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Esterified Whey Proteins Can Protect *Lactococcus lactis* against Bacteriophage Infection. Comparison with the Effect of Native Basic Proteins and L-Polylysines

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Inhibitory action of basic esterified milk whey proteins [methylated (Met) or ethylated (Et) β-lactoglobulin (BLG) and α-lactalbumin (ALA)], basic native proteins (chicken egg white lysozyme and calf thymus histone), and basic protein-like substances (L-polylysines) against the activity and replication of lactococcal bacteriophages (bIL66, bIL67, and bIL170) was tested. Chemical interactions of these proteins with phage DNA were determined as well as their protective effect on the growth of a laboratory plasmid-cured *Lactococcus lactis* subjected to an infection by the bacteriophages. All the proteins studied showed inhibitory activity against the three bacteriophages as tested by marked reduction of their lytic activities and decreasing the replication of studied phages. Histone and Met-BLG were more active toward bIL66 and bIL67, respectively, while both proteins were highly and equally active toward bIL170. Lysozyme showed lower antiviral activity. Antiviral activity of Et-BLG was a little bit lower than that observed in the case of the Met derivative. Esterified ALA also showed considerable but slightly lower antiviral activity as compared to other proteins. L-Polylysines also showed an antiviral effect against the three bacteriophages studied, their influence being highly dependent on their molecular size. The best effective size of L-polylysines was in the range 15−70 kDa. Replication of bIL67 was inhibited by the presence of esterified ALA or BLG and native basic proteins. Complete inhibition of replication of bIL67 occurred when using polylysines with molecular masses in the ranges 4−15, 15−30, and 30−70 kDa, while protein-like substrates with lower molecular masses had only a slight effect. The presence of histone and Met-BLG at a concentration of 0.13 mg/mL in the incubation medium protected *L. lactis* against lysis when it was subjected to an infection by bIL67 (10^5 pfu/mL). The same action was achieved by L-polylysine (15−30 kDa) used at a concentration of 0.03 mg/mL in the incubation medium.

KEYWORDS: Whey proteins; esterification; phage; polylysine

INTRODUCTION

Lactic acid bacteria are the main microorganisms used for the production of traditional or new fermented food products. The Gram-positive bacterium *Lactococcus lactis*, which is the best genetically characterized species of this group, secretes lactic acid as the main fermentation product and is widely used for the production of cheeses. It has a unique set of stress response mechanisms (*1*), but it can be attacked by many phages. Industrial milk fermentation is prone to bacteriophage attacks (*2*, *3*). Phage infection may cause rapid lysis of the starter cultures, diminishing the rate of acid production (*4*). Lytic phages present in pasteurized milk used for the manufacture of dairy fermented products, e.g., buttermilk and cheeses, may cause delayed fermentation or inhibit acid production (*3*, *5*−*7*) and, hence, pose deleterious technical and economical problems. They resist pasteurization, have a short latent period and a large burst size, and can spread quickly within a cheese plant (*8*). Numerous phages can infect *L. lactis* that are classified into 12 genetically distinct groups (*9*). The small isometric-headed species 936 and P335 and the prolate-headed species c2 are frequently isolated in dairy plants (*10*, *11*) and represent more than 80% of all lactococcal phages isolated from the dairy environment (*12*). Moreover, the species c2 was reported as the most common lactococcal phage found in Canadian cheddar cheese (*10*). The lactococcal phages bIL66 and bIL170 (*13*) belong to the 936 species, while bIL67 (*14*) belongs to the c2 species.

Some *L. lactis* strains possess robust phage resistance mechanisms, which are plasmid encoded and can exert their activities through interference during adsorption of the phage, by restriction and modification of phage DNA, by prevention of phage injection, or by abortive phage infection (Abi) (*3*, *5*−*7*).

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The last mechanism occurs thanks to the production of abortive infection proteins (Abi), which shut down the phage lytic cycle. Most Abi systems are plasmid encoded. Some of these Abi systems (e.g., AbiA) exert their action by interfering with the viral DNA replication of isometric phages (*15*−*17*) by promoting rapid degradation of the phage transcript (AbiB) (*18*), while others (e.g., AbiC) inhibit the synthesis of the structural phage proteins (*15*, *19*). Recently, a novel Abi mechanism encoded by plasmid pED1 from *L. lactis* has been characterized (*20*). Domingues et al. (*21*) described a new lactococcal abortive phage infection system (AbiP) effective against some lactococcal phages of one prevalent group, 936, but not against phages from the other two groups (c6A and P335). The same authors (*22*) added that phage bIL66M1 is sensitive to the lactococcal abortive infection mechanism AbiP. Natural Abi systems have already been introduced into industrial phage-sensitive *L. lactis* strains (*23*, *24*), but the extensive use of transformed phage-resistant strains led to the emergence of phage mutants able to escape the antiphage systems (*25*, *26*).

