0273-2289/00/88/0285/$13.50

Antiinfluenza Virus Activity of a Bacteriocin Produced by *Lactobacillus delbrueckii*

Julia Serkedjieva,*[,1] Svetla Danova,[2] and Iskra Ivanova[2]

[1]Institute of Microbiology, Bulgarian Academy of Sciences, Acad. Georgy Bonchev str., bl. 26, 1113 Sofia, Bulgaria, E-mail: viroljul@bas.bg; and [2]Department of Microbiology, Faculty of Biology, Sofia University, Bulgaria

Abstract

A novel antibacterial substance produced by *Lactobacillus delbrueckii* has been isolated and characterized *(1)*. The inhibitory agent corresponded to the criteria for bacteriocins. It was active against lactic acid bacteria (LAB) species and several food-borne pathogens. The cell-free supernatant was purified by HPLC gel-filtration. Three preparations at different purification steps were tested for activity on the reproduction of influenza virus A/chicken/Germany, strain Weybridge (H7N7) and strain Rostock (H7N1) in cell cultures of chicken embryo fibroblasts (CEF). The inhibitory effect was shown to be highly selective and specific. Expression of viral glycoproteins hemagglutinin, neuraminidase, and nucleoprotein on the surface of infected cells, virus-induced cytopathic effect, infectious virus yield, and hemagglutinin production were all reduced at nontoxic concentrations of the crude preparation (B1). B1 did not protect cells from infection, did not affect adsorption, and slightly inhibited viral penetration into infected cells. The purification did not enhance the cellular toxicity and increased about 870-fold the virus-inhibitory activity. No inactivating effect on extracellular virus was found.

Index Entries: Bacteriocin; *Lactobacillus delbrueckii*; influenza virus; viral inhibition.

Introduction

The search for selective antiviral agents has been vigorous in recent years but the need for new antiviral therapies still exists, because many of the problems of the treatment of viral infections, such as generation of viral resistance and undesirable side effects, remain.

Influenza continues to be a major cause of high morbidity and significant mortality both for humans and domestic animals. Rimantadine

*Author to whom all correspondence and reprint requests should be addressed.

hydrochloride, an analog of amantadine hydrochloride, the only approved drug so far, has well-documented prophylactic *(2)* and therapeutic *(3)* activities in the case of noncomplicated influenza A virus infection after oral administration. Rimantadine has no marked antiviral activity or therapeutic effectiveness in established influenza and no specific therapy of proven value currently exists for severe influenza infection. Some adverse effects of rimantadine have also been reported *(4)*, and development of viral resistance to rimantadine has been identified as a problem in the use of this drug *(5)*. Zanamavir, a selective inhibitor of viral neuraminidase, is still under investigation *(6)*. Obviously a need for effective therapies for influenza virus infection continues to exist.

The products of microbial fermentation and secondary microbial metabolites provide an alternative source for substances with virus-inhibitory activities *(7,8)*. Bacteriocins from LAB are antimicrobial peptides of cationic nature. The application of bacteriocins and bacteriocinogenic LAB to eliminate or inhibit the growth of pathogenic or spoilage bacteria in complex food systems has provoked great interest. A novel antibacterial substance produced by a strain isolated from Bulgarian yellow cheese has been characterized *(1)*. The producer strain was identified by molecular typing to belong to the species *Lactobacillus delbrueckii*, which is a rare producer of bacteriocins. The studied bacteriocin was found to have a remarkably broad spectrum including LAB species and several Gram-positive and Gram-negative bacteria. This justified an attempt to investigate its antiinfluenza virus effect.

Materials and Methods

Bacterial Strains and Media

For the cultivation of *Lactobacillus delbrueckii* subsp. bulgaricus 1043, MRS and Elliker broth and agar were used. Before experimental use, cultures of the producer strain were cultivated successively in milk and twice in broth medium at 30°C.

Cells and Media

Primary chick embryo fibroblasts cell cultures (CEF) were prepared according to Portfield *(9)*. The growth medium contained 50% 199 medium and 40% Hanks solution, and was supplemented with 5% hydrolyzed lactoalbumine, 5% calf serum, and antibiotics (streptomycine and penicillin).

Viruses

Influenza viruses A/chicken/Germany, strain Weybridge (H7N7) (A/Weybridge) and strain Rostock (H7N1) (A/Rostock) were from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences. Both viruses were maintained by passages in 11-d old hen's fertile eggs and were used as allantoic fluids. The infectious titers of the viruses were over

the range of 5.0×10^{6}–10^{8} $TCID_{50}$/mL, the HA titers: 1024–2048. The virus stock was stored at –70°C.

