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Antiviral Activity of Esterified α-Lactalbumin and β-Lactoglobulin against Herpes Simplex Virus Type 1. Comparison with the Effect of Acyclovir and L-Polylysines

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The antiviral activity of methylated α-lactalbumin (Met-ALA), methylated and ethylated β-lactoglobulins (Met- and Et-BLG) was evaluated against acyclovir (ACV)-sensitive and -resistant strains of herpes simplex virus type 1 (HSV-1) and compared to that of ACV and L-polylysines (4–15 kDa) using fixed or suspended Vero cell lines. Esterified whey proteins and their peptic hydrolyzates displayed protective action against HSV-1, which was relatively lower than that induced by ACV or L-polylysines. The higher activity of L-polylysines was maintained against an ACV-resistant strain of HSV-1, whereas ACV lost much of its activity. The mean 50% inhibitory concentration (IC_{50}) was about 0.8–0.9 μg/mL for L-polylysines against ACV-sensitive and -resistant strains of HSV-1 when using two concentrations of virus (50% and 100% cytopathic effect, CPE). The IC_{50} values of ACV against the sensitive strain of HSV-1 were 3 and 15 μg/mL when using the low and high concentrations of virus, respectively. When using 50% CPE, IC_{50} values for esterified whey proteins ranged from 20 to 95 μg/mL, depending on the nature of the ester group, the degree of esterification, and the nature of the protein. Using the real-time PCR technique, it was shown that Met-ALA inhibited HSV-1 replication.

KEYWORDS: Whey proteins; alpha-lactalbumin; beta-lactoglobulin; herpes simplex virus; acyclovir; L-polylysine

INTRODUCTION

Herpes simplex virus (HSV) causes a wide spectrum of clinical manifestations in the central nervous system of infants (encephalitis with or without disseminated visceral infection) and adults (*1*). HSV type 1 (HSV-1) is a large enveloped DNA virus replicating in the nuclei of mammalian cells. It is a significant human pathogen causing cutaneous lesions primarily in the oral mucosa but also in other sites. More serious manifestations of HSV-1 infection include encephalitis and blinding keratitis (*2*).

Current antiviral treatments target specific viral replication processes that take place after the virus has entered the cell. Most of the treatment of HSV is based on nucleoside analogs of guanine, for example, acyclovir (9-[2-hydrorulteoxymethyl]guanine: ACV). HSV develops resistance to ACV through mutations in genes coding for thymidine kinase or for DNA polymerase (*3*). Some immunocompromised patients with recurrent HSV lesions develop resistance to ACV after repeated treatments (*4*). A number of new antiviral drugs have been developed during the last decades. However, there is considerable room for improvement because many of the existing compounds are not well-tolerated or particularly efficacious (*5*).

External modified proteins can have an adverse effect on viral activity or replication. A modified milk whey protein, 3-hydroxyphthaloyl-β-lactoglobulin, prevented simian immunodeficiency virus (SIV) transmission in 50% of female rhesus monkeys after intravaginal inoculation with the virus (*6*). Additionally, surface-active agents, such as bile salts and sodium lauryl sulfate, have shown antiviral activity against HSV (*7*, *8*) by disrupting or denaturing the viral envelope proteins. This points to the importance of targeting viral protein components to inhibit virus activity and replication.

Human and bovine lactoferrin (hLF, bLF) interfere with the infection by HSV-1 and HSV-2 in vitro (*9*). A recent study found that a 50% concentration of bLF showed an inhibitory

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effect against several types of HSV-1 and HSV-2. The effects of hLF and goat LF were much reduced. When bLF was combined with drug therapy (ACV), the protective effect increased. This synergistic effect between LF and ACV suggests that the dosage for ACV could be significantly reduced without affecting the results. This is an important finding, as it would enable HSV patients to reduce both their drug dose and the chance of developing a drug-resistant strain of HSV (*10*). The antiviral effect of LF occurs in the early phase of HSV infection, by inhibiting the adsorption of virus onto the target cells and by binding to virus particles (*11*, *12*). The net positive charge and other features, such as hydrophobicity, molecular size, and spatial distribution of charged and lipophilic amino acids, all seem to be important factors for the activity against HSV (*13*). A wide range of medium-sized, highly cationic, α-helical peptides show antiviral activity against HSV (*14*).

