

***In vitro* and *in vivo* Influenza Virus Inhibitory Effects of a Bacterial Protease Inhibitor**

Julia Serkedjieva* and Iskra Ivanova¹

Institute of Microbiology, Bulgarian Academy of Sciences, 26, Academician Georgy Bonchev St., 1113 Sofia, ¹Department of Microbiology, Sofia University, Sofia, Bulgaria.

(Received: 12 June 2009; accepted: 22 July 2009)

The fermentation product, produced by *Streptomyces* sp. 225b (SS 225) selectively inhibited the reproduction of influenza virus A/chicken/Germany/34, strain Rostock (H7N1) in MDCK cells shown by the reduction of viral hemagglutinin (HA) expression on the surface of infected cells, virus-induced cytopathogenic effect, the production of HA and infectious virus. The inhibitory effect was dose-dependent and affected viral penetration and some intracellular stages of viral replication. No inactivating effect of the extracellular virus was found. Using representative strains of influenza virus it was shown that the inhibitory effect was strain-specific; most sensitive to inhibition were A/Rostock and A/PR8/34 (H1N1). In addition the preparation protected mice from mortality in the experimental A/Aichi/2/68 (H3N2) (A/Aichi) influenza virus infection; the mean survival time was prolonged the lung infectious virus titres and the lung consolidation were reduced in comparison with virus control.

SS 225 belongs to the group of serine proteinase inhibitors. It has been subjected to purification by ion exchange chromatography, gel filtration and HPLC analysis. It was a hydrophobic and a thermostable protein, had a molecular mass of 11.1 kDa and a high content of hydrophobic amino acids.

Keywords: Influenza virus, Viral inhibition, Protease inhibitor, *Streptomyces*.

Influenza is a highly contagious, febrile, acute infection of the nose, throat, bronchial tubes, and lungs caused by influenza virus. The infection is usually self-limiting, culminating in a local and systemic reaction. However, there remains a significant proportion of patients, who develop severe illness and complications, such as the

elderly, the very young and the immunocompromised; hence this infection represents one of the serious problems of human health. Even with the development of killed virus vaccines the need for effective anti-influenza therapies still exists. WHO has recently decreed that "another influenza pandemic is inevitable and possibly imminent", an alarming prospect, considering the devastating previous pandemics. Indeed the emergence of a new strain of influenza A H1N1 (swine flu) has led to the first influenza pandemic since 1968. The first step in a potential pandemic is the ability of the virus to enter human cells which is mediated by the viral surface glycoprotein hemagglutinin (HA). The ability of the hemagglutinin protein (HA) of influenza viruses to mediate fusion between viral and

* To whom all correspondence should be addressed.
Tel: (359 2) 979 31 85, Fax: (359 2) 70 01 09
E-mail: jserkedjieva@microbio.bas.bg

endosomal membranes during virus entry into the cell depends on cleavage of fusion-incompetent precursor HA0 into disulfide-linked subunits HA1 and HA2 by a host endoprotease. Cleavage of HA is essential for infection, determines viral pathogenicity and tissue tropism and is necessary for the virus to establish infection in the host as well as to spread within the host. The host enzymes responsible for this cleavage event are believed to correspond with the pathogenicity of the virus and are determined based on the cleavage site sequence¹⁻³. The sequence of the cleavage site which is recognized by host proteases has been linked with pathogenesis of various influenza viruses. The majority of HA subtypes possess a single arginine at their cleavage site which facilitates cleavage by trypsin, a protease mainly localized to the respiratory tract in humans and the gastrointestinal tract in birds. Since protease activities predominate over those of endogenous inhibitory compounds under normal airway conditions, administration of protease inhibitors in the early stage of infection significantly suppresses viral entry and viral multiplication. Proteinase inhibitors may normalize proteolytic balance to limit inflammatory process and development of a syndrome of haemorrhage and coagulation defects and that they may suppress proteolytic activation of virions to reduce virus multiplication and spread.

Proteinase inhibitors are widely distributed in animal, plants and bacteria. They are of a great importance in medicine, agriculture and biotechnology⁴. Proteinase inhibitors are frequently found in *Streptomyces* spp. and are classified as members of the *Streptomyces* subtilisin inhibitor (SSI) family on the basis of their similar structures and protease inhibitory specificities^{5,6}.

As a part of a project on the biological activities of *Streptomyces* strains 100 strains were screened for the production of proteolytic inhibitors. The most active producers were investigated further for antiviral activity. We have examined the virus-inhibitory activity of the most effective fermentation product, produced by *Streptomyces* sp. 225b (SS 225) with respect to the specificity and selectivity of inhibition⁷. Here we present a further detailed characterization of the anti-influenza virus effect together with some

properties of the investigated inhibitor.

MATERIAL AND METHODS

Microbial strain: *Streptomyces* sp. 225 was isolated from a soil sample from Guinea. *Streptomyces* sp. 225b is a morphological variant, producing a blue pigment. In the antiviral experiments partially purified fermentation extract was used (SS 225). The dilutions were made in cell culture medium *ex tempore*.

Media and cultivation conditions

1. Agar maintenance media: mineral medium 1 vs Gause, potato-glucose agar, malt medium for *Streptomyces* (ISP-2), Benet agar. 2. Cultivation media: medium for SSI, optimal for the production of proteinase inhibitors. The cultivation consisted in a two-phase deep fermentation in 500 ml Erlenmeyer flasks at 220 rpm, 28 °C. After incubation for 48 h the fermentation flasks were inoculated with 10% (v/v) of the start culture and were cultivated for 120 h at the same conditions.