Bacteriocin Activity Assays

Antibacterial Activity

Bacteriocin screening was performed by the well diffusion method as described by Tagg and McGiven *(10)*. Usually 1.5% agar was used. The inhibitory activity was studied by using the following test organisms: *Bacillus subtilis* 6633 ATCC; *Escherichia coli* HB101; *Listeria inocua* F (ENITIAA); *Listeria monocytogenes* Scott A (ENITIAA). The antimicrobial activity was expressed in arbitrary units per milliliter (AU/mL). One AU was defined as the reciprocal value of the highest serial twofold dilution showing a clear zone of growth inhibition of the indicator strain. The specific activity (SA) was calculated as the ratio of antimicrobial activity per milligram protein.

Effect of Enzymes and Heat Treatment

The culture supernatant was treated for 2 h at 37°C with the following proteolytic enzymes (1 mg/mL): proteinase K (Boeringer, GmbH); pronase E (Sigma); and trypsin (Sigma). The supernatant was incubated at 100°C for 15 min.

Bacteriocin Purification

The active substance was purified from *Lactobacillus delbrueckii* culture grown in MRS medium, pH 6.5, at 30°C for 32 h. The purification procedure *(1)* included culture centrifugation for 30 min at 12,000*g* and incubation of the supernatant at 90°C for 15 min to get the crude preparation B1. The supernatant was precipitated with ammonium sulfate (80% of saturation) for 2 h at 4°C, then it was centrifuged for 50 min at 12,000*g*. The pellet was resuspended in 10 mL of 25 m*M* ammonium acetate buffer, pH 6.5, and 5 mL of the resuspended liquid was applied on a Cartridge Sep-Pack Waters C_{18} mini-column. Three fractions were obtained; 30, 50, and 80% iso-propanol fractions each of 5 mL volume. The 50% iso-propanol fraction that showed antimicrobial activity was evaporated on a speed-vac concentrator (Jouan, France) and resuspended in 0.2 mL solvent (0.5% *o*-phosphoric acid in 3 *M* urea). The obtained preparation, B2, was tested for antiviral activity. The fraction was applied on a HPLC gel-filtration TSK 2000SW column (7.8 × 300 mm) (Pharmacia-LKB, Uppsala, Sweden). The bacteriocin was eluted with bidistilled water/acetonitril/methanol/*o*-phosphoric acid (500/1/1/1) at a flow rate of 1.5 mL/min. Fractions with high bacteriocin activity were combined and evaporated on a speed-vac concentrator. The purification factors were calculated from the ratios SA_{B2}/SA_{B1} and TI_{B2}/TI_{B1}.

HPLC Gel-Filtration

The molecular weight of the purified bacteriocin was estimated by HPLC gel-filtration (TSK 2000SW) using bidestilled water/acetonitril/

methanol/*o*-phosphoric acid (500/1/1/1) at a flow rate of 0.5 mL/min. The Pharmacia kit of molecular weight markers (2600–17,200 Da) was used.

Reverse-Phase Chromatography

The evaporated fractions were resuspended in 0.05 mL solvent (0.1% trifluoroacetic acid in 3 *M* urea) and analyzed by HPLC (Shimadzu 4A, Kyoto, Japan) with a Rapid Spectral Detector 2140 (LKB-Pharmacia, Uppsala, Sweden) using Eurosil Bioselect 300 C_{18}, 5 μm column (250 × 8 mm) (Knauer, Germany). The column was maintained at 30°C with a column heater.The column was equilibrated with solvent A (0.1% trifluoroacetic acid in water) at a flow rate of 1 mL/min and peptides were eluted by increasing the concentration gradient of solvent B (0.1% trifluoroacetic acid in acetonitril). Peptides were monitored spectrophotometrically at 212 and 260 nm. Peak fractions representing all peaks were collected manually in vials, evaporated, and assayed for bacteriocin and antiviral activity (preparation B3). The purification factors were calculated from the ratios SA_{B3}/SA_{B1} and TI_{B3}/TI_{B1}.

For the antiviral experiments the preparations were diluted in a serum-free medium (10-fold dilutions) *ex tempore*, pH was adjusted to 7.2 by the addition of a saturated sodium bicarbonate. Aliquots of preparation B1 were frozen and stored at –20°C.

Protein Determination

Protein content was determined by the method of Lowry (*11*).