Milk whey proteins acquire positive charges when esterified with methanol or ethanol, enabling them to interact with negatively charged macromolecules such as nucleic acids (*15*) or proteins. Such positively charged proteins are expected to play a role in viral activity by creating interactions with viral DNA or proteins. Esterified milk proteins have been reported to inhibit bacteriophages infecting *Escherichia coli* and lactic acid bacteria (*16*, *17*), and enteroviruses of the *Picornaviridae* family (Coxsackie virus and poliovirus (*18*)). Both methylated (Met)- ALA and Met-BLG, as well as their peptic hydrolyzates, decreased the infectious activity of human cytomegalovirus (HCMV, member of the *Herpesviridae* family) in MRC-5 fibroblast cells (*19*).

The aim of this work was to investigate the influence of Met-ALA, Met- and Et-BLG, and of their peptic hydrolyzates on the activity and replication of two strains of HSV-1 (ACV-resistant and ACV-sensitive) as compared to ACV and to basic peptide-like components, L-polylysines.

MATERIALS AND METHODS

The BLG (Protarmor 907 NK) was purchased from Armor Protéines (Saint-Brice-en-Coglès, France) and purified according to Mailliart and Ribadeau Dumas (*20*). ALA (Armor Protéines) was further purified by anion-exchange chromatography on a DEAE-Sepharose Fast Flow column (50 × 300 mm). The elution was performed with a 50 mM Tris and 10 mM $CaCl_2$ buffer at pH 7, with a gradient from 10 to 40 mM NaCl. Porcine pepsin (EC 3.4.23.1; 4550 U/mg) and L-polylysines (4–15 kDa) were purchased from Sigma Chemical Co. (St. Quentin-Fallavier, France).

Protein Esterification. The general procedure of Sitohy et al. (*21*) was used with some modifications in protein and acid concentrations.

The BLG (5% w/v) was esterified with methanol and ethanol by dispersing an appropriate amount in concentrated alcohols (concentration >99.5%). Hydrochloric acid was added dropwise at the start of the reaction to give a molar ratio (MR) of acid to carboxyl group equivalent to 60. The reaction mixture was stirred at 4 °C for 10 h, centrifuged at 10,000*g* for 10 min, and then washed three times with an equal amount of alcohol, followed by the same centrifugation treatment. The final precipitate obtained was dissolved in an appropriate amount of distilled water and was kept at −80 °C until freeze-drying. The lyophilized samples were kept at −20 °C until analyzed. The same technique was followed to esterify ALA with methanol except that the time of reaction was 6 h.

Extent of Esterification. The color reaction using hydroxylamine hydrochloride developed by Halpin and Richardson (*22*) was used with modification according to Bertrand–Harb et al. (*23*) to quantify the extent of protein esterification.

Peptic Hydrolyzis of Esterified Proteins. Weighed amounts (10 mg/mL) of native and esterified proteins were dispersed in 20 mM citric acid buffer, pH 2.6. Appropriate aliquots of pepsin solution (initial concentration: 2 mg/mL H_2O) were added to give an enzyme/substrate (E/S) ratio of 2%. The mixture was incubated at 37 °C for 10 h. An appropriate volume of concentrated disodium phosphate solution was added to stop the proteolysis and to bring the pH to 7.6. The degree of hydrolyzis (% DH) was determined by quantifying the increase in free amino groups in the resulting hydrolyzates by the reaction with ortho-phthalaldehyde (OPA) by measuring the absorbance at 340 nm (*24*). The % DH was obtained by dividing the amount of liberated amino groups by the total amount of bound α-amino groups in the starting substrate and multiplying by 100.

Characterization of Peptic Peptides. Peptic peptides were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) according to the procedure of Schägger and von Jagow (*25*) and by reversed-phase high performance liquid chromatography (RP-HPLC) on a Nucleosil C_{18} column (4.6 mm i.d. × 25 cm, SFCC, Gagny, France) equilibrated with solvent A (0.11% trifluoroacetic acid (TFA) in H_2O) and eluted with a linear gradient of 65% solvent A and 35% solvent B (60% acetonitrile, 40% H_2O, 0.09% TFA) for the first 4 min, 30% A and 70% B for the following 30 min, and 100% B for the last 6 min. The flow-rate was 1 mL/min, and the absorbance was recorded at 214 nm. The temperature of the column was kept at 60 °C.

Cells and Virus. Vero cell lines ATCC CCL81 (African green monkey kidney cells) were grown in Eagle's minimum essential medium (MEM, Eurobio, Courtabœuf, France) containing 8% fetal calf serum (FCS, Eurobio) and 1% PCS (penicillin 10 000 U, colimycin 25 000 U, and streptomycin 10 mg). A virus stock of HSV-1, wild 17 strain ACV-sensitive and PCS-sensitive was obtained from the Laboratoire de Virologie de Nantes (France). The HSV-1 strain was propagated in Vero cells and was stored at −70 °C until use.