Partial purification of the fermentation product

This was performed according to Hiraga *et al.*, 2000⁸. After centrifugation, 40 min, 12 000 rpm/min at 4 °C, the supernatants were precipitated with 80% (NH₄)₂SO₄ for two days at 4 °C, centrifuged for 40 min at 4 °C, 12 000 rpm. The precipitates were re-suspended in 20 mM Tris HCl (pH 7.0) and dialyzed against the same buffer for two days. Samples for analysis of the proteinase inhibitory activity were taken after each step of the treatment.

Protein determination

The protein content was determined according to Bradford, 1976⁹, using the Bio-Rad assay reagent (Bio-Rad, Munich, Germany) and bovine serum albumin as the standard. It was expressed as mg/ml.

Amino acid analysis was performed according to Bidlingmeyer *et al.*, 1984¹⁰ and the results are provided by Ms. Michelle Dalgalarondo, Institut National de la Recherche Agronomique-BIA-FIPL, Nantes, France.

Preparation of lung tissue homogenates

On days 2 and 6 post infection (p.i.) 3 mice of each group were anaesthetized with ether and exsanguinated by section of the subclavian arteries. Lungs were removed aseptically, washed

in cold PBS, blotted dry; tissue pieces of about 1 g were disintegrated mechanically in ice-cold PBS and subsequently by an ultrasound disintegrator (MSE, England) for 3 min (interrupting sonication every 15 sec). The homogenates were centrifuged (8 000 rpm, 30 min, 4°C) and the supernatants were examined for protease and protease-inhibitory activities.

Protease activity in the lungs used the method, utilizing BAPNA as the substrate. One unit of proteolytic activity (U) was defined as the amount of the enzyme, producing one μmol *p*-nitroanilide for 1 min at 37°C. The specific proteolytic activity was evaluated (U/mg protein).

Protease inhibitory activity in the lungs was determined as described above. The specific protease-inhibitory activity was evaluated (TIU/mg protein).

Ion exchange filtration and affinity chromatography were as in Angelova *et al.*, 2006⁷.

Polyacrylamide gel electrophoresis (PAGE). A standard 16% acrylamide SDS-PAGE was performed for molecular mass determination.

Virology

Compounds

Ribavirin, *e*-amino caproic acid (ACA), trypsin and N-benzyloxycarbonyl-L-arginine-*p*-nitroanilide (BAPNA), were purchased from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany.

Cells and Media

Madin-Darby canine kidney (MDCK) cells were passaged in Dulbecco's Eagle medium (GibcoBRL, Scotland, UK), supplemented with 5% fetal calf serum (FCS) (BioWhittaker Europe, Germany) and antibiotics (100 IU/ml benzylpenicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin). MDCK were cultivated at 37°C in the presence of 5% CO_2 till the formation of confluent monolayers. In the antiviral experiments 0.5% FCS was added. MDCK cells were supplied by Dr. I. Roeva (Institute of Microbiology, Bulgarian Academy of Sciences). A/Aichi was cultivated after pre-treatment with trypsin.

Viruses

Avian influenza viruses A/chicken/Germany/34, strain Rostock (H7N1) (A/Rostock) and A/chicken/Germany/27, strain Weybridge (H7N7) (A/Weybridge) and human influenza viruses - A/PR/8/34 (H1N1) (A/PR8), A/PR/8/34, rimantadine resistant (H1N1-R), A/Aichi/2/68

(H3N2) (A/Aichi) and B/Lee/40 were adapted to MDCK cells. In the animal experiments A/Aichi, adapted to mice lungs was used (A/Aichi-a). The virus infectious titres were over the range of 10^6 - 10^7 TCID₅₀/ml (50% tissue culture infectious doses/ml), the hemagglutination (HA) titres - 1024-2048. The virus stocks were stored at -70°C. The strains are from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia.

Mice

Inbred ICR mice (16-18 g) were obtained from the Experimental Animal Station of the BAS in Slivnitsa, Sofia. The experimental groups were of 10 - 12 animals each. At the end of the experiments, surviving mice were sacrificed by cervical dislocation. They were quarantined 24 h prior to use and maintained on standard laboratory chow and tap water *ad libitum*. The number of experimental animals was reduced as much as possible, depending on statistical significance. Refinement of the tests with animals was achieved by careful planning of multifactor experiments. The animals were bred under standard conditions, accepted by the Bulgarian Veterinary Health Service.

Cellular toxicity

Confluent MDCK cell monolayers in 96-well plastic plates were overlaid with four fold dilutions of the samples in growth medium and were observed microscopically for changes in cell morphology and viability at 24, 48, 72 h of incubation. The cytopathic effect (CPE) was scored under an inverted microscope (score 0=0% CPE, score 1=0-25% CPE, score 2=25-50% CPE, score 3=50-75% CPE, score 4=75-100% CPE). The dilution, reducing CPE by 50% (TC₅₀) with respect to cell control was estimated from graphic plots.

Virucidal activity

Stock virus suspension was treated with equal volume of 1:2 dilution of SS 225 in phosphate buffered saline (PBS) for 15 - 60 min at the room temperature. The differences in the biological activities of treated and control viruses were determined on the basis of infectivity and HA- production.