Cell Toxicity

Cell toxicity was monitored according to the effect of the preparations on cell morphology and viability. Serial 10-fold dilutions of the preparations in a tissue culture medium were added to confluent cell monolayers and the cells were cultivated at 37°C for 72 h. The morphology of the cells was inspected daily to observe microscopically detectable alterations, i.e., loss of monolayer, rounding, shrinking of cells, granulation, vacuolization in the cytoplasm. The cytopathic effect (CPE) was scored at 72 h under inverted microscope (scores: 0 = 0% CPE; 1 = 0–25% CPE; 2 = 25–50% CPE; 3 = 50–75% CPE; 4 = 75–100% CPE). Alternatively, in some experiments monolayers were stained with 0.5% neutral red after the end of incubation and cell viability was estimated. The concentration required to cause visible changes in 50% of intact cells, 50% toxic concentration (TC_{50}), was evaluated from graphic plots. TC_{50} was expressed with respect to protein content.

Hemagglutination Assay

Stock virus suspension (0.05 mL) was diluted 12 times by serial two-fold dilutions and incubated with an equal volume of a 1% hen erythrocytes suspension for 30 min at the room temperature. The hemagglutination titer was estimated as the reciprocal value of the last dilution exhibiting hemagglutination (HA) and expressed as $\log_2$ HA.

Virucidal Activity

Virucidal activity was tested by a direct contact assay. Ten-fold diluted stock virus suspension was treated with equal volumes of increasing 10-fold dilutions of the preparation in phosphate-buffered saline (PBS) for 1 h at room temperature. The final concentrations were over the range of 0.008–80 mg/mL. The difference in biological activities of treated and control viruses (treated with PBS only) was determined on the basis of infectivity and HA production. Surviving infectious virus titers (50% tissue culture infectious doses/mL, $TCID_{50}$/mL) were determined in CPE assay using the method of Reed and Muench *(12)* and expressed as $\log_{10} TCID_{50}$/mL. The minimum concentration required to reduce the infectious titer by 1 $\log_{10} TCID_{50}$/mL (by 90%) was estimated (MIC_{90}).

Antiviral Assays

Cytopathic Effect Reduction Assay

Quadruplicate confluent monolayers in 96-well plates were overlaid with serial 10-fold dilutions of the preparations in a tissue culture medium (0.05 mL) and an equal volume of virus suspension (100 $TCID_{50}$/mL). The virus-induced CPE was examined daily and scored at 48–72 h after infection as described above in Cell Toxicity. The reduction of virus multiplication was calculated as percentage of virus control (% of virus control = $CPE_{exp}/CPE_{virus\ control} \times 100$). The concentration reducing CPE by 50% with respect to virus control was estimated from graphic plots and was defined as 50% effective concentration (EC_{50}). EC_{50} was expressed relative to protein content. The therapeutic index (TI) was calculated from the ratio TC_{50}/EC_{50}.

50% End-Point Titration Technique

Fifty percent end-point titration technique (EPTT) was used according to Vanden Berghe et al. *(13)*. Monolayers in 96-well microtiter plates were infected with 0.05-mL serial 10-fold dilutions of the virus suspension, then serial 10-fold dilutions of the preparation in a tissue culture medium (0.05 mL) were added. The cultures were incubated at 37°C and examined microscopically daily for CPE. CPE was scored as described above. The antiviral activity of the preparations was determined by the difference in the virus infectious titers ($\delta \log_{10} TCID_{50}$/mL) in the absence and in the presence of the preparation.

Reduction of the Viral Glycoprotein Expression on the Infected Cell Surface

The reduction of the expression of the viral glycoproteins, i.e., hemagglutinin (HA), neuraminidase (NA), and nucleoprotein (NP), on the surface of the infected cells was evaluated. The assay is described in Serkedjieva and Hay *(14)*. Quadruplicate monolayers in 96-well microtiter plates were overlaid with serial 10-fold dilutions of the preparation in a tissue culture medium (0.05 mL) 30 min prior to inoculation with an equal volume of serial 10-fold dilutions of infectious virus. Monolayers were fixed with

0.1% glutaraldehyde in PBS and assayed for HA, NA, and NP after 16–20 h incubation at 37°C. ELISA was performed with monoclonal antibodies (MAb) to HA, NA, and NP of the corresponding viral strain and protein A-horseradish peroxidase conjugate (Bio-Rad, Hercules, CA). Cells were permeabilized with 1% solution of Triton X-100 prior to ELISA for NP. The ELISA was used as described in ref. *15*. The optical densities (OD_{450}) were measured and expressed as percentage of nondrug-treated virus-infected cells (virus control). MAb were kindly provided by Dr. Alan Douglas from the World Centre of Influenza, Mill Hill, London. The concentration causing 50% reduction in optical density values was evaluated (EC_{50}) from graphic plots. EC_{50} was expressed with respect to protein content.

