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Proteinase inhibitors from *Streptomyces* with antiviral activity

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An extensive screening study for the production of proteolytic inhibitors has been carried out on 75 *Streptomyces* strains. It was found that 18 of the strains and/or their variants (24%) produced proteinaceous substances, which belonged to the group of typical serine protease inhibitors. 23 samples were tested for inhibitory activity on the replication of influenza virus A/Germany/34, strain Rostock (H7N1) (A/Rostock) in chicken embryonic fibroblast (CEF) cells. Eleven of the tested samples (52.2%) significantly inhibited viral growth. Further the specific inhibitory effect on the replication of influenza virus A/Aichi/2/68 (H3N2) (A/Aichi) in Madin-Darby canine kidney (MDCK) cells and on the growth of herpes simplex virus type 1, strain DA (HSV-1) in Madin-Darby bovine kidney (MDBK) cells was tested. Nine samples significantly inhibited A/Aichi and four – HSV-1. The most effective inhibitors, produced by *Streptomyces* sp. 225b (SS 225b) and *Streptomyces chromofuscus* 34-1 (SS 34-1) protected mice from mortality in the experimental influenza A/Aichi virus infection.

Influenza and herpes simplex viruses are important human pathogens. Viral proteases are an absolute requirement in the life cycle of these viruses (PATICK and POTTS 1998). In the case of influenza virus, the virulence of a particular influenza virus strain depends on the ability of its hemagglutinin precursor (HA0) to be cleaved post translation to subunits HA1 and HA2 by trypsin-like proteases of the host (LOZITSKY *et al*. 1987, ZHIRNOV *et al*. 1985). Therefore it seems reasonable that inhibition of this cleavage would result in inhibition of the subsequent rounds of viral replication (LOZITSKY *et al*. 1987). There is experimental evidence presented by LOZITSKI *et al*. (2002) that the replication of influenza virus was efficiently inhibited by a proteinase inhibitor MI 0114, produced by *Streptomyces* sp.

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 nature. The present paper focuses on the study of the virus-inhibitory effects of selected proteinase inhibitors, produced by *Streptomyces*.

Materials and methods

Microbial strains: 18 *Streptomyces* strains, isolated from different soil samples, obtained from the collection of the Department of Microbiology, Sofia University.

Media and cultivation: Agar maintenance media: mineral medium 1vs Gause, potato-glucose agar, malt medium ISP-2, routinely used in the laboratory for *Streptomyces*.

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Cultivation medium: medium for SSI, optimal for the production of proteinase inhibitors (TAGUCHI *et al.* 1992).

The cultivation consisted in a two-phase deep fermentation in 500 ml Erlenmayer flasks at 220 rpm/min and 28 °C. After incubation for 48 h the fermentation flasks were inoculated with 10% (v/v) of the starter culture and cultivated for 120 h at the same conditions. Samples for analysis of the proteinase inhibitory activity were taken every 24 h.

Partial purification of the fermentation products: This experiment performed according to HIRAGA *et al*. (2000). After centrifugation, 40 min, 12.000 rpm min^{-1} at 4 °C, the supernatants were precipitated with 80% $(NH_4)_2SO_4$ 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.

Trypsin inhibitory assay: Done as described by ANGELOVA *et al*. (2003). This method is based on the suppression of the cleavage of N-bensoil-DL-arginine-*p*-nitroanilide (BPN^I), determined at 405 nm on UV-VIS spectrophotometer Shimadzu; the specific inhibitory activity is presented as Kunitz inhibitory units mg^{-1} protein (KIU mg^{-1} protein).

Protein determination: Bio-Rad assay reagent (BIO-RAD, Munich, Germany) and bovine serum albumin as the standard were used and the protein content was expressed as mg ml^{-1} (BRADFORD 1976).

Amino acid analysis was performed according to BIDLINGMEYER *et al*. (1984) and the results are provided by Ms. MICHELLE DALGALARRONDO, Institut National de la Recherche Agronomique-BIA-FIPL, Nantes, France.

Virology – compounds: The cell-free extracts, used in the antiviral experiments are listed in Table 1. Four-fold dilutions of the preparations were made in cell culture medium *ex tempore*. Rimantadine hydrochloride (Rimantadine) was purchased from HOFFMAN-LA ROCHE Inc., Nutley, NJ, USA; Acyclovir and Ribavirin (Rib) were obtained from Sigma, Diesenhofen, Germany, ε-amino caproic acid (ACA) was a gift from Prof. V. P. LOZITSKY from the Odessa Anti-plague Institute, Ukraine.