Antiviral assays

The antiviral effect was studied in multicycle and single cycle experiments of viral

growth and the following antiviral assays were used. 1. *Cytopathogenic effect (CPE) reduction assay* was as in Serkedjieva and Hay, 1998¹¹. In short - quadruplicate confluent monolayers in 96-well plates were overlaid with 2x drug-containing medium (0.1 ml) and an equal volume of virus suspension (100 TCID₅₀/ml). The virus-induced CPE was scored after 72 h as described above. The dilution, reducing CPE by 50% (EC₅₀) with respect to virus control was estimated. The selectivity index (SI) was determined by the ratio TC₅₀/EC₅₀. SI ³ 4 was considered to stand for a significant selective inhibition. 2. *50% end point titration technique (EPTT)* was as in Vanden Berghe *et al.*, 1986¹². The antiviral activity was determined by the difference between the virus titres of control and treated viruses (d log₁₀ TCID₅₀/ml). The selective anti-influenza drug rimantadine hydrochloride was used as a positive control. 3. *Time of addition study*. Confluent monolayers in 96-well plates were incubated with plain or drug-containing medium for 1 h at 37°C (effect of cell pre-treatment), washed twice with PBS and challenged with serial ten-fold dilutions of infectious virus. Adsorption was carried out for 1 h at 4°C 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 penetration into cells (effect on penetration). Then plain or drug-containing medium was added and cells were cultivated for 48 - 72 h at 37°C (effect on replication). At the end of cultivation the CPE was scored as described above and EC₅₀-s were evaluated. 4. Enzyme-linked immunosorbent assay (ELISA) was carried out according to Belshe *et al.*, 1988¹³. The monoclonal antibody (MAb) to viral hemagglutinin (HA) HC58 was kindly provided by Dr. A.J. Hay, the WHO Collaborative Centre of Influenza, Mill Hill, London, UK.

Virus infection in mice

To produce lethal infection, mice were infected nasally with 5-10 LD₅₀ of A/Aichi-a in the volume of 0.05 ml physiological saline (PBS) per mouse under light ether anesthesia.

Viral infection

The infection was induced under light ether anesthesia by intranasal (i.n.) inoculation of A/Aichi-a. To induce lethal infection, mice were

challenged with 10 LD₅₀ (50% lethal doses), corresponding to 10³ TCID₅₀/ml, in the volume of 0.05 ml phosphate buffered saline (PBS) per mice.

Experimental design

Mice were treated orally 24 and 2 h before and 24, 48 and 72 hours after viral exposure with SS 225 (0.14 mg/mouse/day) in 0.2 ml PBS and were observed for death daily for 14 days. The known proteolytic inhibitor ACA and the antiviral drug ribavirin were used for comparative reasons and were inoculated according to the same treatment schedule in the daily doses of 1 mg/mouse/day and 0.125 mg/mouse/day respectively. The protective effect was evaluated by the increase of the rate of survival (%) and mean survival time (MST). The protective index (PI) was determined from the equation $(PR - 1)/PR \times 100$, where PR (protective ratio) is Mcontrol/Mexperiment and M is mortality and MST as described in Serkedjieva and Ivanova, 1997¹⁴.

To determine the lung parameters 3 animals from each experimental group were sacrificed on day 6 after infection, lungs were removed aseptically, weighed and lung consolidation (score) was scored as described in Serkedjieva and Ivanova, 1997¹⁴. Lungs were homogenized to a 10% suspension in PBS and ten-fold dilutions (0.2 ml) were assayed for infectivity in MDCK cells in the presence of 2 µg/ml trypsin (Sigma). Virus-induced CPE was scored as described above; infectious virus titres were evaluated according to Reed and Muench, 1938¹⁵ and expressed in log₁₀ TCID₅₀/ml.

The results are the mean values from 3 - 4 independent experiments. Increase of survival rates compared to placebo controls were evaluated using Fisher's exact test. Student's *t* - test was employed to analyze differences in survival times and infectious titres. *p<0.05

RESULTS AND DISCUSSION

Current anti-influenza drugs (M2 ion channel blockers and neuraminidase inhibitors) target viral components. Recently, cellular proteins are emerging as potential targets for new anti-viral drugs. The principal idea is to affect the mechanisms underlying virus-cell interactions and favoring viral replication in virus-infected

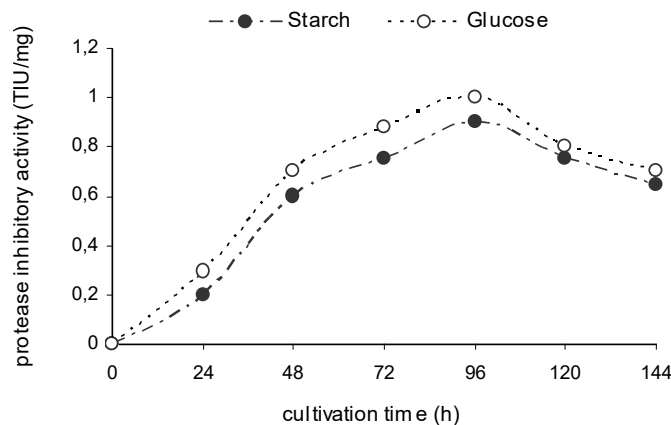


Fig. 1. Dynamics of the proteinase inhibitory activity during the submerged cultivation of *Streptomyces sp.* 225b with glucose and starch as carbon sources

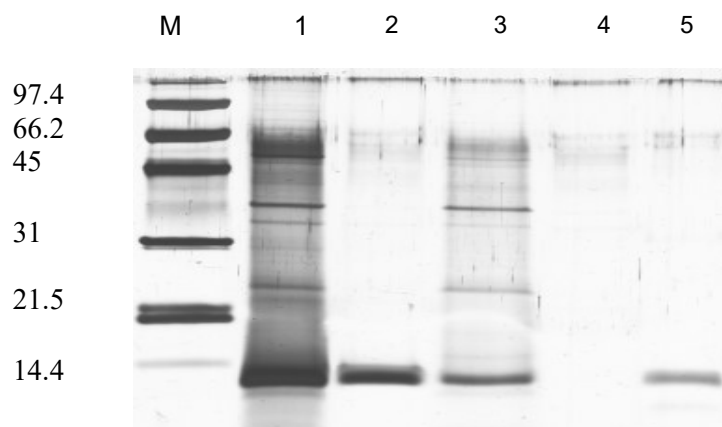


Fig. 2. SDS PAGE electrophoresis of the purification stages of SS 225. M, molecular markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa, soybean trypsin inhibitor, 21.5 kDa and lysozyme, 14.4 kDa); 1, culture supernatant; 2, ion exchange filtration, unbound fraction ; 3, ion exchange filtration, bound fraction; 4, affinity chromatography, bound fraction; 5, affinity chromatography, unbound fraction