Time of Addition Studies

The inhibitory effect of B1 was studied following addition of the preparation at different times relative to viral infection. Confluent monolayers in 96-well plates were incubated with plain or drug-containing medium (serial 10-fold dilutions of the preparation) for 1 h (effect of cell pretreatment), washed twice with PBS, and challenged with serial 10-fold dilutions of infectious virus. Adsorption was carried out for 1 h at 4°C to prevent virus internalization in the presence or in the absence of drug-containing medium (effect on adsorption). The cells were washed twice with PBS and overlaid with plain or drug-containing medium for 1 h to allow viral particles to penetrate into cells (effect on penetration). Then plain or drug-containing medium was added and cells were cultivated for 48 h at 37°C (effect on replication). At the end of cultivation CPE was scored as described above and infectious titers of control and treated viruses were evaluated according to EPTT *(13)* and by HA endpoint. A viral titer reduction by 1 $\log_{10}$ $TCID_{50}$/mL (by 90%) was considered to indicate a significant antiviral effect. The significance of the difference in infectious titers was estimated (Student's *t*-test).

In all antiviral experiments nondrug-treated, mock infected cells were used as cell control and nondrug-treated, virus-infected cells were used as virus control. Rimantadine hydrochloride (Hoffman-La Roche, Nutley, NJ) (1 μg/mL) was used as a positive control. The results are the mean of three to six experiments.

Results

The cell-free supernatant obtained after the cultivation of *Lactobacillus delbrueckii* was purified. The results are summarized in Table 1. The overall purification procedure resulted in more than a 3400-fold increase in specific antibacterial activity and about an 870-fold increase in antiviral effect with a recovery yield of about 17%. C_{18} reverse-phase HPLC, the final step of purification, gave a single symmetrical peak with antibacterial activity. The molecular mass of the purified bacteriocin was estimated as 6 kDa by HPLC gel-filtration. The homogeneity of the purified bacteriocin

Table 1
Purification of a Bacteriocin Produced by *Lactobacillus delbrueckii*[a]

Sample (signature)	Antimicrobial activity[b]			Antiviral effect[c]		Yield recovery, %
	Protein (μg/mL)	AU/mL	PF	TI	PF	
Cell-free supernatant (B1)	80.5	4	1	1.1×10^3	1	100
50% Iso-propanol fraction (B2)	123	0.24×10^4	390	8.2×10^4	75	30
Rechromatography after HPLC (B3)	780	1.36×10^5	3480	9.6×10^5	870	17

[a]Part of the results are presented in Miteva et al. *(1)*.
[b]Indicator strain *Listeria inocua* F (ENITIAA).
[c]A/chicken/Germany/27, strain Weybridge (H7N1).

was further confirmed by a single diffuse band with a molecular mass of 5 kDa on SDS-PAGE (data not shown).

Bacteriocine Activity

Antibacterial Activity

The cell-free supernatant (B1) was assayed for antibacterial activity on the growth of *Bacillus subtilis*, *Escherichia coli*, *Listeria inocua*, and *Listeria monocytogenes*. All tested strains were susceptible to the inhibitory action of the preparation.

Effect of Enzymes and Heat Treatment

B1 was assayed for heat sensitivity and susceptibility to the action of proteolytic enzymes. The preparation was treated with proteinase K (1 mg/mL), pronase E (1 mg/mL), and trypsin (1 mg/mL), and was tested against A/Weybridge. There was elimination of the virus-inhibitory activity by proteinase K and pronase E. Trypsin reduced the virus-inhibitory effect of B1 slightly, but it remained stable after heating at 100°C for 15 min.

Cellular Toxicity

By two separate toxicity assays, CPE and neutral red dye uptake, it was shown that confluent CEF monolayers treated for 72 h with the preparations in doses up to 25 mg/mL did not show any visible changes in cell viability and morphology or cell sheet density. Higher concentrations caused alterations in cell morphology and loss of monolayer, microscopically detectable at 72 h of incubation. The results are presented in Table 2. TC_{50} for CEF cells was over the range of 6.4–19.2 mg/mL.

Virucidal Activity

The ability of the crude preparation B1 to produce a direct virucidal effect in the contact assay was studied. In doses up to 8 μg/mL the prepa-

Table 2
Cell-Toxic and Virus-Inhibitory Effects of Bacteriocin Preparations on the Reproduction of A/Weybridge and A/Rostock[a] in CEF

Preparation	Cytotoxicity TC_{50} (ng/mL)	Virus-inhibitory effect			
		A/Weybridge		A/Rostock	
		EC_{50} (ng/mL)	TI	EC_{50} (ng/mL)	TI
B1	6400	5.6	1143	4.2	1524
B2	14,800	0.18	82,200	0.16	92,500
B3	19,200	0.02	960,000	0.02	960,000

[a]Virus inoculum 10–100 $TCID_{50}$/mL.

ration B1 did not exhibit any direct inactivating effect on A/Weybridge, i.e., no detectable reduction of viral biological activity (infectivity and HA production) was found.

