). The signal obtained with muramidase-1 antibody was too weak to be reliable and may be due to nonspecific binding (data not shown). Thus, only muramidase-2 antibody was used for further experiments.

Protein from *L. bulgaricus* cells was found to cross-react with muramidase-2 antibody on slot blot (Figure 2), the strongest signal being obtained after 12 h of growth (Figure 2a). This signal was present only in the cell pellet fraction, suggesting that the protein is cell-wall-bound (Figure 2, lanes a vs. c). For autolysed cells, the strongest signal was obtained after only 4 h of growth (Figure 2, lane b), indicating a relationship between antibody binding and *L. bulgaricus* cell wall state. The signal from autolysed cells was associated mainly with cell pellet fractions (Figure 2, lanes b vs. d), but in contrast with untreated cells, small amounts of cross-reacting proteins were detected in supernatants. Autolysis may, therefore, expose the target protein for antibody binding although another possibility is binding to soluble protein fragments produced by a cellular protease (Kariyama and Shockman, 1992; Bust et al., 1998). Protein degradation may also be involved in the decrease in signal with time after autolysis induction.

Cell Wall Hydrolase Molecular Mass Determination

The protein extracts from *L. bulgaricus* were subjected to SDS-PAGE containing *Micrococcus lysodeikti-*

Figure 3. Analysis of *L. bulgaricus* protein extracts under reducing conditions on denaturing PAGE for detection of bacteriolytic activities. A) Coomassie Blue stained supernatant (1) proteins (5 μ g) and pellet (2) from guanidine-HCl treated cells. B) Corresponding peptidoglycan hydrolase activities on *M. lysodeikticus* cells as substrate. Arrowheads indicate the position of lytic bands. The relative mobility of molecular mass markers in kilodaltons (kDa) is shown on the left.

cus cells for detection of bacteriolytic hydrolases. After renaturation in a Triton X-100 buffer, hydrolases were visualized as dark bands against a dark background (Potvin et al., 1988). Two proteins, with molecular masses of about 35 and 80 kDa could be detected and were recovered in both soluble and insoluble fractions (Figure 3B). This electrophoretic pattern is different from those obtained by Lortal et al. (1997) with the same species. The difference could be due to the extraction procedure or to a natural variation in the number of lytic bands for this species (Lortal et al., 1997). The protein content of our samples, evaluated by Coomassie blue staining (Figure 3A), indicates that peptidoglycan hydrolases are not abundant proteins in *L. bulgaricus*.

A Western blot was done to determine if the cell wall hydrolases detected by SDS-PAGE match the cross-reacting protein found in the slot blot experiment. In addition, different treatments of *L. bulgaricus* cells were done to evaluate their effect on hydrolase detection. The results of extracts from sonicated, autolysed or mechanically disrupted cells and cell walls are presented in Figure 4. Following gel staining, only two protein bands (probably abundant structural proteins) were observed in control cells whereas several polypeptides were released in all other cases (Figure 4A). This result suggests that structural integrity is retained in this control. Multiple proteins were also observed with *E. hirae* cells treated in the same way, suggesting that these cells are easier to break than cells of *L. bulgaricus*.

Figure 4. Analysis of *L. bulgaricus* protein extracts under reducing conditions on denaturing PAGE for immunodetection. A) Coomassie blue stained protein extracts from cultures at exponential and stationary growth phases (4 and 12 h respectively) were obtained as follow: lane a, control cells; lane b, sonicated cells; lane c, cells autolysed in NaCl (pellet); lane d, mechanically disrupted cells (suspension); lane e, lyophilized cell walls; lane f and g, supernatant and pellet respectively, of Triton X-100 treated cell walls. The amount of protein in samples was about 10 μ g except for sonicated cells (50 μ g). B) Same samples in Western blot probed with monoclonal antibody against *E. hirae* muramidase-2. *Enterococcus hirae* cell extract (*Eh*) used as positive control was obtained from a culture grown according to Kariyama and Shockman (1992). Numbers on the left refer to molecular mass markers (M).

Western blotting (Figure 4B) carried out using the same samples and probed with anti-muramidase-2 revealed only one cross-reacting protein at a mass of about 80 kDa. This band was observed in Triton X-100-treated cell wall samples from exponentially growing culture (Figure 4, lanes f and g). Triton X-100 appeared to be required for protein detection by Western blotting. The influence of the Triton X-100 may be explained by detergent action on various membrane components and is consistent with the slot blot results which revealed an association between *L. bulgaricus* peptidoglycan hydrolase and its cell wall. Interactions between the cell wall and hydrolase could interfere with the ability of the antibody to bind its target protein. Triton X-100 may increase the availability of the protein epitopes by reducing protein interactions with the cell walls. Moreover, the proteins found in the corresponding supernatant indicate that this detergent, particularly ef-

Figure 5. Light microscopic observations of *L. bulgaricus* cells. Micrographs of FITC-GAM labeled cells were taken under differential interference contrast (A, C and E) or blue irradiation (B, D and F) microscopic conditions. Sections A, B, diluted cells without anti-muramidase-2; C, D, concentrated cells treated with anti-muramidase-2 and FITC conjugated antibodies; E, F, concentrated and NaCl autolysed cells with both antibodies. Arrows on Figures C and D point out an isolated cell. Arrowheads indicate the difference in fluorescence signal between whole and lysed cells. Scale bar: 10 μ m.