The aim of the present work was to study the influence of basic proteins on the activity and replication of lactococcal phages (bIL phages) and, hence, their protective effect on *L. lactis* cultures (strain IL1403) as a possible substitute for the Abi proteins. Native basic proteins used in this study were chicken egg white lysozyme and calf thymus histone. Basic esterified milk whey proteins were Met- and Et-ALA and -BLG. L-Polylysines with different molecular masses (0.5−70 kDa) were used as basic protein-like polypeptides.

MATERIALS AND METHODS

BLG was purchased from Armor Proteins (Saint-Brice-en-Coglès, France) and purified according to Mailliart and Ribadeau Dumas (*27*). ALA (Armor Proteins) was further purified by ion-exchange chromatography using a DEAE-Sepharose fast-flow column (50 × 300 mm). The elution was performed with a 50 mM Tris, 10 mM $CaCl_2$ buffer at pH 8, with a gradient from 10 to 40 mM NaCl. Chicken egg white lysozyme (EC 3.2.1.17), calf thymus histone type II-AS, and polylysines (molecular masses of 0.5−2, 1−4, 4−15, 15−30, and 30−70 kDa) were purchased from Sigma Chemical Co. The medium MRS broth was purchased from Biokar Diagnostics (Beauvais, France), and the medium M17 agar was from Difco Laboratories (Detroit, MI). A culture of *L. lactis* strain IL1403 and stock solutions of bacteriophages bIL66 (∼21 kbp), bIL67 (22195 bp), and bIL170 (31754 bp) were kind gifts from Dr. Alexandra Gruss, Unité de Recherches Laitières et Génétique Appliquée, Institut National de la Recherche Agronomique (INRA), Jouy-en-Josas, France. All solvents and chemical reagents were of analytical grade.

Protein Esterification. Native BLG and ALA were turned into basic proteins by esterification according to the general procedure of Sitohy et al. (*28*) as follows. An amount of native protein was dispersed in concentrated (>99.5%) alcohols at a concentration of 5%, and an amount of hydrochloric acid equivalent to a 50 molar ratio (MR, mol of acid/mol of carboxyl group) was added dropwise at the start of the reaction time. All the reaction mixtures were kept at 4 °C under continuous stirring. At the end of the reaction (6 h), the samples were centrifuged at 10000*g* for 10 min. The resulting supernatant was discarded, and the residue was dispersed in a volume of alcohol (99.7% ethanol) equal to that of the discarded supernatant, and well mixed before recentrifugation in the same conditions. This washing step was repeated three times. The final precipitate was dissolved in an appropriate amount of distilled water and kept at −80 °C until freeze-drying. The lyophilized samples were kept at −20 °C until analysis. To quantify the extent of esterification of proteins, the color reaction using hydroxylamine hydrochloride developed by Halpin and Richardson (*29*) was used with modification according to Bertrand-Harb et al. (*30*).

Influence of Basic Proteins on the Viral Activity. The following procedure was generally adopted with some modifications that will be indicated in the Results and Discussion. Aliquots (40 μL) of protein solutions (original concentration 360 μM) were mixed with equal volumes (10 μL) of virus stock diluted in MRS to 10^4−10^5 plaque-forming units (pfu)/mL, and an activated culture of *L. lactis* strain IL1403 (OD_{600} = 0.2−0.3) containing 10 mM $CaCl_2$ was added directly before infection. The mixtures were incubated at 37 °C for 30 min to obtain the maximal action of protein before dilution with addition of 100 μL of the same previous bacterial culture (OD_{600} = 0.3−0.4; the OD value of this culture was higher than the OD of the original culture since IL403 was growing during the incubation step) and 4 mL of MRS broth medium containing 0.7% agar and 10 mM $CaCl_2$ (kept at 50 °C) and then pouring the resulting mixture on top of the M17 agar medium containing 0.5% glucose in Petri dishes warmed to 30 °C. A positive control was similarly formulated but in the absence of the tested proteins. After one night of incubation, the Petri dishes were observed and scanned for the viral inhibition zones. The relative reduction in the number of these inhibition zones relative to the positive control was taken as a measure of the antiviral effect of the tested proteins. All plaque assays have been done at least in duplicate and show reproducible results.