Effect on the Reproduction of A/Chicken/Germany in CEF

The effect of the preparation B1 and its purified active component (B2 and B3) on the reproduction of two influenza virus strains A/Weybridge and A/Rostock in CEF was studied. The virus-induced CPE and the production of HA as measures of viral reproduction were considerably reduced by all three preparations (Table 2). The antiviral effect was highly selective since the therapeutic indices were over the range 10^3–10^5. The drug susceptibility of the two tested strains was of comparable degree (Table 2). The antiviral activity of the crude preparation B1 was investigated in more detail. B1 inhibited in a dose-dependent manner the replication of A/Weybridge in CEF in multicycle viral growth experiments (Fig. 1). The preparation B1 abolished completely viral reproduction measured by the production of infectious virus and HA in doses >0.1 mg/mL (Fig. 1). B1 inhibited HA, NA, and NP expression on the surface of the infected CEF cells in cell-ELISA with the corresponding MAb (Fig. 2). HA expresion was most sensitive to inhibition with B1. The EC_{50} values in the ELISA assay were 0.7 ng/mL (HA), 6.4 ng/mL (NA), and 8 ng/mL (NP). The influence of the different parameters such as drug concentration and virus inoculum on the virus-inhibitory effect was determined. The expression of viral glycoproteins was entirely reduced by the preparation (8–800 ng/mL) depending on the multiplicity of infection (Fig. 3). The effect was dose-dependent in all assays used, i.e., CPE-reduction, EPTT, and ELISA (Table 2, Figs. 1–3).

Time of Addition Study

To investigate the effect of B1 on different steps of viral replication the preparation (in a dose 0.08 μg/mL) was added at various times relative to viral infection. The results are presented in Table 3. The pretreatment of cells (A) as well as the addition at the time of adsorption (B) did not result in decrease of virus infectivity. Virus replication was slightly reduced when

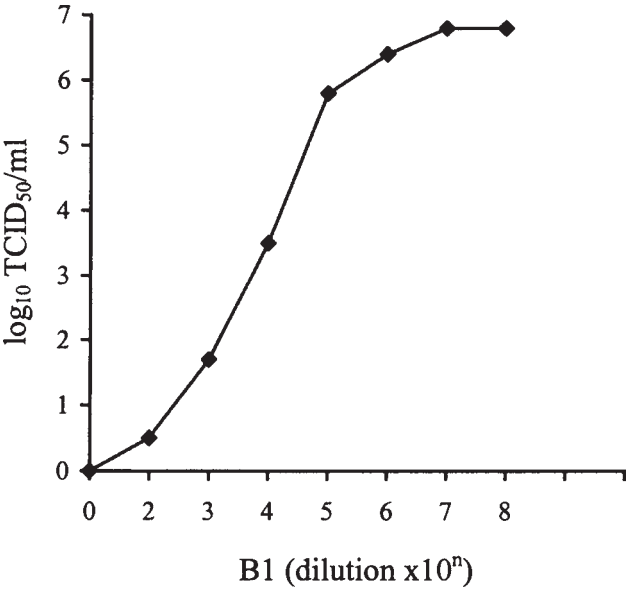

Fig. 1. Reproduction of A/Weybridge* in CEF in the presence of B1 (dose-dependence of inhibition). *, virus control: 6.8 $\log_{10}$ $TCID_{50}$/mL.

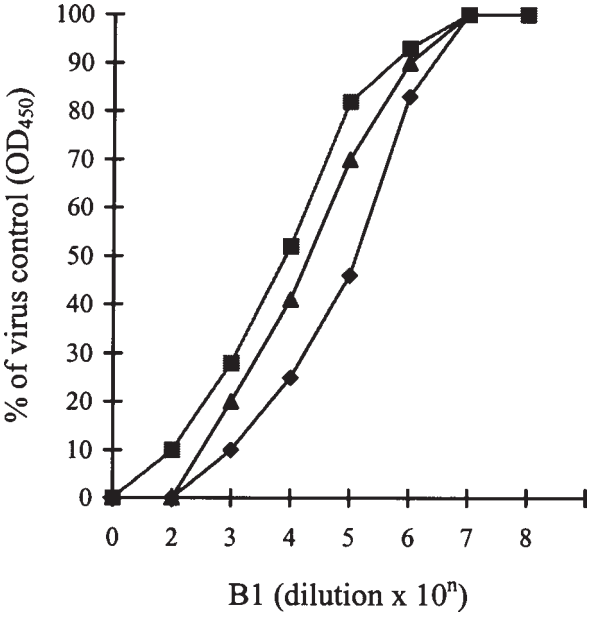

Fig. 2. Expression of HA, NA, and NP on CEF cell surface infected with A/Weybridge* and A/Rostock* in the presence of B1. *, virus inoculum: 100 $TCID_{50}$/mL. -◆-, HA (A/Weybridge); -■ -, NP (A/Weybridge); -▲ - NA (A/Rostock).