Anti-HSV-1 Activity and Cytotoxicity Assay. Aliquots (100 μL) of various concentrations of esterified whey proteins diluted with MEM containing 8% FCS were added to the 96 wells containing 50 μL of Vero cell suspension (10^5 cells/mL). To test the anti-HSV-1 activity, 50 μL of virus suspension was added to cells at a dose equivalent to 50% and 100% viral cytopathic effect (CPE), equivalent to a range of 0.1–1.0 multiplicity of infection (MOI)/cell, in the presence of different concentrations of the tested esterified whey proteins. To test the toxicity, 50 μL of MEM containing 8% FCS was added to the wells containing no virus in the presence of different concentrations of proteins. Cell and virus controls were performed simultaneously. The sodium salt of acyclovir (Glaxo-Wellcome, Marly le-Roi, France) was used as reference. After 72 h of incubation at 37 °C with 5% CO_2, the antiviral activity was followed by evaluating the viability of the cells by the neutral red method (*26*). The OD is directly proportional to the amount of viable cells, which is inversely proportional to the CPE ratio. The percentage of antiviral activity was calculated using the following equation,

$$\text{Antiviral activity (\%)} = [(\text{ODs} - \text{Odv})/(\text{ODc} - \text{Odv})] \times 100$$

where ODs is the optical density of the cells infected by the virus and protected by the esterified whey proteins; ODv is the optical density of the cells infected by the virus, and ODc is the optical density of the control cells (not infected by the virus but treated with the esterified whey proteins).

The cytotoxicity of the tested substance was calculated by comparing the viability of the cells grown in the presence of the esterified whey proteins to that of the control cells (in its absence) as follows:

$$\text{Cytotoxicity (\%)} = [(\text{ODc} - \text{Ods})/(\text{ODc})] \times 100$$

The same procedure was performed on fixed cells by incubating 50 μL of the cell suspension distributed in each well of the plate for 24 h. The next day, both virus and esterified whey proteins to be tested were added as previously defined. The plate was incubated for a further 3 days before determining the cellular viability.

Real-time (RT) PCR. The relative quantity of HSV-1 DNA was determined by RT-PCR after incubating the HSV-1-infected Vero cells at 37 °C for 3 days in the presence or absence of different concentrations of Met-ALA. RT-PCR was performed on an LC instrument (Rotor-Gene RG-3000, Corbett Research, Mortlake, Australia) using two primers (Genosys, Pampisford, England), HSV-1/2RG1(5′-GTG-GAT-

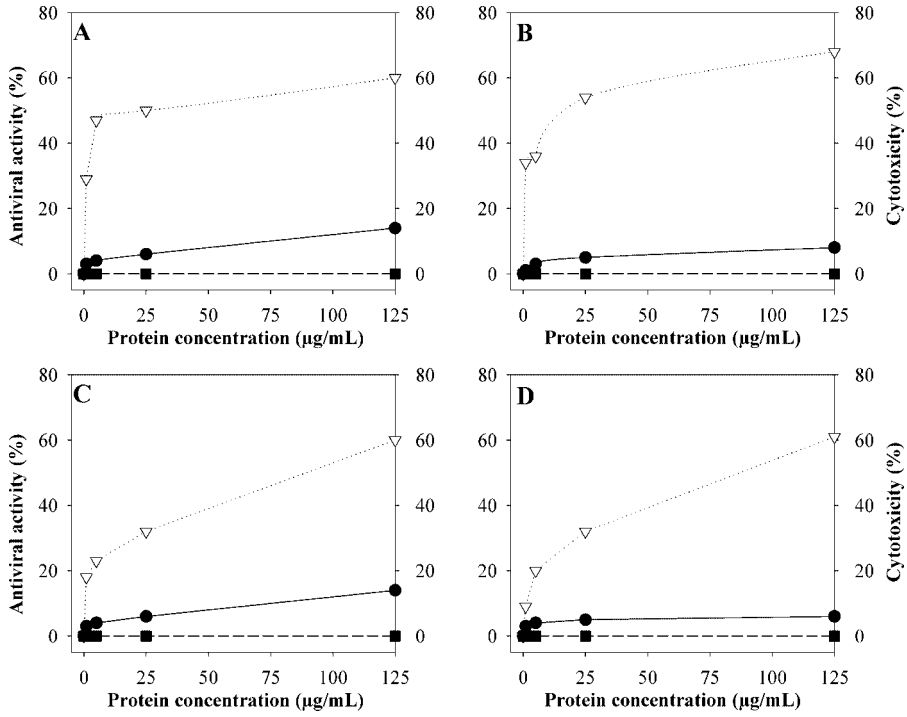

Figure 1. Antiviral activity of 60% Met-ALA (**A**) and its 10 h peptic hydrolyzate (**B**) and 100% Met-BLG (**C**) and its 10 h peptic hydrolyzate (**D**) against HSV-1 [(●) 100% CPE, (▽) 50% CPE] inoculated into Vero cells and after 72 h incubation at 37 °C. (■): cytotoxicity. Results are the mean of three determinations; the error did not exceed the size of the data symbols.