Cells and Media: Primary CEF cell cultures were prepared by a standard procedure and maintained in a growth medium, containing minimal Eagle's medium 45%, Hank's solution 45%, LAH 5% (lacto-albumin hydrolizate) and supplemented with calf serum and antibiotics 5% (100 IU ml^{-1} benzyl-penicillin and 100 μg ml^{-1} streptomycin). MDCK and MDBK cells were supplied by Dr. I. ROEVA (IM, BAS). The cell cultures were cultivated at 37 °C in the presence of 5% CO_2 till the formation of confluent monolayers. In the antiviral experiments FCS 0.5% was added. A/Aichi was cultivated in the presence of 2 μg ml^{-1} trypsin (SIGMA, Diesenhofen, Germany).

Viruses: The avian influenza virus A/Rostock and the human influenza virus A/Aichi were grown in 11-days old fertile hen's eggs and the allantoic fluids were used as virus inoculum. In the animal experiments A/Aichi, adapted to mice lungs was used (A/Aichi-a). The viral infectious titres were 10^6–10^7 $TCID_{50}$ ml^{-1} (50% tissue culture infectious doses ml^{-1}), the hemagglutination (HA) titres – 1024-2048. HSV-1 was adapted to MDBK cells; its infectious titre was $10^{6.5}$ $TCID_{50}$ ml^{-1}. The strains were from the collection of the Institute of Microbiology, BAS. The viral stocks were stored at –70 °C.

Mice: Inbred ICR mice (16–18 g) were obtained from the Experimental Animal Station of the BAS in Slivnitza, Sofia. The experimental groups were of 10–12 animals each. At the end of the experiments, surviving mice were sacrificed by cervical dislocation. The animals were bred under standard conditions, accepted by the Bulgarian Veterinary Health Service.

Cellular toxicity: Confluent CEF, MDCK and MDBK cell monolayers in 96-well plastic plates were overlaid with two-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 50% toxic concentration (TC_{50}) was estimated from graphic plots.

Antiviral assay: The antiviral effect was studied in multicycle experiments of viral growth and the virus-induced cytopathic effect (CPE) was used as a measure of viral growth.

Cytopathic effect reduction assay: Quadruplicate confluent monolayers in 96-well plates were overlaid with 2× drug-containing medium (0.1 ml) and an equal volume of virus suspension (100 $TCID_{50}$ ml^{-1}). The virus-induced CPE was scored after 48–72 h of incubation as described above. The dose reducing CPE by 50% with respect to virus control (50% effective concentration, EC_{50}) was estimated from graphic plots. The selectivity index (SI) was determined from the ratio TC_{50}/EC_{50}; $SI \geq 4$ was considered to stand for a significant selective inhibition.

Endpoint titration technique (EPTT, VANDEN BERGHE *et al.* 1986): In some experiments EC_{90} (90% effective concentration, the dose, reducing infectious virus titre with 1 $\log_{10}$ $TCID_{50}$ ml^{-1}) was evaluated by the EPTT. The anti-influenza drug Rimantadine and the antiherpetic drug Acyclovir were used as positive controls.

Virus infection in mice: To produce lethal infection, mice were infected nasally with 5–10 LD_{50} of A/Aichi-a in the volume of 0.05 ml physiological saline (PBS) per mouse under light ether anesthesia.

Experimental design: Mice were treated orally 24 and 2 h before and 24, 48 and 72 hours after viral exposure with the partially purified fermentation extracts from *Streptomyces* sp. 225b (0.14 mg/mouse/day) and *Streptomyces chromofuscus* 34-1 (0.16 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 50 mg/mouse and 0.125 mg/mouse 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 $M_{control}/M_{experiment}$ and M is mortality and MST as described in SERKEDJIEVA and IVANOVA (1997).

To determine 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 (1997). 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^{-1} trypsin (SIGMA). Virus-induced CPE was scored as described above; infectious virus titres were evaluated (REED and MUENCH 1938) and expressed in $\log_{10} TCID_{50}$/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

Protease inhibitory activity of the Streptomyces strains

75 *Streptomyces* strains were screened for protease inhibitory activity. 15 strains did not show any inhibitory effect. The specific inhibitory activity of the remaining 60 (80%) strains varied from 62.7 to 1280.0 KIU mg^{-1} 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 are summarized in Table 1.