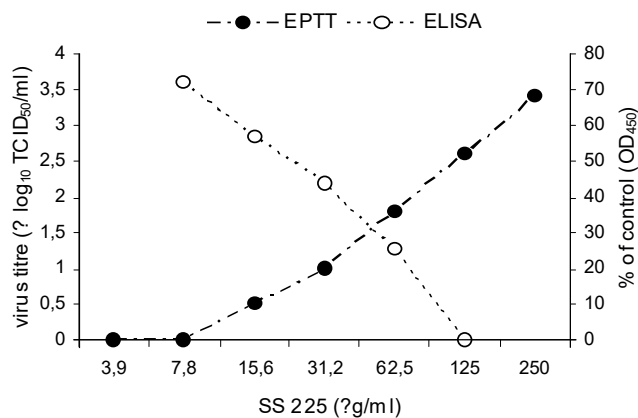


Fig. 3. Dose-dependence of the inhibitory effect of SS 225 on the replication of A/Rostock in MDCK cells (EPTT assay) and on the expression of viral HA on the surface of infected cells (ELISA assay)

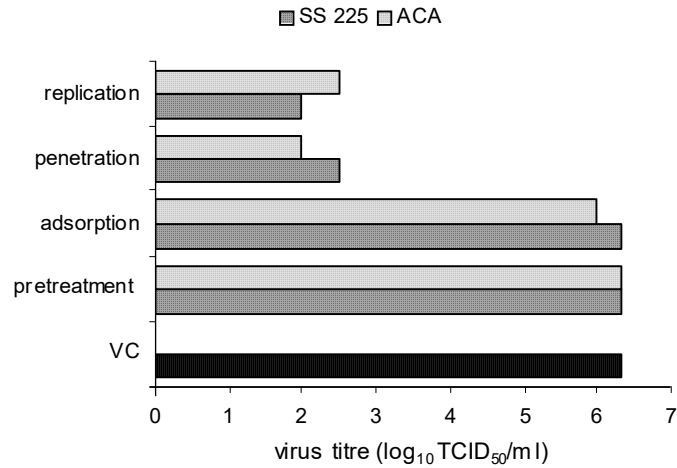


Fig. 4. Inhibitory effect of SS 225 (125 µg/ml) on the replication of A/Rostock in MDCK cells in a time-of-addition study (IVY assay)

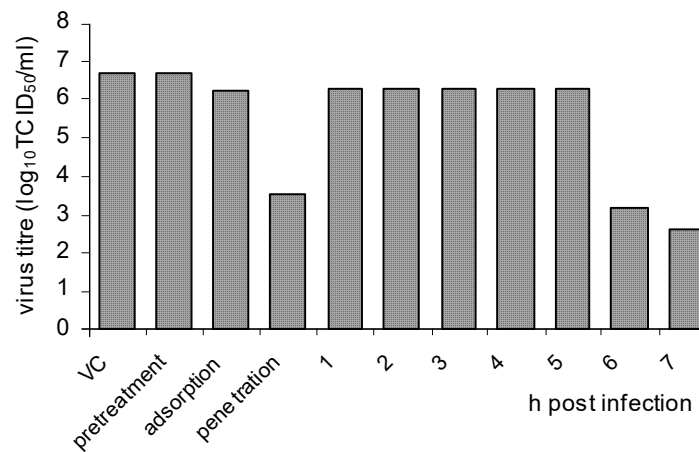


Fig. 5. Inhibitory effect of SS 225 (250 µg/ml) on a single cycle of A/Rostock replication in MDCK cells (single cycle of viral replication)

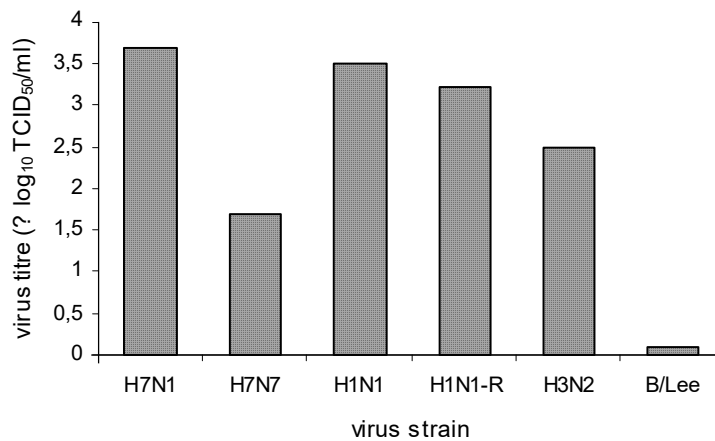


Fig. 6. Susceptibility of various influenza viruses to the inhibitory effect of SS 225 (125.0 µg/ml)

cells. This kind of strategy instigates interest as it could eliminate the selection of viral resistance and would be suitable for different influenza viruses independently on the viral type and strain. In the case of influenza viruses, the virulence of a particular influenza virus strain depends on the ability of its haemagglutinin precursor HA0 to be cleaved post translation to subunits HA1 and HA2 by trypsin-like proteases of the host¹⁶⁻¹⁹. Cleavage of influenza virus HA is essential to infectivity,

Table 1. Amino acid composition of the protease inhibitor SS 225*.