Influence of Basic Proteins on Virus Replication. Aliquots (40 μL) of protein solutions (360 μM) were mixed with 10 μL of virus (10^4−10^5 pfu/mL) and 10 μL of an activated culture of *L. lactis* strain IL1403 (OD_{600} = 0.2−0.3), and incubated for 30 min at 30 °C (first infection medium). Then, a further 100 μL of the same previous bacterial culture (OD_{600} = 0.3−0.4) was added to the incubated mixtures and kept at 30 °C for another 30 min (second infection medium). Additional fresh MRS medium (2 and 8 mL) was added to the incubated mixture after 1 or 2 h (from the end of the second incubation time), respectively. The last mixture was kept at 30 °C for up to 18 h. A positive control was performed similarly but in the absence of tested protein, and a negative control was obtained in the absence of both tested proteins and virus. To isolate the synthesized phage DNA, the general method outlined by Sambrook et al. (*31*) was followed with slight modifications as follows. The final mixture was centrifuged at 9000 rpm to remove the bacterial cells. A 2 mL sample of 20% PEG 8000 containing 2.5 M NaCl was added to the supernatant and kept at 4 °C overnight. Then, the mixture was centrifuged for 10 min at 9000 rpm, and the obtained precipitate was dissolved in 0.4 mL of 1× TE buffer (10 mM Tris−HCl pH 8, 1 mM EDTA) and combined with 0.2 mL of phenol saturated with 10 mM Tris−HCl pH 8, 1 mM EDTA. After centrifugation at 9000 rpm for 2 min, the aqueous phase (0.39 mL) was recovered, and viral DNA was precipitated by the addition of 1.2 mL of a mixture of 99.7% ethanol and 3 M sodium acetate (25:1, v/v), previously kept at −20 °C. After centrifugation at 12000 rpm for 10 min, the resulting precipitate was washed with 70% ethanol kept at −20 °C. After recentrifugation in the same conditions, the final precipitate was dried under vacuum before being dissolved in 100 μL of 1× TE buffer, and 10 μL of that solution was used for electrophoresis on 0.6% agarose containing 1 μg/mL ethidium bromide in 1× TBE buffer (89 mM Tris−borate, 2 mM EDTA, pH 8). Synthesized viral DNA was quantified by measuring the intensity of its corresponding band with Quantity One quantitation software (Bio-Rad, Marnes-la Coquette, France), and the extent of inhibition of replication was estimated by the relative difference of the band intensity compared to that of the positive control.

Interactions between Viral DNA and Esterified Proteins. Produced viral DNA was extracted as described above except that no protein was added. The concentration of the obtained DNA was calculated by measuring the optical density at 260 nm, and then the sample was diluted to a concentration of 40 ng/μL. An 8 μL sample of such a solution was added to 4 μL of tested protein solutions with different concentrations to give protein:DNA ratios (w/w) of 0:1, 0.38:1, 0.75:1, 1.5:1, 3:1, 6:1, and 12:1, and then the reaction mixtures were kept at room temperature for 20 min before analysis by electrophoresis as previously described.

Influence of Basic Proteins on the Growth of *L. lactis* Infected by Phages. Aliquots (40 μL) of protein solutions with different concentrations were mixed with 10 μL of virus (10^4−10^5 pfu/mL) and 10 μL of an activated culture of *L. lactis* culture strain IL1403 (OD_{600} = 0.2−0.3) and then incubated for 30 min at 30 °C. A further 100 μL

Table 1. Antiviral Activity of 100% Met-BLG, Histone, and Lysozyme against bIL66 (10^4 pfu/mL), bIL67 (10^5 pfu/mL), and bIL170 (10^5 pfu/mL)

protein sample	antiviral activity[a] (%)		
	bIL66	bIL67	bIL170
Met-BLG	55 ± 3	97 ± 2	100 ± 1
histone	100 ± 0	85 ± 3	100 ± 0
lysozyme	72 ± 5	59 ± 4	0 ± 1

[a] (Number of lytic zones in the control − number of lytic zones in the sample)/number of lytic zones in the control.

of the same previous bacterial culture (OD_{600} = 0.3−0.4) was added to the incubated mixtures and kept at 30 °C for another 30 min. An additional 4 mL of fresh MRS medium was added, and incubation was continued for another 5 h. The turbidity of the resulting mixtures was measured at 600 nm and taken as an index for bacterial growth.

RESULTS AND DISCUSSION

Antiviral Activity. *Effect of Native Basic Proteins or Esterified Milk Whey Proteins*. Data in **Table 1** show the antiviral activity of native basic proteins (chicken egg white lysozyme and calf thymus histone, p*I* 9.5 and 10.5, respectively) and basified milk protein (100% Met-BLG, p$I \approx 10$) against the bacteriophage bIL66 as compared to a control performed in the absence of the tested proteins. The original protein concentration of 360 μM decreased to 240 μM and then to 3.5 μM in the first and final infection media, respectively. The protein concentration was further decreased by the addition of 4 mL of MRS. However, the conditions used during the infection period are the main factors determining the subsequent antiviral activity. Short time periods were adopted for infection since replication of bacteriophage c6A, which is very similar to that of c2 phage, begins 5−6 min after phage infection and increases gradually and constantly during the phage cycle (*32*). Histone showed the highest antiviral activity (100% inhibition). Lysozyme and 100% Met-BLG induced 72% and 55% inhibition, respectively. When the tested proteins were added directly to the final diluted medium at the same final concentration (3.5 μM), their actions were either very slight or null; the antiviral activity was in the range 0−5% (data not shown). Consequently, the protein concentration during initiation of bacterial culture must be high enough to induce inhibition of the viral activity.