CTG-GTG-CGC-AAA-A-3′) and HSV-1/2RG2 (5′-TCC-GGA-TAC-GGT-ATC-GTC-GTA-A-3′), and the probe HSV12RG (Eurogentec, Serraing, Belgium), 5′-CAA-CTG-CGC-GTT-TAT-CAA-CCG-CAC-3′. The samples were initially prepared for RT-PCR by extracting the viral RNA using the Viral RNA Isolation kit from Macherey–Nagel (Düren, Germany) (*27*). Because the cycle number at which the reporter dye emission intensity rises above the background noise (the threshold cycle, C_t) is inversely proportional to the copy number of the target template, the relative amount of viral RNA was calculated as $1000/C_t$.

RESULTS AND DISCUSSION

Extent of Esterification. BLG and ALA (5%, w/v) were esterified with >99.5% alcohols, a MR of 60, and at 4 °C for 10 and 6 h, respectively. The extent of esterification of BLG with methanol (100%) was significantly higher than that obtained with ethanol (73%). This results from the higher reactivity of methanol. After 6 h of reaction, the extent of esterification of ALA did not exceed 60%. The observed extents of such esterification are in accordance with previous results (*21*).

Peptic Hydrolysis. The influence of enzyme concentration (E/S ratios of 0.125, 0.25, 0.5, 1, 2, and 4%), protein concentration (2.5, 5, 10, and 20 mg protein/mL solution), temperature of hydrolysis (4, 10, 20, 30, and 37 °C), and time of pepsinolysis (30 min, 1, 2, 8, 10, and 24 h) on the DH of esterified BLG and ALA was previously studied (*28, 29*). Optimal conditions giving the highest DH were used in this study (2% E/S, 10 mg/mL protein solution, 10 h hydrolysis at 37 °C). After 10 h hydrolysis of 60% Met-ALA (60% extent of esterification), 100% Met-BLG (100% extent of esterification), and 73% Et-BLG (73% extent of esterification), the maximum expected degree of hydrolysis was obtained (about 25% DH). SDS-PAGE showed that esterification of ALA and BLG facilitates their pepsinolysis, resulting in peptides with molecular mass <1 kDa (data not shown). RP-HPLC profiles of peptic hydrolyzates of esterified BLG and ALA showed a broad population of hydrophilic peptides independent of the nature of the ester group or of the extent of esterification (data not shown). This is mainly due to the increased availability and to the spread of the peptic cleavage sites throughout the esterified molecules, leading to their lysis into small hydrophilic peptides.

Antiviral Activity Against HSV-1. The data presented in **Figure 1** show the antiherpetic activity of 60% Met-ALA (**Figure 1A**) and its 10 h peptic hydrolyzate (**Figure 1B**) against an ACV-sensitive strain of HSV-1 inoculated into Vero cells by using 50 and 100% CPE. Both 60% Met-ALA and its peptic hydrolyzate exerted a more pronounced antiviral activity against the smaller quantity of virus used (50% CPE). The peptic hydrolyzate was as efficient as the intact esterified whey protein. The average protein concentrations leading to 50% inhibition (IC_{50}) were similar for the esterified whey protein and its hydrolyzate (20 ± 1 μg/mL). A comparable behavior of 100% Met-BLG (**Figure 1C**) and its peptic hydrolyzate (**Figure 1D**) was observed with IC_{50} values of 95 ± 1.5 and 85 ± 1.5 μg/mL, respectively. The greater antiviral activity observed in the case of 60% Met-ALA, in spite of its lower degree of modification as compared to Met-BLG (100% esterification extent), might be due to their different molecular properties. The antiviral activities of the esterified whey proteins are mainly due to the increase in positive charges resulting from their esterification. These positive charges can induce DNA-binding properties, (*15*) modifying the viral DNA–protein interactions or the viral protein–cellular protein interactions, thus affecting virus infectivity or replication. Neither native ALA nor native BLG showed any antiviral activity against HSV-1 (data not shown), thus confirming the role of esterification and of the resulting increase in positively charged molecules in the antiviral activity of the modified whey proteins. Within the protein concentration range used in this experiment (0–125 μg/mL), no sign of cytotoxicity was observed.