Amino acids	Residues (%) SS 225b
Asx	3.7
Glx	6.9
Ser	7.1
Gly	8.0
His	2.3
Arg	6.2
Thr	8.5
Ala	21.3
Pro	6.9
Tyr	2.7
Val	10.3
Met	0.8
Ile	2.0
Leu	0.7
Phe	7.6
Lys	3.0

*the results are provided by Ms. Michelle Dalgarrondo, Institut National de la Recherche Agronomique-BIA-FIPL, Nantes, France and are partially published in Angelova *et al.*, 2006

Table 2. Comparative sensitivity of A/Rostock to inhibition by SS 225

Assay	EC ₅₀ (µg/ml)
Contact assay ^a	> 1 200
ELISA	24.5
CPE reduction	16.8
EPTT	15.6
IVY reduction ^b	31.2

a MIC, minimal inhibitory concentration

b EC₉₀, 90% effective concentration

allowing fusion of viral and hosts cell membranes prior to the release of nucleocapsid into the cytoplasm. The mammalian and the non-pathogenic avian influenza virus strains have HAs with monobasic cleavage site and are usually cleaved only in a restricted number of cell types so that these viruses cause local infection. In general the glycoproteins are activated by secreted proteases like serum plasmin, kallikrein, urokinase, thrombin, acrosin, tryptase Clara and mini-plasmin in rat lungs, mast cell tryptase and tryptase TC30, found in porcine lungs (¹⁸ and references cited in). Cleavage activation of influenza monobasic HAs by host proteases is generally thought to occur extracellularly on the surface and/or in the lumen of the respiratory tract. The induction of influenza virus infectivity by host cell proteases is strictly regulated by inhibitors of the serine proteases such as human mucus protease inhibitor (MPI) in the upper respiratory tract²⁰ and pulmonary surfactant²¹ in the lower respiratory tract.

Taking into account the important role of proteolytic processing for viral reproduction, one of the possible targets for chemotherapy of influenza virus infection is the blocking of proteolytic cleavage of viral proteins; it is believed that this would result in inhibition of subsequent rounds of viral replication and spread in the respiratory tract. For influenza viruses with monobasic HAs, exogenous inhibitors of serine proteases, including ACA¹⁷, aprotonin²² and ambroxol²³ have been shown to reduce HA-cleavage and virus activation in cultured cells, in chick embryos and in the lungs of infected mice.

From another side the proteinase inhibitors are widely distributed in the plant and animal kingdoms as well as in bacteria and some viruses. They take part in the processes of control and regulation of cell metabolism by activation and deactivation of cell proteases. The bacteria of the genus *Streptomyces* also produce proteinase inhibitors with interesting properties, belonging in large part to the family of streptomyces subtilisin inhibitors (SSI). Proteinase inhibitors of microbial origin could be used successfully as antiviral agents. There is experimental evidence presented by Lozitski *et al.*, 2002²⁴ that the replication of influenza virus was efficiently inhibited by a proteinase inhibitor MI 0114,

produced by *Streptomyces* sp. Our group has found antiviral activity for a proteinaceous protease inhibitor, produced by *Streptomyces chromofuscus* 34-1²⁵.

In the light of these findings we investigated the potential virus-inhibitory effect of proteolytic inhibitors from *Streptomyces*. As a first approach an extensive screening study for producers of proteinase inhibitors has been carried out on 75 strains. 18 of them and/or their variants (24%) produced inhibitors of proteinaceous nature⁷. 15 strains did not show any inhibitory effect. The specific inhibitory activity of the remaining 60 (80%) strains varied from 62.7 to 1 280.0 KIU/mg protein. It should be noted that nearly half of the strains produced the maximum of proteinase inhibitors at 96th hour of cultivation. Further the most active 18 strains and their variants, 23 samples in total, were characterized with respect to their protein content, specific activity towards trypsin, cell-toxicity and virus-inhibitory effects.

The results from Tricin-SDS-PAGE analysis of the most promising preparations (SIA>500 KIU/mg) indicated the presence of proteins with molecular mass between 6.5 – 14.5 kDa, which is characteristic of the proteinaceous proteolytic inhibitors.

The most active protease inhibitors were produced by *Streptomyces* sp. 225b (SS 225, SIA 653.9 KIU/mg) and *Streptomyces chromofuscus*

34-1 (SS 34-1, SIA 625.0 KIU/mg)⁷. The production of the proteinase inhibitors reached its maximum values at 72 - 96 h of cultivation and was closely related to mycelial growth. The dynamics the proteinase inhibitory activity during the submerged cultivation of *Streptomyces* sp. with glucose and starch as carbon sources as a carbon source followed the same pattern (Fig. 1).

The chemical nature of SS 225 was further studied. The protein was subjected to purification by ion exchange chromatography, gel filtration and HPLC analysis. The major unbound fraction of SS 225 consisted of two proteins, representing variants of one and the same protein with Mw 11.1 and 10.9 kDa (Fig. 2). The protein was hydrophobic and thermostable. The amino acid analysis showed that the protein was very rich in Ala (21%) and Val (10%) residues and very poor in Ile (Table 1).

SS 225 was classified as members of the *Streptomyces* subtilisin inhibitor (SSI) family on the basis of its properties, the similarity of their structures and protease inhibitory specificities. SSI share several common physico-chemical properties: they have small molecular masses and are stable at low pH and high temperatures^{5,6}.

Virology

Cellular toxicity

Firstly the toxicity of SS 225 to MDCK cells was tested. This was necessary for evaluating the specificity of the virus-inhibitory effect.