Similar antiviral activities against the phage bIL67 are shown in **Table 1**. However, 100% Met-BLG was more active than histone, inhibiting about 97% of the phage activity as compared to 85% inhibition obtained in the case of histone. Lysozyme showed lower antiviral activity (59%) against this phage. Differences observed between antiviral activities of 100% Met-BLG and histone may demonstrate some specificity or adaptability of each protein to each bacteriophage.

By using phage bIL170, 100% Met-BLG and histone induced 100% inhibition of the bacteriophage activity (**Table 1**). Lysozyme did not show any antiviral effect against this phage, even increasing its activity (data not shown). Some bacteriophages such as T7, T4, and λ express lysozyme to help them in their transcriptional mechanisms (*33*−*35*). Lysozymes are ubiquitous enzymes with specific hydrolytic activities directed against cell-wall peptidoglycan (*36*). The lower antiviral activity in the case of lysozyme might also be due to its lytic effect on the bacterial cell walls, which helps the virus internalization rather than inhibiting its activity. Egg white lysozyme has been previously found to exert antimicrobial activity against *L. lactis* (*37*). However, this activity is lactococcal strain-dependent since

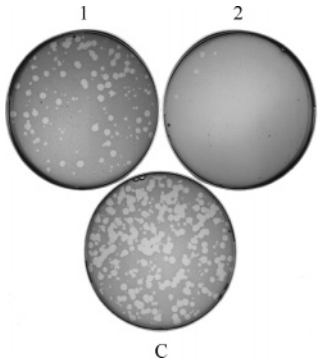

Figure 1. Influence of the concentration (1, 180 μM; 2, 240 μM) of 100% Met-BLG in the preincubation medium on its antiviral activity against phage bIL67 (10^5 pfu/mL), as compared with a control (C) performed in the absence of protein.

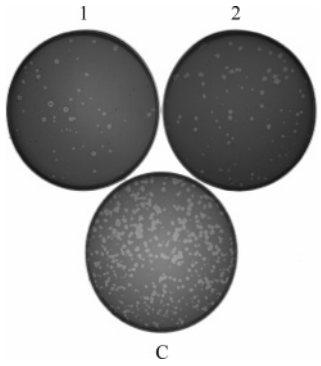

Figure 2. Antiviral activity of (1) 100% Met-BLG and (2) 73% Et-BLG dissolved at pH 6 against phage bIL67 (10^5 pfu/mL), as compared with a control (C) performed in the absence of protein. The protein concentration in the preincubation medium was 200 μM.

the exopolysaccharide (EPS)-producing strain was more tolerant of lysozyme than the non-EPS-producing strains (*38*). In our study, lysozyme added to the infection medium might play a role both in the attack of the cell wall and in the liberation of viral DNA. The final result will depend on the relative availability of both the viral DNA liberated during the replication cycle and the bacterial cell wall. By using phages bIL66 and bIL67, some antiviral activities of lysozyme were observed, demonstrating the relatively high availability of these phages to lysozyme attack. In the case of phage bIL170, the results suggest that lysozyme was mainly oriented toward the bacterial cell walls, probably because the viral DNA was not available to viral attack.

The influence of protein concentration on the magnitude of the antiviral activity was determined by using 240 and 180 μM 100% Met-BLG in the first infection medium, showing 97% and 63% inhibition of viral activity, respectively (**Figure 1**). Lower protein concentrations further decreased the antiviral activity. Hence, protein concentration is the main factor, which should be appropriately determined to obtain a nearly complete inhibition with the minimal quantities of added modified proteins.

To control the influence of the grafted group on the antiviral activity of the modified protein, experiments were performed with 200 μM 100% Met-BLG and 73% Et-BLG dissolved at pH 6 in the original solution, showing 91% and 82% viral inhibition, respectively (**Figure 2**). This difference might be due to the higher net positive charge of the methylated derivative.

Although esterified ALA did not show antiviral activity at pH 7, due to its very low solubility, it could show an important

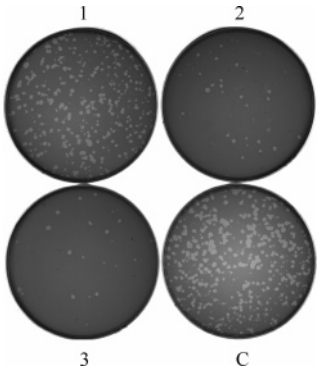

Figure 3. Antiviral activity of (1) native, (2) 60% Met-ALA and (3) 40% Et-ALA dissolved at pH 5 against phage bIL67 (10^5 pfu/mL), as compared with a control (C) performed in the absence of protein. The protein concentration in the preincubation medium was 200 μM.

activity against phage bIL67 at pH 5. In these conditions, 60% Met- and 40% Et-ALA led to 94% viral inhibition when used at a concentration of 240 μM in the preinfection medium. Native ALA was completely devoid of such an activity (**Figure 3**).