The data in **Figure 2** show the antiviral activity of L-polylysines (4–15 kDa), of highly basic polypeptides, and of

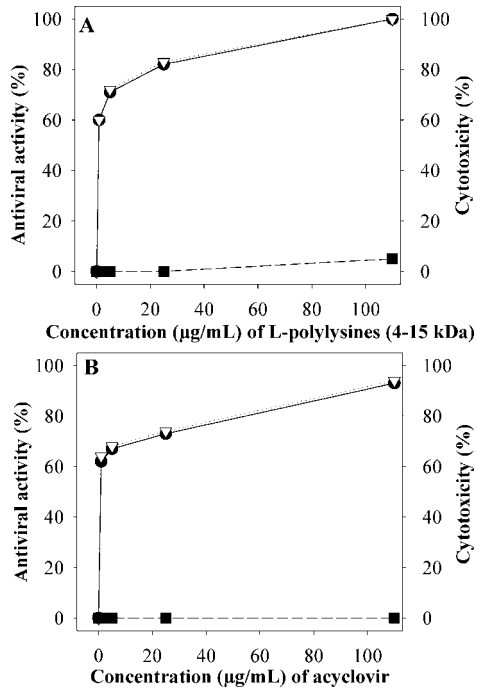

Figure 2. Antiviral activity of (**A**) L-polylysines (4–15 kDa) and (**B**) acyclovir against the ACV-sensitive HSV-1 strain [(●) 100% CPE, (▽) 50% CPE] inoculated into Vero cells and after 72 h incubation at 37 °C. (■): cytotoxicity. Results are the mean of three determinations; the error did not exceed the size of the data symbols.

ACV against an ACV-sensitive strain of HSV-1 inoculated into Vero cells using 50 and 100% CPE. The two substances showed a similar activity against the two concentrations of virus used (50 and 100% CPE) with an IC_{50} of 0.8 ± 0.1 μg/mL. The high antiviral activity of L-polylysines against the greater virus level (100% CPE) is due to their extremely high basicity, that is, their high ratio of positive charges involved in interactions with viral DNA or viral proteins. A similar mechanism may be involved in the antiviral activity of esterified whey proteins. The greater the positive charge of the protein, the higher its antiviral activity is. The entry of HSV-1 into host cells occurs through the fusion of the viral envelope with the plasma membrane, which involves numerous interactions between several viral and cellular proteins (*2*). The presence of esterified whey proteins at the site of these interactions may inhibit them, thus limiting virus entry and protecting the cells. This possible mechanism of action of positively charged modified proteins or of L-polylysines is similar to the previously described action of a bioactive peptide called entry blocker (EB), which was proven to block HSV-1 infection at the entry stage and during cell-to-cell spreading (*2*). This peptide was thought to interact with the virions, inactivating them irreversibly by disrupting their physical integrity. HSV is reported to code for 12 glycoproteins, which are necessary as structural components and are essential for virus entry (*30*). Because of the presence of their positive charges, the esterified whey proteins or L-polylysines may be able to complex or to compete with these glycoproteins for DNA binding, thus inactivating the virus, because it was previously shown that the absence (due to mutations) of these glycoproteins results in noninfectious virus (*7, 31*). It has also been reported that HSV-1 glycoprotein E (gE) functions as an immunoglobulin G (IgG) Fc binding protein and is involved in the spread of the virus (*32*). The positive charges, as well as the hydrophobic groups, on the esterified whey protein molecules may also give rise to interactions between the esterified whey protein and gE, inhibiting viral activities.

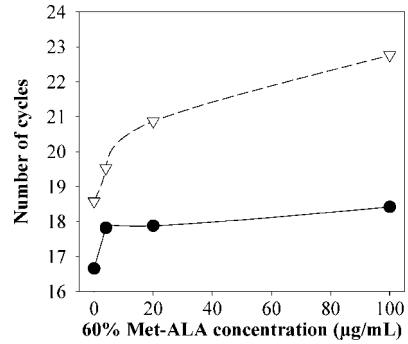

Figure 3. Inhibitory activity of 60% Met-ALA on the replication of HSV-1 [(●) 100% CPE, (▽) 50% CPE] inoculated into Vero cells, after 72 h incubation at 37 °C, detected by RT-PCR. Results are the mean of three determinations; the error did not exceed the size of the data symbols.