Table 3. Protective effect of SS 225 in EIVI in mice

Treatment group	Protection index* (%)	MST ^b	Lung parameters (6 days p.i.)					
			Weight (g)	Lung index ^c (%)	Score ^d	Virus titre (log ₁₀ TCID ₅₀ /ml)	Lung protease activity (U/mg)	Lung protease inhibitory activity (TIU/lmg)
Virus control		6.9	0.25	1.18	3.5	6.7	200.0	320.0
SS 225 ^s	64.9	12.4	0.21	0.96	0	1.5	16.0	128.0
Ribavirin ^{&}	62.4	12.7	0.24	1.02	0.5	1.0	14.0	124.0
ACA [*]	56.1	11.2	0.21	0.95	2.3	3.1	21.0	146.0
Healthy control			0.21	1.0			10.0	120.0

a PR=1/PR x 100, where PR protective ratio is $M_{\text{control}}/M_{\text{experiment}}$ and M is mortality. Mortality in the VC group is 85.2%; b mean survival time; c lung weight/body weight x 100; d scores 0-4, assigned to % visible consolidation; ^s SS 225, cell-free filtrate from *Streptomyces* sp. 225b, 5x7 mg/kg, p.o.; & Ribavirin, 5x10 mg/kg, p.o.; * ACA, e-amino caproic acid, 5x50 mg/kg, p.o.; \$, &, * administered 24, 2 h before and 24, 48, 72 h after viral infection

Confluent MDCK monolayers treated for 72 h with SS 225 at concentrations of 10-1 400 µg/ml did not show any visible changes in cell morphology or cell density; thus TC_{50} exceeded 1 400 µg/ml. It is important to note that the cell-toxic effect was cell-specific, CEF (chicken embryonic fibroblast) cells were more sensitive than MDCK and MDBK (Madin-Darby canine kidney) cells⁷.

Virucidal activity

To investigate the direct inactivating effect of SS 225, A/Rostock was treated for 1 h at room temperature with concentrations of SS 225 ranging from 10 to 1 200 µg/ml. In doses up to 1 200 µg/ml the preparation did not exhibit any direct inactivating effect on A/Rostock, no detectable reduction of viral biological activity (infectivity and HA-production) was found.

Effect of SS 225 on virus replication

In all experiments non drug treated, mock infected cells were used as cell control and non drug treated, virus infected cells as virus control. Rimantadine hydrochloride (0.1-1 µg/ml) was used as a positive control for inhibition of virus replication.

The *in vitro* anti-influenza virus effect of SS 225 was studied in a number of assays of the replication of A/Rostock in MDCK. The results are presented in Table 2. The expression of HA on the surface of infected cells, determined by an ELISA with anti-HA MAb, was inhibited by SS 225. In experiments employing multiple cycles of virus replication the respective EC_{50} -s were 24.5 µg/ml. SS 225 inhibited the virus-induced CPE of A/Rostock in MDCK with an EC_{50} of 16.8 µg/ml. Similar result was obtained applying the EPTT assay (EC_{50} = 15.6 µg/ml). In an IVY reduction assay, the concentration of SS 225 which partially inhibited HA production and reduced the infectious titre by 1 \log_{10} $TCID_{50}$ /ml (EC_{90}) was 31.2 µg/ml; the reduction of infectivity increased up to 3.5 \log_{10} at concentration of approx 250 µg/ml (Fig. 3).

The experiments on the dose-effect relationship were performed by the EPTT and the EISA assay; the results are presented in Fig. 3. Both assays revealed a dose-dependence of the virus-inhibitory effect.

We have tested 10 samples - crude and partially purified fermentation extracts as well

as extracts from different fermentations for inhibitory activity on A/Rostock virus replication²⁶. Although in general the partial purification of the crude fermentation extracts lead to an increase of the protease-inhibitory activity, the virus-inhibitory effect was diminished.

Time of addition studies

To investigate the effect of SS 225 on different steps of viral replication the preparation (in a dose 125 µg/ml) was added at varying times relative to viral infection. The experiments were performed by the end point titration technique and the results are presented in Fig. 4.

The pre-treatment of cells as well as the addition at the time of adsorption did not result in decrease of virus infectivity. A slight inhibition observed when the adsorption was performed at 37°C, allowing internalisation of viral particles, was not significant. Virus replication was significantly reduced when SS 225 was added at the time of penetration, $\delta \log_{10} TCID_{50}$ /ml = 3.6, and markedly inhibited when the preparation was inoculated after virus infection, $\delta \log_{10} TCID_{50}$ /ml = 4.5. The results were comparable with the effect of ACA. These observations were confirmed in experiments, following a single cycle of virus replication (Fig. 5). Pre-treatment of cells with the preparation for 1 h and its presence during adsorption did not reduce virus production.

Susceptibility of different influenza viruses to SS 225

The sensitivities of representative influenza viruses to the inhibitory action of SS 225 was assessed by the reduction of the virus-induced CPE after multiple cycles of virus replication. The concentration dependence of the inhibitory effect of SS 225 was strain-dependent (EC_{50} = 16.8-125.0 mg/ml; SI = 11.3-83.3). Most sensitive to inhibition by SS 225 was the replication of A/Rostock in MDCK cells (EC_{50} = 16.8 mg/ml; SI = 83.3) and least sensitive was that of B/Lee. The rimantadine-resistant mutant A/PR8-R was as susceptible to the virus-inhibitory effect of SS 225 as that of the parent virus.