B1 was added at the time of penetration (C), δ $\log_{10}$ $TCID_{50}$/mL = 1.1, and significantly inhibited when the preparation was inoculated after virus infection (D), δ $\log_{10}$ $TCID_{50}$/mL = 5.1. When the substance was present in

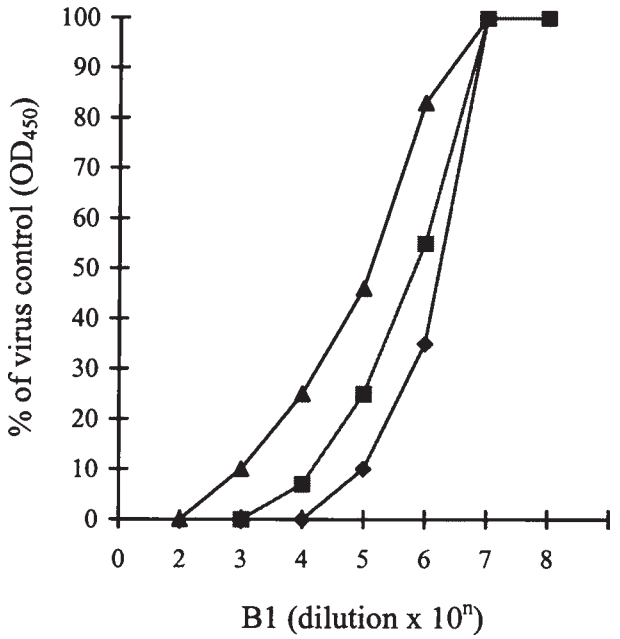

Fig. 3. Expression of HA on CEF cell surface infected with A/Weybridge in the presence of B1 (dependence on virus inoculum). -◆-, 1 $TCID_{50}$/mL; -■-, 10 $TCID_{50}$/mL; -▲-, 100 $TCID_{50}$/mL.

Table 3
Reproduction of A/Weybridge in CEF in the Presence of B1 (Timing of Addition)

	Virus-inhibitory effect		
Inoculation of B1 (0.08 μg/mL)[a]	$\log_{10}$ $TCID_{50}$/mL	δ $\log_{10}$ $TCID_{50}$/mL	Significance[b]
A. Pretreatment of cells, 1 h, 37°C	6.2	0.53	—
B. Adsorption 1 h, 4°C	6.5	0.23	—
C. Penetration, 1 h, 37°C	5.63	1.1	+
D. After infection, 48 h, 37°C	1.63	5.1	+
A + B + C + D	1.21	5.52	+

[a]The dose is expressed with respect to protein content.
[b]$p < 0.05$.

the growth medium during the whole replicative cycle (A + B + C + D) the viral inhibition reached maximal values, δ $\log_{10}$ $TCID_{50}$/mL = 5.5.

Discussion

The main objective of the study was to characterize the influenza virus-inhibitory effect of the cell-free supernatant from *Lactobacillus delbrueckii* fermentation and an active component derived from it (at two purification steps). The culture supernatant was subjected to a multistep purification procedure (Table 1). The overall purification resulted in more than a

3400-fold increase in specific antibacterial activity and about a 870-fold increase in antiviral effect with a yield recovery of about 17%.