Antireplicative Effect. After 3 days of Vero cell growth infected with two concentrations of HSV-1 (50 and 100% CPE) in the absence or presence of different concentrations of 60% Met-ALA, virus replication was followed by RT-PCR. The data presented in **Figure 3** show that the number of PCR cycles giving rise to a detection of amplified viral DNA increased with increased concentrations of 60% Met-ALA. This demonstrates that the original quantity of viral DNA was reduced, because the number of cycles is inversely proportional to the amount of starting DNA template. Hence, it can be concluded that the extent of inhibition of replication is directly related to the concentration of 60% Met-ALA. It can also be concluded that the previously observed protective effect of esterified whey proteins toward the Vero cells originates effectively from their antireplicative action against HSV-1. The action of esterified whey proteins on viral replication may occur by a modification of the protein–protein or DNA–protein interactions. Protein–protein interactions are involved in the function of viral DNA polymerase through hydrophobic interactions that are crucial for viral replication (*33*). Their disruption may diminish or stop the replication. The condensed positive charges or the hydrophobic ester groups distributed over the esterified whey protein molecules enable them to interact with one or more protein components interrupting hydrogen bonds. Moreover, the HSV genome codes for seven viral proteins essential for its replication by their binding to the viral DNA as well as for their own replication (*34*). Hence, perturbation of such DNA–protein interactions by the presence of active antiviral proteins will inhibit viral replication. The DNA binding properties of esterified whey proteins have been described previously (*15*). Moreover, it was demonstrated that the transcription of immediate early genes (IE genes) of HSV is activated by the virion protein VP16, which is a 65 kDa phosphoprotein containing a potent C-terminal acid transcriptional domain (*35*). The positively charged esterified whey proteins may interact with that negatively charged protein or its acid domain, inhibiting all of the viral transcriptional activities.

Antiviral Activity of L-polylysines and ACV Against the ACV-resistant HSV-1 Strain. The data in **Figure 4** show the antiviral activity of L-polylysines and ACV against an ACV-resistant strain of HSV-1 inoculated into a Vero cell suspension or Vero fixed cells. As expected, L-polylysines are much more efficient than ACV at inhibiting the ACV-resistant HSV-1 strain inoculated into fixed or suspended Vero cells. The IC_{50} was 0.9 ± 0.1 μg/mL for L-polylysines for the two concentrations

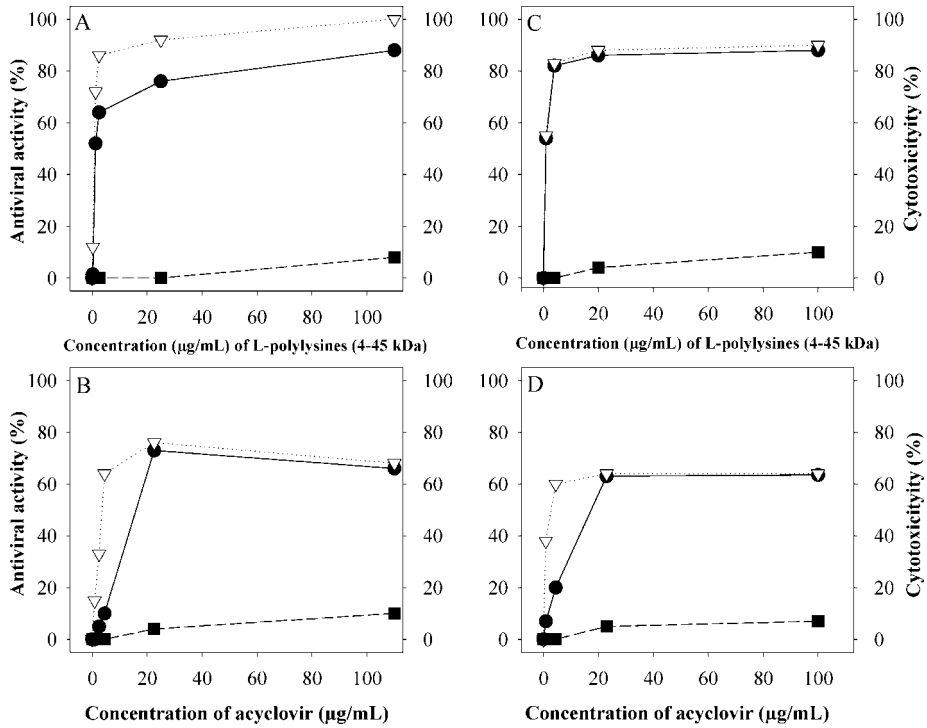

Figure 4. Antiviral activity of (**A** and **C**) L-polylysines (4–15 kDa) and of (**B** and **D**) acyclovir against the ACV-resistant HSV-1 strain [(●) 100% CPE, (▽) 50% CPE] inoculated into Vero cells suspensions (**A** and **B**) or fixed Vero cells (**C** and **D**), after 72 h incubation at 37 °C. (■): cytotoxicity. Results are the mean of three determinations; the error did not exceed the size of the data symbols.