The obtained results show that the fermentation extract do not inactivate influenza virus A/Rostock but is able to interfere with the penetration of viral particles into susceptible cells as well as with the late stages of viral replication

(post-translational processing of viral lipoproteins, assembly and release of newly formed viral particles from infected cells). Both the internalization and release of influenza virus particles depend on the post-translational cleavage of the haemagglutinin precursor (HA0) to subunits HA1 and HA2²⁷. Thus, the antiviral effect of SS 225 is presumably due to inhibition of this cleavage through inhibition of the host cells serine type proteases. Production of HA was also reduced significantly (results not shown). Further studies concerning the mode of its antiviral effect are in progress and are carried out with a purified preparation of the inhibitor.

In connection with the envisaged experiments *in vivo* (the experimental infection is induced by virus A/Aichi), it was important to overcome the difficulty, caused by the necessity to grow A/Aichi in MDCK cells only in the presence of trypsin; thus the inhibitory effect of SS 225 could be diminished. A separate experiment was set up in order to verify this possibility. A/Aichi was pre-treated for 1 h with trypsin (1 mg/ml), the effect of trypsin was stopped by the addition of lime bean inhibitor (1 mg/ml) (A/Aichi-pr)²⁸. A/Aichi-pr was cultivated in a medium without trypsin containing a purified preparation of SS 225. The inhibitory effect of SS 225 was tested in parallel on A/Aichi (cultivated in a medium supplemented with 2 µg/ml trypsin) and A/Aichi-pr. The virus-inhibitory effect towards A/Aichi-pr was stronger: EC₅₀-s were 110.0 and 240.0 µg/ml respectively. Thus the presence of trypsin in the culture medium reduced the antiviral effect of SS 225 and the experiments were performed with A/Aichi-pr).

Protective effect of SS 225 in the murine EIVI

Further we investigated the protective effect of SS 225 in the murine model of experimental influenza infection, induced by A/Aichi virus. The intranasal inoculation of the virus to mice produces a damaging infection of the lungs, causing viral pneumonitis. PBS was used for a placebo control and ribavirin (5x10 mg/kg) - as a positive control. For comparative reasons the known proteolytic inhibitor ACA¹⁷ was used as well in the dosage 5x50 mg/kg. ACA, an analog of lisin, inhibits plasmin activity *in vivo* by competing for the interaction of fibrin, plasminogen and plasminogen activator. In any

case this inhibitor interferes with the replication of viruses that are activated by secreted proteases. Applied according to a prophylactic-therapeutic schedule, SS 225 protected mice from mortality; this protection reached 64.9%. The preparation was not toxic to the experimental animals and the protection was comparable to that shown by ribavirin (PI = 62.4%) and surpassed that, exhibited by ACA (PI = 56.1%), both inoculated according to the same treatment schedule (Table 3). Both the rate of survival (80%) and MST (+5.5 days) were increased; the effect was dose-related. The mice receiving SS 225 treatment showed minimal pathological lesions in the lungs whereas control untreated animals had total hemorrhagic pneumonia. Lung parameters (infectious virus titres, consolidation and weights) were all significantly reduced in the SS 225-treated mice (Table 3).

The lung protease and protease-inhibitory activities were estimated on day 6 p.i. Influenza virus infection triggered a marked increase of both activities at 6 day p.i. SS 225-treatment brought both activities to normal levels. The restoration of the examined parameters was consistent with amelioration of lung injury (Table 3).

Clarifying the anti-influenza virus effects of the protease inhibitor SS 225 might open the way to utilize this substance in the control of influenza infection. Equally the information, obtained from our investigation may facilitate the studies on the development of novel antiviral agents of bacterial origin.

In conclusion the virus-inhibitory effect of a fermentation product, produced by *Streptomyces* sp. 225b was found to be specific and selective, strain-related and dose-dependent. The growth of representative influenza viruses was repressed considerably; virus-induced cytopathogenic effect, the production of hemagglutinin and infectious virus were all reduced significantly. By time of addition studies it was established that SS 225 affects adsorption and some intracellular stages of the replication of influenza virus A/Rostock in MDCK cells. In addition the preparation protected mice from mortality in the experimental influenza virus infection; the mean survival time was prolonged the lung infectious virus titres and the lung

consolidation were reduced in comparison with virus control. The present results are in accordance with the findings that proteinase inhibitors of microbial origin could be used as antiviral agents. The replication of human immunodeficiency virus type 1 (for review see De Clercq, 2001²⁹), cytomegalovirus³⁰ and influenza virus²⁴ were all efficiently inhibited by proteinase inhibitors, produced by *Streptomyces* species.

ACKNOWLEDGMENTS

The authors acknowledge the skillful technical assistance of Mrs. K. Todorova, Institute of Microbiology, Bulgarian Academy of Sciences. This study was supported by research grants L-1518 and D002-0188 from the National Science Council, Bulgaria.