The study on the antiinfluenza virus effect was conducted with respect to the specificity and selectivity of viral inhibition. The preparations were tested for antiviral activity on the reproduction of two influenza virus strains in CEF by two separate assays (CPE- and HA-reduction). All tested preparations showed pronounced activity (Table 2). The inhibitory effect was shown to be highly selective, therapeutic indices ranged over 10^3–10^5. Three more assays (contact assay, ELISA, and EPTT) were introduced to investigate the virus-inhibitory effect of the crude preparation B1. Expression of viral glycoproteins hemagglutinin, neuraminidase, and nucleoprotein on the surface of infected cells, virus-induced cytopathic effect, infectious virus yield, and hemagglutinin production were all reduced in a dose-dependent manner at nontoxic concentrations of B1. The purification increased the virus-inhibitory activity but not the cellular toxicity (Table 2). Antiviral activity appeared as corresponding to a selective virus inhibition since no virucidal effect of B1 was detected. The inhibition was dose-related and depended on virus inoculum (Figs. 1–3). B1 failed to inactivate virus but was able to interfere with the interaction between viral particles and the cell. The time of addition experiments, studying the possible mode of action of B1, indicated that the significant inhibitory effect cannot account for the protection of cell cultures or decrease in cell sensitivity to virus, adsorption was not the target of inhibition, and internalization of viral particles was slightly affected (Table 3). The inhibition was most pronounced when the extract was added after virus infection. We assumed that some of the intracellular specific steps in the viral reproduction must be inhibited. The presence of B1 throughout the whole replicative cycle was necessary for the full expression of the antiviral effect. It has been shown that a partially purified extract from *Melia azedarach* L. leaves, whose active inhibitory component appeared to be of proteinaceous nature, did not affect viral adsorption to or penetration into BHK cells following Sindbis virus infection while virus-specific synthetic stages were inhibited *(16)*. The mechanism of virus inhibition by B1 remains subject to further studies. The antiviral activity of B1 was sensitive to digestion with proteinase K and pronase E, which indicated its proteinaceous nature. Treatment with trypsin or boiling at 100°C did not abolish the inhibitory effect. It seemed likely that the active component(s) was protein in nature and appeared to be resistant to treatment with selected proteolytic enzymes and boiling, as reported for bacteriocins *(17)*. Investigations on the chemical nature of the purified active substance (B3) showed that it had a peptide structure containing 10% carbohydrate moieties *(1)*.

There are scarce literature data on the virus-inhibitory activity of peptides and proteins of different origin, i.e., microbial *(7,18,19)*, plant *(16,20,21)*, mammalian *(22,23)*, and synthetic *(24,25)*. Only few of the papers deal with the influenza virus-inhibitory effect of preparations of proteinceous nature *(18,20,21,26)*. It has been shown that the pokeweed

antiviral protein from *Phytolacca americana* in n*M* concentrations reduced protein synthesis in virus-infected cells by inactivation of host cell ribosomes *(27)*. Synthetic peptide, corresponding to a Zn finger region of the M1 sequence of influenza virus, inhibited viral transcription through direct interaction with viral RNA *(24)*. A polypeptide isolated from *Pseudomonas fluorescens* inactivated influenza virus A/PR8 *(18)*, whereas the whole bacterial extract did not show any virucidal effect. Some bacterial enzymes, endotoxines, and polypeptide antibiotics also inhibited influenza virus replication (for review see ref. *7*). The presented results identify the bacterial preparation B1 and its purified biologically active component B3 as promising candidates for further investigation. The mechanism(s) of the antiviral effect as well as their efficacy in experimental influenza infection are under current examination. The active component of B1 corresponds to the criteria for bacteriocins *(17)*; its identification and characterization has been published in detail in ref. *1*.

A large number of bacteriocins with different properties have already been described and characterized with respect to biochemical properties and structure, activity spectrum, genetic determinants, and mechanism of action. Four different classes of bacteriocins in LAB have been established: I, lantibiotics; II, small heat stable non-lantibiotic bacteriocins; III, large heat labile bacteriocins; and IV, complex bacteriocins with lipid and carbohydrate moiety *(28,29)*. The bacteriocin produced by *Lactobacillus delbrueckii* could be classified as class IV bacteriocin according to its chemical structure *(1)*.

The mode of action of various bacteriocins has been extensively studied in membrane vesicles and artificial membranes recently *(29,30)*. They seem to affect the membrane permeability barrier by forming water-filled channels or pores, probably by a barrel-stave mechanism *(17)*. This leads to subsequent cell lysis.

It has been found by the patch-clamp method that the bacteriocin produced by *Lactobacillus delbrueckii* induced membrane permeability leading to a vast increase in cation flux through the membranes of smooth muscle cells isolated from human intestines. A 700- to 2100-fold increase of the outward current amplitudes was observed; the effect was voltage-dependent. It was suggested that the preparation incorporated into the native cell membranes possibly by forming voltage-dependent pores just like nigericin and calcium ionophores do. According to the voltage-dependency of its pore-forming action, the bacteriocin could be classified as a class I bacteriocin (unpublished data). At the present stage of investigation, we cannot relate the effect of the bacteriocin produced by *Lactobacillus delbrueckii* on cell membrane permeability to its virus-inhibitory activity. Dundarov et al. *(31)* established a strong antiherpes virus effect of the ionophore antibiotic pandavir (nigericin). The drug inhibited considerably viral reproduction when applied in 0.01–0.02 ng/mL doses. It seemed that the antiviral effect was connected with specific inhibition of virus DNA synthesis. Monensin and A-23187, ionophore antibiotics similar to nigericin,

inhibited some RNA viruses by blocking the viral glycoproteins on the surface of the infected cells *(32)*.

The present study supports the concept that naturally occurring products and specifically microbial metabolites in addition to known chemical compounds, can be used as alternative antiviral agents.