Figure 6. Immunogold labeling of *L. bulgaricus* cells. Micrographs of GAF₁₀ labeled cells, embedded in LRW (A, B and C) or in Epon (D and E). Cells were observed before (A) and after NaCl-induced autolysis (B, C, D and E) with (A, B and D) or without (C and E) anti-muramidase-2 antibody. Scale bar: 200 nm.

fective in protein extraction, may liberate peptidoglycan hydrolase completely from cell wall interactions.

A weak antibody-binding signal was also detected after cell sonication, which may be expected since this sample contained five times as much protein as the others. Cells from *E. hirae* were used as positive control and revealed the presence of two immunodetectable proteins. The immunological similarity between these two proteins indicates a common epitope for muramidase-2 antibodies. Two different proteins with the same epitope may be involved, or a single protein may be cleaved by a cellular proteolytic enzyme (Kariyama and Shockman, 1992; Buist et al., 1998).

DIC and Fluorescence Microscopy

The cross-reactivity between *E. hirae* anti-muramidase-2 and *L. bulgaricus* peptidoglycan hydrolase was also used to estimate the state of the *L. bulgaricus* cell wall in different culture preparations. The thick peptidoglycan layer of the cell wall presents a barrier to the entry of labeled probes (Bidnenko et al., 1998). Autolysis may permeabilize the *L. bulgaricus* cell wall and make the hydrolase available for anti-muramidase-2 binding and subsequently FITC-GAM antibody binding for fluorescence. The endogenous fluorescence of *L. bulgaricus* cells under blue irradiation in absence of antibodies is shown in Figure 5b. When a sample with higher cell density was probed with anti-muramidase-2 and FITC conjugated antibodies, a diffuse fluorescence (probably background) signal was obtained (Figure 5d). However, the strong signal detected on a few spots may correspond to lysed cells (Figure 5d), since a strong confluent signal (Figure 5f) was obtained following autolysis treatment. These results suggest that the 80 kDa peptidoglycan hydrolase of *L. bulgaricus* is intracellular.

Cell structural integrity was confirmed by DIC microscopy (Bidnenko et al., 1998). The rod morphology of untreated *L. bulgaricus* cells (Figure 5a) is intact and essentially unaffected by antibody treatments (Figure 5c). The loss of this characteristic morphology is apparent in autolysed samples (Figure 5e), as observed by FLM.

Immunogold Localization

A procedure analogous to FLM, electron microscopic imaging with gold-conjugated immuno-markers was performed using anti-muramidase-2 in order to study the distribution of peptidoglycan hydrolase in *L. bulgaricus* cells. The same monoclonal muramidase-2 antibody was used to label electron microscopic ultra-thin sections of whole and lysed cells, but a gold-conjugated marker was used as secondary antibody.

Proper preparation of embedded material for TEM immunological localization is a critical step (Slot and Geuze, 1984). To maximize the preservation of antigenic sites, a combination of glutaraldehyde and paraformaldehyde was used as a fixing agent, combining the high quality of the first with the speed of the second. Samples were also treated with Na-borohydrate to block free aldehyde groups formed during the fixing process, since they may decrease antigenic site accessibility (Reynolds, 1963). Different resins such as epoxy (Epon 812), which is hydrophobic with a high molecular weight, and an acrylic resin (LRW), which is hydrophilic with a low molecular weight, were used to embed samples (Newman et al., 1982).

Labeling is another difficult process due to the ability of the monoclonal antibody to recognize only one epitope. Immunological recognition occurs only at exposed antigenic sites on the surface of the resin section (Slot and Geuze, 1984). Laan et al. (1996) used a pool of monoclonal antibodies to achieve increased labeling of ultra-thin sections, even for low levels of target. In our study, a single monoclonal antibody was used. As shown in Figure 6, good signals were detected with both resins compared to their negative controls without anti-muramidase-2 (Figures 6c and 6e). For whole cells embedded in LRW, accumulation of gold particles was limited mainly to near the plasma membrane, while a few are also found in the cytoplasm (arrow heads, Figure 6a). A stronger signal is present on fibrous material trapped between two adjacent autolysed cells (arrowheads, Figure 6b). Autolysed cells embedded in Epon 812 exhibit gold particles localized near the cytoplasmic membrane (arrowheads, Figure 6d). This pattern of enzyme distribution suggests that the peptidoglycan hydrolase from *L. bulgaricus* is bound between the membrane and the cell wall.

CONCLUSIONS

We have detected a peptidoglycan hydrolase in *Lactobacillus delbrueckii* subsp. *bulgaricus* able to degrade *Micrococcus lysodeikticus* cells. We have shown that this hydrolase with an estimated molecular mass of 80 kDa cross-reacts with monoclonal antibodies raised against muramidase-2 from *Enterococcus hirae*. Microscopic observations suggest that this bacteriolytic enzyme is essentially localized in the cell wall near the membrane.

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