of HSV-1 used, whereas it was 3.3 ± 0.5 and 15 ± 2 μg/mL for ACV depending on the concentration of virus used. As expected, the low inhibitory effect of ACV against this strain is due to its resistance. It is established that HSV produces mutations in genes coding for thymidine kinase or mutations in DNA polymerase (*3*). ACV, which is a nucleoside analog of guanine, requires the viral thymidine kinase for its phosphorylation, whereas ACV triphosphate inhibits the viral DNA polymerase (*36*). Consequently, a lack of activity of this thymidine kinase renders ACV inactive against the viral DNA polymerase. The greater efficiency of L-polylysines over ACV is especially evident when using a low concentration range of tested esterified whey proteins (0–20 μg/mL). Moreover, the maximum of antiviral activity of ACV was clearly lower than that of L-polylysines when using the two forms of cell lines. The maximum value of antiviral activity of ACV was never higher than 65%, whereas that of L-polylysines was almost 90%. It was previously demonstrated that L-polylysines exert their antiviral action through a different mechanism than that of ACV, and L-polylysines can induce more or less the same potential of antiviral inhibition as that observed for the ACV-sensitive strain. Hence, the highly basic polypeptides, L-polylysines, can inhibit the ACV-resistant strain of HSV-1 as efficiently as the sensitive strain, independent of the developed resistance system and independent of the stage of cellular activity (division or differentiation).

Antiviral Activity of Esterified Whey Proteins against the ACV-resistant HSV-1 Strain. The data presented in **Figure 5** show that 60% Met-ALA (**Figure 5A**) and its peptic hydrolyzate (**Figure 5B**) were both active against the ACV-resistant strain of HSV-1 with an IC_{50} of 58 ± 1.6 μg/mL for 60% Met-ALA when using 50% CPE. This antiviral activity is slightly lower than that observed for the ACV-sensitive HSV-1 strain (IC_{50} = 20 ± 1 μg/mL). However, contrary to what was observed for the ACV-sensitive HSV-1 strain, the IC_{50} was not measurable for the peptic hydrolyzate of 60% Met-ALA in the peptide concentration range used with 100% CPE. The efficiency of 60% Met-ALA toward both the ACV-resistant and the ACV-sensitive strains of HSV-1 indicates that the antiviral activity of esterified ALA functions through pathways that differ from those utilized by the guanine-based drugs. The same phenomenon was observed with 100% Met- and 73% Et-BLG and their peptic hydrolyzates (**Figure 5**, panels **C**–**F**, respectively). The IC_{50} values were 8 ± 1 and 75 ± 2 μg/mL for 100% Met-BLG and its peptic hydrolyzate, respectively, and 62 ± 3 μg/mL for 73% Et-BLG, when using 50% CPE. As observed for 60% Met-ALA, the IC_{50} was not measurable for the peptic hydrolyzate of 73% Et-BLG. The difference observed between the antiviral activity of the two ester forms of BLG is apparently because of their different extents of esterification (73 and 100% for the ethylated and methylated forms, respectively) and, consequently, to the amount of positive charges present on the modified molecules. The antiviral activity of esterified whey proteins against the ACV-resistant strain of HSV-1 is less than that observed for L-polylysines, because of the large difference in the density of positive charges. The use of extrinsic modified proteins to counteract virus activity may be useful because many viruses have developed strategies to bypass the inhibitory effects of the natural antiviral protein, interferon (IFN), by producing interferon receptor decoys, which prevent IFN signal transduction (*37, 38*), in addition to the evolving ACV-resistant HSV mutants.