REFERENCES

- Chen, J., Lee, K.H., Steinhauer, D.A., Stevens, D.J., Skehel, J.J., Wiley, D.C. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell*, 1998; **95**: 409-17.
- Russell, C.J., Webster, R.G. The genesis of a pandemic influenza virus. *Cell*, 2005; **123**: 368-71.
- Webster, R.G., Rott, R. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. *Cell*, 1987; **50**: 665-6.
- Rawlings, N.D., Tolle, D.P., Barrett, A.J. Review Article: Evolutionary families of peptidase inhibitors. *Biochem. J.*, 2004; **378**: 705-16.
- Laskowski, M., Kato, I. Protein inhibitors of proteinases. *Annu. Rev. Biochem.* 1980; **49**: 593-627.
- Taguchi, S., Kikuchi, H., Suzuki, M., Kojima, S., Terabe, M., Miura, K-I., Nakase, T., Momose, H. *Streptomyces* subtilisin inhibitor-like proteins are distributed widely in *Streptomyces*. *Appl. Environ. Microbiol.* 1993; **59**: 4338-41.
- Angelova, L., Remichkova, M., Ivanova, I., Serkedjieva, J. Antiviral effects of proteolytic inhibitors from *Streptomyces*. *J. Basic Microbiol.*, 2006; **46**(6): 504-12.
- Hiraga, K., Suzuki, T., Oda, K. A novel double-headed proteinaceous inhibitor for metalloproteinase and serine proteinase. *J. Biol. Chem.*, 2000; **275**: 25173-79.
- Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 1976; **72**: 248-54.
- Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L. Rapid analysis of amino acids using precolumn derivatization. *J. Chromatogr.*, 1984; **336**: 93-104.
- Serkedjieva, J., Hay, A.J. *In vitro* antiinfluenza virus activity of a plant preparation from *Geranium sanguineum* L. *Antiviral Res.*, 1998; **37**(2): 121-30.
- Vanden Berghe, D.A., Vlietink, A.J., Van Hoof, L. Plant products as potential antiviral agents. *Bull. Inst. Pasteur*, 1986; **84**: 101-47.
- Belshe, R.B., Smith, M.H., Hall, C.B., Betts, R., Hay, A.J. Genetic bases of resistance to rimantadine emerging during treatment of influenza virus infection. *J. Virol.* 1988; **62**: 1508-12.
- Serkedjieva, J. and Ivanova, E. Combined protective effect of an immunostimulatory bacterial preparation and rimantadine hydrochloride in experimental influenza A virus infection. *Acta virol.*, 1997; **41**: 65-70.
- Reed, L. J., Muench, H. A simple method of estimating fifty per cent end points. *Am. J. Hyg.*, 1938; **27**: 493-8.
- Kido, H., Niwa, Y., Beppu, Y., Towatari, T. Cellular proteases involved in the pathogenicity of enveloped animal viruses human immunodeficiency virus influenza virus A and Sendai virus. *Advances in Enzyme Regulation*, 1966; **36**: 325-47.
- Lozitsky, V.P., Fedchuk, A.S., Puzis, L.E., Buiko, V.P., Gurlia, I.I. Participation of the proteolysis system in promoting the virulence of the influenza virus and development of the infectious process: effect of protease inhibitors. *Voprosi Virusologii*, 1987; **32**: 413-9.
- Steinhauer, D.A. Role of haemagglutinin cleavage for the pathogenicity of influenza virus. *Virology*, 1999; **258**: 1-20.
- Zhirnov, O.P., Ikizler, M.R., Wright, P.F. Cleavage of influenza A virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. *J. Virol.* 2002; **76**(17): 8682-9.
- Kido, H., Beppu, Y., Imamura, Y., Chen, Y., Murakami, M., Oda, K., Towatari, T. The human mucus protease inhibitor and its mutants are novel defensive compounds against infection with influenza A and Sendai viruses. *Biopolymers*, 1999; **51**: 79-86.
- Kido, H., Sakai, K., Kishino, Y., Tashiro, M. *J. Pure & Appl. Microbiol.*, **3**(2), Oct. 2009.

- Pulmonary surfactant is a potential endogenous inhibitor of proteolytic activation of Sendai virus and influenza A virus. *FEBS Letters*, 1993; **322**: 115-9.
22. Ovcharenko, A.V., Zhirnov, O.P. Aprotinin aerosol treatment of influenza and paramyxovirus bronchopneumonia of mice. *Antiviral Res.*, 1994; **23**: 107-18.
 23. Yang, B., Yao, D.F., Ohuchi, M., Ide, M., Yano, M., Okumura, Y., Kido, H. Ambroxol suppresses influenza-virus proliferation in the mouse airway by increasing antiviral factor levels. *Eur. Resp. J.*, 2002; **19**: 952-8.
 24. Lozitsky, V.I., Kashkin, A.P., Shneider, M.A., Fedchuk, A.S., Puzis, L.E., Alexander, N.G. Antiviral action of proteolysis inhibitor from *Streptomyces* species. In: Abstracts 15th ICAR, 17-21 March Prague, Czech Republic, 2002; A75.
 25. Angelova, L., Dalgalarondo, M., Minkov, I., Danova, S., Kirilov, N., Chobert, J.-M., Haertle, T., Serkedjieva, J., Ivanova, I. A novel proteinaceous protease inhibitor - PISC-2002 isolated from *Streptomyces chromofuscus* 34-1 with antiviral activity. *BBA – General Subjects*, 2006; **1760** (8): 1210-16.
 26. Serkedjieva, J., Roeva, I., Ivanova, I. Anti-influenza virus effects of a proteolytic inhibitor, produced by *Streptomyces* species. *Biotechnol. Biotechnol. Eq.*, 2009; **23**: Suppl., 585-8.
 27. Zhirnov, O.P., Ovcharenko, A.V., Bukrinskaja, A.G. Suppression of the proteolytic activation of myxoviruses in infected chick embryos by using aprotinin. *Voprosi virusologii*, 1985; **30**: 207-14.
 28. Noma, K., Kiyotani, K., Kouchi, H., Fujii, Y., Egi, Y., Tanaka, K., Yoshida, T. Endogenous protease-dependent replication of human influenza viruses in two MDCK cell lines. *Arch Virol.* 1998; **143**(10): 1893-909.
 29. De Clercq, E., New developments in anti-HIV chemotherapy. *Curr. Med. Chem.*, 2001; **8**: 1543-72.
 30. Shu, Y.Z., Ye, Q., Kolb, J.M., Huang, S., Veitch, J.A., Lowe, S.E. and Manly, S.P. Bripiodionen, a new inhibitor of human cytomegalovirus protease from *Streptomyces* sp. WC76599. *J. Nat. Prod.*, 1997; **60**: 529-32.