Consequently, increasing the basicity of milk whey proteins by esterification could endow them with antiviral activities against lactococcal bacteriophages. This property could be exploited in designing specific modified proteins used as protective agents against the viral attack occurring probably during bacterial cultures, during stocking, or during production of fermented dairy foods. The inhibitory action of basic proteins on the activity of lactococcal bacteriophages may occur by a direct inhibition of the phage replication through its binding to the phage DNA, or by inhibition of the phage transcription by binding to some promoters on the viral DNA similarly to what was observed during the action of the phage-resistant protein AbiB, which leads to the degradation of phage mRNAs 10−15 min after infection (*39*). This may lead to a reduction in the synthesis of phage capsid proteins (*15*) and consequently affect the phage assembly (*40*). Although some phage DNA can escape to the formation of complexes with basic proteins during its replication, it will be submitted to the same action during its transcription, decreasing its antibacterial activity. In such a case, the activity of basic protein can simulate that of AbiK, which did not prevent the replication of phage p2 inside the $AbiK^+$ cells but prevented phage DNA maturation (*41*).

Effects of L*-Polylysines.* L-Polylysines, synthetic polymers of the basic amino acid L-lysine, are highly basic as compared to any native basic protein, which contains a limited extent of basic amino acids. The molecular masses of L-polylysines are not well controlled since they are prepared as mixtures of polymers. Consequently, concentrations of polylysine solutions used in this study are determined as weight per volume (w/v, mg/mL). Keeping a common w/v concentration for all the polylysine types independently of their molecular sizes will mean an equal magnitude of positive charges in all solutions. Hence, the only variable between the solutions of L-polylysine with the same w/v concentration (7.2 mg/mL) is the molecular size. These conditions were deliberately adopted to control the impact of the molecular size of basic protein-like material on its antiviral activity. Data in **Table 2** show that antiviral activity against phage bIL66 increased gradually with increased molecular mass of L-polylysine although the treatment was performed in the presence of the same amount of positive charges in each experiment. The antiviral inhibitory activities of L-polylysines were 0%, 43%, 92%, 96%, and 99% when the molecular size ranges were 0.5−2, 1−4, 4−15, 15−30, and 30−70 kDa, respectively. A molecular mass range of 15−30 kDa is enough to induce a maximal antiviral activity. Similar data were obtained when activity was measured against the phage bIL67 (10%, 41%, 96%, 98%, and 99% inhibition when using L-polylysines in 0.5−2, 1−4, 4−15, 15−30, and 30−70 kDa ranges, respectively (**Table 2**). Similarly, polylysines with high molecular masses (15−70 kDa) achieved 100% inhibition of phage bIL170.

Table 2. Antiviral Activity of L-Polylysine of Different Molecular Masses against bIL66 (10^4 pfu/mL) and bIL67 (10^5 pfu/mL)

molecular mass (kDa)	antiviral activity[a] (%)		molecular mass (kDa)	antiviral activity[a] (%)	
	bIL66	bIL67		bIL66	bIL67
0.5–2	0 ± 0	10 ± 2	15–30	96 ± 3	97 ± 2
1–4	43 ± 2	41 ± 3	30–70	99 ± 1	98 ± 2
4–15	92 ± 5	96 ± 3			

[a] (Number of lytic zones in the control − number of lytic zones in the sample)/number of lytic zones in the control.

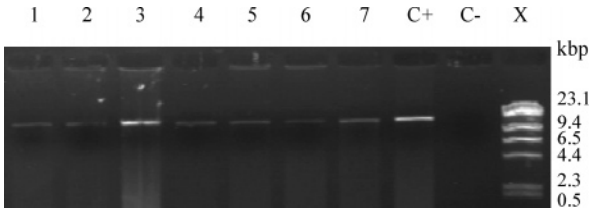

Figure 4. Agarose gel electrophoresis of replicated DNA of phage bIL67. Replication was performed in the presence of (1) Met-BLG, (2) Et-BLG, (3) native ALA, (4) Met-ALA, (5) Et-ALA, (6) histone, and (7) lysozyme applied at a concentration of 240 μM in the preincubation medium. The preincubation medium was composed of 40 μL of protein (360 μM), 10 μL of virus (10^5 pfu/mL), and 10 μL of an activated culture of *L. lactis* strain IL1403 (OD_{600} = 0.2–0.3) and kept for 30 min before being added to the agar medium. C^+ is a positive control performed in the presence of phage and in the absence of protein. C^- is a negative control performed in the absence of phage and protein. X is a standard of DNA sizes (kb).

The probable mechanism of action of L-polylysine may occur through complexing the viral DNA during its release from its protein envelope. Small molecular-sized polylysines with less than 20 residues (2.9 kDa) were previously reported unable to bind to DNA (*42*). Extensive peptic hydrolysis of Met-BLG into low molecular mass fragments (∼1 kDa) terminates the DNA binding capacity obtained with the whole protein after its esterification. Limited tryptic hydrolysis into medium molecular mass fragments (5−15 kDa) maintains this property (unpublished data). As the size of basic protein or basic protein-like material is important for the antiviral effect, the mechanism of inhibition should pass through an interaction between the basic agent and viral DNA. Hence, a molecular mass of 15−30 kDa will be sufficient for a basic protein to show nearly maximal antiviral activity.