In summary, esterified whey proteins acquire antiviral activity against HSV-1 thanks to the presence of large amounts of positive charges revealed during the esterification reaction. Even greater antiviral activity was observed for L-polylysines (4–15 kDa), which have a much higher density of positive charges than esterified whey proteins. The positive charges confer considerable activity on the protein molecules, enabling them to interact with either viral proteins or viral DNA affecting viral replication, transcription or translation and, consequently, viral infectivity. L-Polylysines and esterified whey proteins may exert

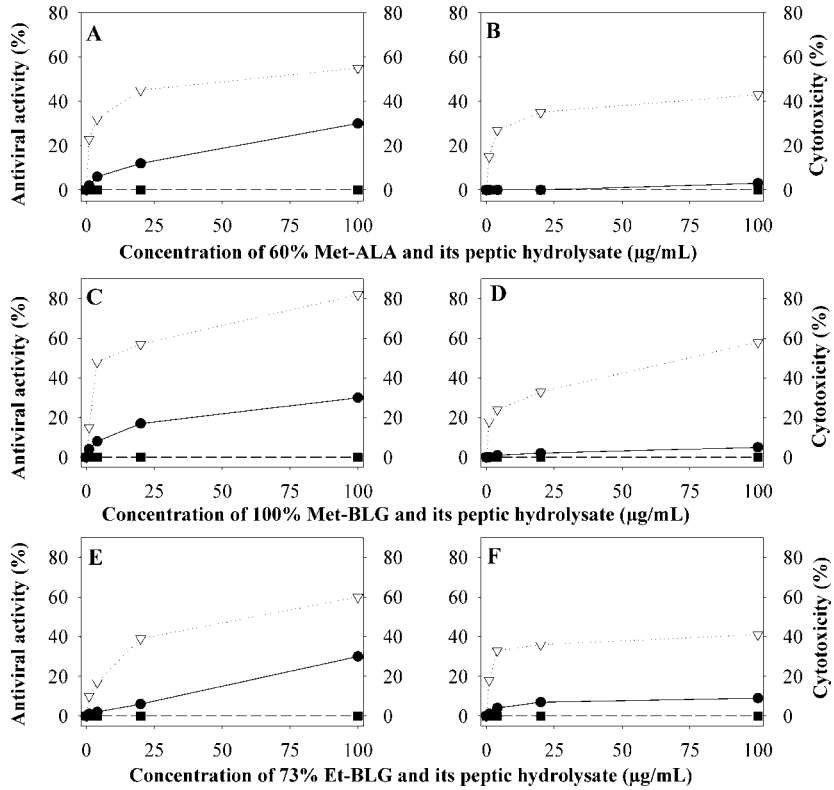

Figure 5. Antiviral activity of (**A**) 60% Met-ALA and (**B**) its 10 h peptic hydrolyzate, (**C**) 100% Met-BLG and (**D**) its 10 h peptic hydrolyzate, and (**E**) 73% Et-BLG and (**F**) its 10 h peptic hydrolyzate against the ACV-resistant HSV-1 strain [(●) 100% CPE, (▽) 50% CPE] inoculated into fixed Vero cells, after 72 h incubation at 37 °C. (■): cytotoxicity. Results are the mean of three determinations; the error did not exceed the size of the data symbols.

their antiviral activities through various possible mechanisms: (i) disruption of the viral envelope or capsid protein constituents affecting capsid integrity; (ii) interference with the viral protein–cellular protein interactions essential for viral infectivity; (iii) binding to the negatively charged virion phosphoprotein VP16, inhibiting the overall transcriptional activities of the immediate early genes; (iv) binding of viral DNA, inhibiting its transcription and replication; and (v) inhibition of the hydrolytic activity of the herpes virus protease essential for capsid formation (*39*).

Whereas ACV was less efficient against some resistant mutants, L-polylysines and esterified whey proteins were still able to inhibit the infectivity of the ACV-resistant HSV-1 strain with almost the same efficiency because they use a mechanism of antiviral action that is different from that of ACV. Hence, L-polylysines and esterified whey proteins can be used in the fight against the ACV-resistant virus mutants emerging after prolonged treatment with ACV and leading to ACV treatment failure. L-Polylysines were generally more efficient than ACV or esterified whey proteins against both ACV-sensitive and ACV-resistant mutants of HSV-1. Consequently, esterified whey proteins or L-polylysines could possibly be used topically against a viral infection or the treatment of HSV-1 infections because no cell toxicity was observed within the concentration range used in this study.

ABBREVIATIONS USED

ALA, α-lactalbumin; BLG, β-lactoglobulin; Met, methylated; Et, ethylated; ACV, acyclovir; HSV, herpes simplex virus, CPE, cytopathic effect; MOI, multiplicity of infection; IC, inhibitory concentration; hLF, human lactoferrin; bLF, bovine lactoferrin; HCMV, human cytomegalovirus; ATCC, African green monkey kidney cells; MEM, Eagle's minimum essential medium; FCS, fetal calf serum; PCS, penicillin, colimycin, streptomycin; MR, molar ratio; DH, degree of hydrolysis.

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