Acknowledgments

The present work has been partially supported by an EC Copernicus grant CIPA-CT 0202/95 and a research grant K-429/94 from The National Science Fund, Bulgaria.

References

1. Miteva, V., Ivanova, I., Budakov, I., et al. (1998), *J. Appl. Microbiol*. **85,** 603–614.
2. Dolin, R., Reichman, R. C., Madora, H. P., Maynard, R., and Linton, P. N. (1982), *New Engl. J. Med*. **307,** 580–584.
3. Van Voris, L. P., Betts, R. F., Hayden, F. G., Christmas, W. A., and Douglas, Jr., R. G. (1981), *J. Am. Med. Assoc*. **245**, 1128–1131.
4. Herrmann, J. E., West, K., Bruns, M., and Ennis F. A. (1990), *J. Infect. Dis*. **161,** 180–184.
5. Hayden, F. G. and Couch, R. B. (1992), *Rev. Med. Virol.* **2,** 89–96.
6. Hayden, F. G. (1997), *J. Infect. Dis*. **176,** S56–S61.
7. Aizenman, B. Ju. and Milchenko, K. P. (1973), *Microbi1ologicheski J.* (Kiev), **35,** 660–672 (in Ukrainian).
8. Demain, A. L. (1992), *CIBA Foundation Sym.* **171,** 3–23.
9. Portfield, J. S. (1960), *Bull. WHO* **22,** 373–380.
10. Tagg, J. R. and McGiven, A. R. (1971), *Appl. Microbiol*. **21,** 943–948.
11. Lowry, O. H., Rosenbough, N. J., and Farr, A. L. (1951), *J. Biol. Chem.* **193,** 265–272.
12. Reed, L. J. and Muench, H. (1938), *Am. J. Hyg*. **27,** 493–498.
13. Vanden Berghe, D. A., Vlietinck, A. J., and Van Hoof, L. (1986), *Bull. Inst. Pasteur* **84,** 101–147.
14. Serkedjieva, J. and Hay A. J. (1998), *Antiviral Res*. **37,** 221–230.
15. Belshe, R. B., Smith, M. H., Hall, C. B., Betts, R., and Hay, A. J. (1988), *J. Virol*. **62,** 1508–1512.
16. Wachsman, M. B., Damonte, E. B., Coto, C. E., and de Torres, R. A. (1987), *Antiviral Res.* **8,** 1–13.
17. Jack, R. W., Tagg, J. R., and Ray, B. (1995), *Microbiol. Rev.* **59,** 171–200.
18. Bickel, H. (1961), *Arch. Hyg. Bacteriol*. **145,** 475–478.
19. Kimura. T., Yoshimizu, M., Ezura, Y.. and Kamei, Y. (1990), *Aquat. Animal Health* **2,** 12–20.
20. Ahmad, A., Davies, J., Randall, S., and Skinner, G. R. B. (1996), *Antiviral Res.* **30,** 75–87.
21. Tomlinson, J. A., Walker, V. M., Flewett, T. H., and Barkley, G. R. (1974), *J. Gen. Virol*. **22,** 225–232.
22. Schoen, P., Corver, J., Meijer, D. K. F., and Swart, P. J. (1997), *Biochem. Pharmacol*. **53,** 995–1003.
23. Tomas, E. and Samuel, I. (1980), *Rev. Roum. Med. Virol.* **31,** 131–138.
24. Judd, A. K., Sanchez, A, Bucher, D. J., Huffman, J. H., Bailey, K., and Sidwell, R. W. (1997), *Antimicrob. Ag. Chemother.* **41,** 687–692.
25. Wlieklik, M., Panasiak, W., Luczak, M., and Konopinska, D. (1988), *Acta Virologica* **32,** 79–82.
26. Yokota, Y., Watanabe, Y., Wakai, Y., and Mine Y. (1992), *J. Antibiotics* **45,** 258–265.

27. Watanabe, K., Kawasaki, T., Sako, N., and Funatsu G. (1997), *Biosci. Biotechnol. Biochem.* **61,** 994–997.
28. Piard, J. and Desmazeaud M. (1992), *Lait* **72,** 113–142.
29. Nes, I., Diep, D., Havarstein, L., Bruberg, M., Eijsink, V., and Holo H. (1996), *Antonie van Leuwenhoek* **70,** 113–128.
30. Kordel, M., Schuller, F., and Sahl, H.G. (1989), *FEBS Lett.* **244,** 99–102.
31. Dundarov, S. and Andonov, P. (1989), *Medico-Biological Info.* **4,** 3–27.
32. Johnson, D. and Schlesinger, M. (1980), *Virology* **103,** 407–424.