Inhibitory Action on Viral Replication. *Basic Esterified Whey Proteins Compared to Native Basic Proteins.* Viral (bIL67) replication was followed by agarose gel electrophoresis in the presence of Met- and Et-BLG or -ALA and native basic proteins (chicken egg white lysozyme and calf thymus histone) (**Figure 4**). A negative control containing no virus and no protein did not show any DNA band, while a positive control containing added virus in the absence of tested proteins showed a band migrating near the level of migration of a marker of 23.1 kb, which is in accordance with the size of the bIL67 genome (22195 bp). In the presence of native ALA, a band appeared with an intensity similar to that of the positive control,

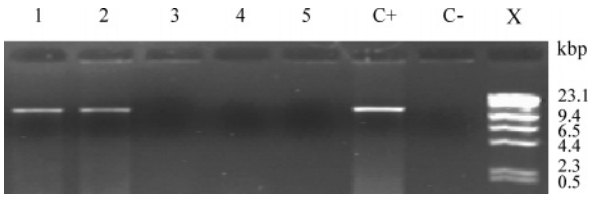

Figure 5. Agarose gel electrophoresis of replicated DNA of phage bIL67. Replication was performed in the presence of L-polylysine with different molecular mass ranges: (1) 0.5–2, (2) 1–4, (3) 4–15, (4) 15–30, and (5) 30–70 kDa. C^+ is a positive control performed in the presence of phage and in the absence of protein. C^- is a negative control performed in the absence of phage and protein. X is a standard of DNA sizes (kb).

demonstrating no inhibitory effect of native protein on viral replication. In contrast, when the replication of the virus was performed in the presence of modified proteins, a band representing viral DNA appeared with a very low intensity as compared to the band obtained in the case of the positive control or native protein. According to the intensity of the band obtained by electrophoresis, the extent of inhibition of replication could be estimated to be 94% and 86% in the presence of Met- and Et-BLG, respectively, and to be 87% and 67% in the presence of Met- and Et-ALA, respectively. The difference observed between the two whey proteins might be due to a higher extent of esterification in the case of BLG (100% methylated and 73% ethylated) as compared to ALA (60% methylated and 40% ethylated). Viral replication was also inhibited in the presence of native basic proteins, chicken egg white lysozyme and calf thymus histones, showing 66% and 84% inhibition of replication, respectively. However, whatever the protein used during the replication, the band corresponding to the DNA of phage bIL67 did not completely disappear, showing that protein concentrations used during incubation were not sufficient to completely inhibit viral replication. To obtain a complete inhibition of viral replication, the protein concentration should be increased. An inhibitory effect of esterified proteins on in vitro DNA replication was previously reported and attributed to the acquired DNA binding properties of such modified proteins (*43*).

Antiviral Activities of L*-Polylysines*. Viral (bIL67) replication was also studied in the presence of L-polylysines with different molecular mass ranges (0.5−70 kDa) applied at the same w/v concentration in the infection and incubation media (**Figure 5**). The band representing the DNA of phage bIL67 was very slightly affected (6−7% inhibition) by the presence of polylysines with sizes in the range 0.5−4 kDa, while it completely disappeared when the sizes of the L-polylysines were in the ranges 4−15, 15−30, and 30−70 kDa. Consequently, L-polylysines of large sizes only were able to completely inhibit viral DNA replication. This result agrees with the previous observation that L-polylysines with high molecular masses were the only ones showing antiviral activity against phage bIL67. As compared with native basic proteins or basic esterified milk whey protein samples, polylysines show a more potent inhibitory effect on viral replication. This may be due to higher amounts of positive charges in the case of polylysines than in the case of native basic proteins or basified milk whey proteins when used at the same concentration and when tested protein-like samples were of large size (14−30 kDa).

Viral DNA−Protein Binding Properties. *Histones and Methylated β-Lactoglobulin*. The electrophoregrams in **Figure 6** show that both 100% Met-BLG and calf thymus histone were able to form complexes with the DNA of phage bIL67 by using

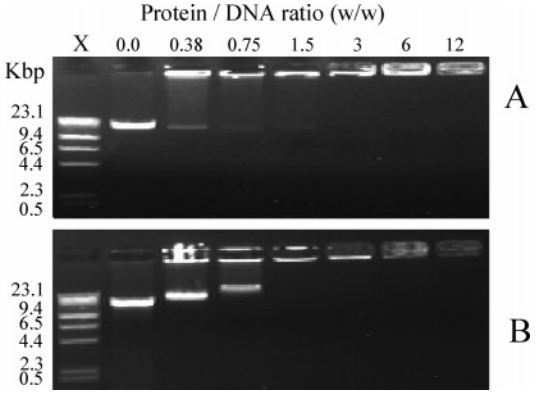

Figure 6. Agarose gel electrophoresis of the DNA of phage bIL67 after interaction with (**A**) 100% Met-BLG or (**B**) calf thymus histone when using different ratios of protein to DNA (w/w), as indicated at the top of the gel. X is a standard of DNA sizes (kb).

a protein:DNA ratio of 0.38 (w/w). Complete retardation of DNA migration in agarose gel occurred at a ratio of protein to DNA of 3 in the case of 100% Met-BLG and at a ratio of protein to DNA of 1.5 in the case of histone. Binding of Met-BLG at low protein concentrations to viral DNA showed a complete retardation of the complexed DNA in the gel wells, while the DNA part not involved in complexes with protein migrated at the same level as free DNA. In the case of histone applied at low concentrations, complexes formed with DNA showed a relatively retarded mobility. Gradual DNA retardation of migration on agarose gel can be observed when the protein:DNA concentration ratio is increased from 0.0 to 0.38 (w/w) and then to 0.75. This difference in behavior of the protein−DNA complexes made with BLG and histone might be due to different physicochemical properties of the formed complexes. The tertiary structure of BLG is affected by esterification (unpublished data), and thus, the esterified derivative may form rather random complexes, which do not enter easily into the agarose gel. Moreover, complexes made between DNA and Met-BLG might be of hydrophobic nature, due to the grafted methyl groups, which might lead to voluminous hydrophobic aggregates completely stopping the DNA−protein complex from entering the gel. Complexes formed between plasmid DNA and esterified milk proteins or native basic proteins were previously reported (*44*). Because of their specific conformation, histone molecules are known to be used for wrapping DNA in limited size complexes. Such complexes were still able to enter the gel as long as the viral DNA was still only partially neutralized by histone and, hence, still carrying a negative net charge. However, increasing the ratio of added histone leads to a complete neutralization of the complexed DNA and, hence, to a complete retention in the gel wells.

L*-Polylysines*. The potentiality of L-polylysines to complex the DNA of phage bIL67 was studied as a function of their molecular sizes (**Figure 7**). L-Polylysines of molecular mass ranges 0.5−2 and 1−4 kDa showed a slight viral DNA binding activity when using a ratio of polylysine to DNA (w/w) as high as 0.75. L-Polylysines less than 20 residues long (∼2.9 kDa) were previously reported unable to bind to DNA (*42*). Maximum DNA binding activity can be observed with high molecular mass L-polylysines (15−30 and 30−70 kDa) when using ratios of polylysine to DNA as low as 0.037 (w/w), which is 20 times lower than the ratio needed to obtain complexes with low molecular mass L-polylysines. The higher the molecular mass, the higher the stability of the formed polylysine−DNA complexes. This result confirms the previous conclusions that

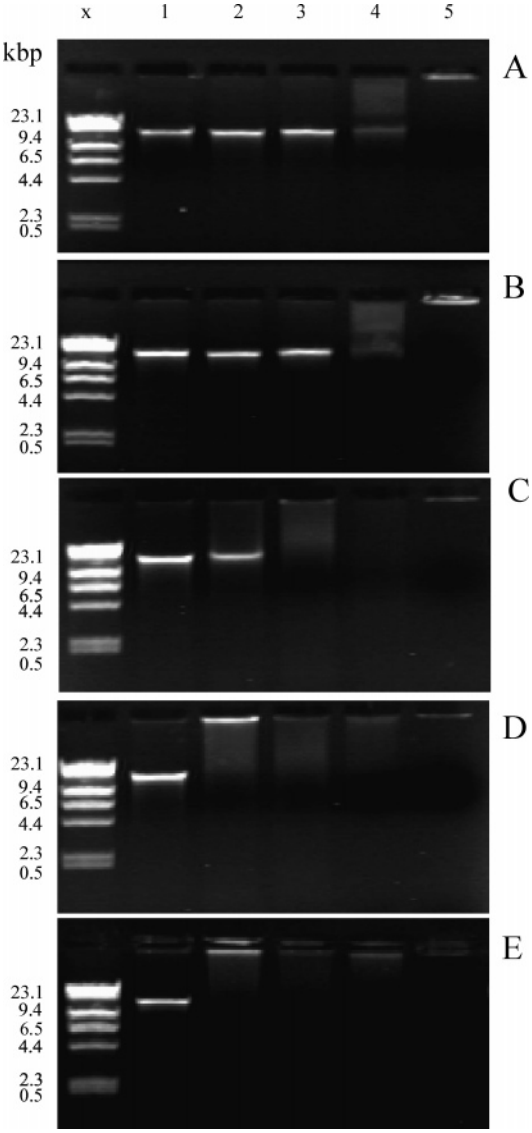

Figure 7. Agarose gel electrophoresis of the DNA of phage bIL67 after interaction with L-polylysine with different molecular mass ranges: (**A**) 0.5–2, (**B**) 1–4, (**C**) 4–15, (**D**) 15–30, and (**E**) 30–70 kDa when using different ratios of protein to DNA (w/w): (1) 0, (2) 0.037, (3) 0.075, (4) 0.15, and (5) 0.75. X is a standard of DNA sizes (kb).

although the charges are the driving force for initiating electrostatic interactions between basic protein and negatively charged DNA molecules, it is the size of the protein which can give stability to the formed complexes. In this study it has been shown that a molecular mass in the range of 15−70 kDa is required to give the polylysine its maximum DNA binding activity. Extensive peptic hydrolysis of Met-BLG into small fragments of about 1 kDa led to a complete loss of its DNA binding capacity (data not shown). As compared to Met-BLG or histone, polylysines with high molecular mass (15−70 kDa) have a higher DNA binding capacity since polylysine:DNA ratios of 0.037−0.075 (w/w) were sufficient to induce an inhibition, compared to protein:DNA ratios of 1.5−3 (w/w) in the case of proteins. This difference is due to the presence of a cluster of positive charges in the case of L-polylysine, the amount of positively charged residues being only 11% of the total amino acids in the case of Met-BLG.

Protective Effect on *L. lactis*. Data presented in **Figure 8** show the growth of *L. lactis* strain IL1403 after 6 h of incubation in the presence of 100% Met-BLG, histone, or L-polylysine

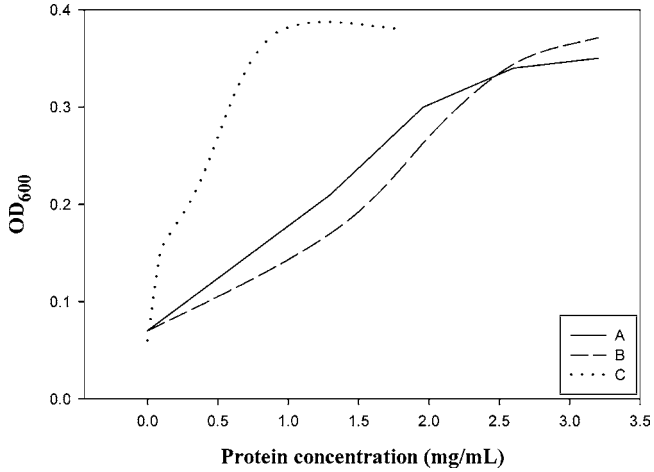

Figure 8. Growth of *L. lactis* strain IL1403 infected by phage bIL67, in the presence of (**A**) Met-BLG, (**B**) histone, and (**C**) L-polylysine (15–30 kDa) added at different concentrations in the preinfection medium after 6 h of incubation.

(15−30 kDa) added to the infection medium at different concentrations. It is evident that addition of esterified protein, native basic protein, or a basic protein-like substance to the incubation medium of *L. lactis* strain IL1403 could protect it against the attack by bacteriophage bIL67 inoculated at a concentration of 10^5 pfu/mL. Relative maximum growth of *L. lactis* strain IL1403 was obtained when the concentration of Met-BLG or histone was about 2.5 mg/mL in the infection medium (0.13 mg/mL in the final incubation medium). The same growth of *L. lactis* strain IL1403 was obtained when L-polylysine was added at a concentration of 0.8 mg/mL in the infection medium (0.03 mg/mL in the incubation medium). This higher effect of polylysine as compared to proteins is mainly due to its relatively higher DNA binding capacity as previously stated. However, although polylysine may protect *L. lactis* strain IL1403 efficiently against phage bIL67, its reported cytotoxicity (*45*) may prevent its wide application in the food industry. The same restriction may be applied for histones. Whey proteins can be used to prepare such basic proteins by a simplified fast esterification technique (*46*). Modified (ethylated) milk proteins may be acceptable agents to protect *L. lactis* cultures during stocking or production of fermented dairy products. Esterified BLG or ALA did not show any cytotoxicity against the monkey kidney fibroblast COS-7 cell line (unpublished results) and, hence, may be preferred for food applications. The maximal bacterial growth obtained in the presence of basic proteins or a protein-like substance is about 10% higher than that of the negative control obtained in the absence of both virus and basic proteins. A negative control, made in the absence of virus and in the presence of basic proteins, also showed enhanced bacterial growth resulting from an increase in N source in the medium. Consequently, basic proteins, which do not show any antibacterial effects, can be used for protecting *L. lactis* cultures against bacteriophage attack. Solutions of such proteins should also be used for disinfecting the phage-infected material at low cost and short time without the hazards of using nonfood synthetic disinfectants. Using modified milk proteins as protecting agents during the manufacture of fermented dairy products should have the advantage of not affecting the properties of the final product. Addition of esterified whey proteins to the traditional detergents may widen their spectrum of antimicroorganism activity.

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