The most promising preparations (SIA > 500 KIU mg^{-1}) were studied by Tricin-SDS-PAGE. The received data indicated the presence of proteins with molecular mass between 6.5–12.5 kDa, which is characteristic of the proteinaceous proteolytic inhibitors.

Table 1
Protease-inhibitory activity*, cell toxicity and virus-inhibitory effects of cell-free extracts from *Streptomyces* on A/Rostock virus replication in CEF cells

Name of the strain	Specific inhibitory activity (KIU mg^{-1} protein) [a]	Cell toxicity TC_{50} [b] ($\mu g\ ml^{-1}$)	Virus inhibition	
			EC_{50} [c] ($\mu g\ ml^{-1}$)	SI [d] (TC_{50}/EC_{50})
Streptomyces fradiae	347.1	20.0	3.1	6.4
Streptomyces hygroscopicus subsp. *hygroscopicus*	430.2	5.0	> TC_{50}	
Streptomyces lincolnensis	195.9	7.0	1	6.4
Streptomyces sp. 2–15	62.7	15.0	>TC_{50}	
Streptomyces sp. 7–12[i]	684.9	183.0	11.4	16.0
Streptomyces sp. 19-B[i]	1280.0	67.0	17	4.0
Streptomyces sp. 32	166.3	34.0	1.3	25.6
Streptomyces sp. 33	227.4	53.0	>TC_{50}	
Streptomyces chromofuscus 34-1[i]	625.0	>1600[e]	12.5	128.0
Streptomyces sp. 35	102.3	14.0	>TC_{50}	
Streptomyces sp. 35p[g]	276.1	10.0	>TC_{50}	
Streptomyces sp. 39	270.6	25.0	>TC_{50}	
Streptomyces hygroscopicus 155+	453.4	6.0	>TC_{50}	
Streptomyces hygroscopicus 155o	576.9	8.0	2	4
Streptomyces sp. 225	596.4	3.0	0.4	6.4
Streptomyces sp. 225b[f]	653.9	136.0	0.01	256
Streptomyces albogriseolus 444+	355.8	243.0	= TC_{50}	1
Streptomyces albogriseolus 444o	150.2	9.0	2.2	4
Streptomyces sp. 695[e]	490.2	0.3	>TC_{50}	
Streptomyces sp. 695-2-206[e]	469.5	0.3	>TC_{50}	
Streptomyces sp. A-12[i]	598.8	>1400[e]	>TC_{50}	
Streptomyces sp. DSB-3[i]	970.9	26.0	8	3.2
Streptomyces sp. DSB-3b[h,i]	796.9	>1 024[e]	32	32.0
ε-ACA		50 mg ml^{-1}	2 mg ml^{-1}	25
Rimantadine		>32	0.4	>90

*part of the results are provided by Dr. B. GOCHEVA (Dept. Microbiology, Sofia University), [a] specific proteinase-inhibitory activity (KIU mg^{-1} protein), [b] TC_{50}, the dose that caused morphological alterations in 50% of intact cells, [c] EC_{50}, the dose that caused 50% reduction of infectivity, [d] SI – selectivity index, [e] not toxic, [f] morphological variant of *Streptomyces* sp. 225, producing a blue pigment, [g]morphological variant of *Streptomyces* sp. 35, producing a pink pigment, [h] morphological variant of *Streptomyces* sp. DSB-3, producing a blue pigment, + active and o inactive variant with respect to antibiotic synthesis, [i] part of the results are presented in ANGELOVA *et al*. (2003)

The most active protease inhibitors were produced by *Streptomyces* sp. 225b (SS 225b, SIA 653.9 KIU mg^{-1}) and *Streptomyces chromofuscus* 34-1 (SS 34-1, SIA 625.0 KIU mg^{-1}). 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 of the proteinase inhibitory activity during the submerged cultivation of both strains with glucose as a carbon source followed the same pattern (Fig. 1)

It has been shown that the procedures of partial purification of both protease inhibitors lead to a significant increase of their specific proteinase inhibitory activity; in contrast, the purification resulted in decrease of the virus-inhibitory effect (IVANOVA *et al*. 2003). The chemical nature of SS 225b and SS 34-1 was further studied. Both proteins were subjected to purification by ion exchange chromatography, gel filtration and HPLC analysis.

The major unbound fraction of SS 225b consisted of two proteins, representing variants of one and the same protein with Mw 11.1 and 10.9 kDa. SS 34-1 had a molecular mass of

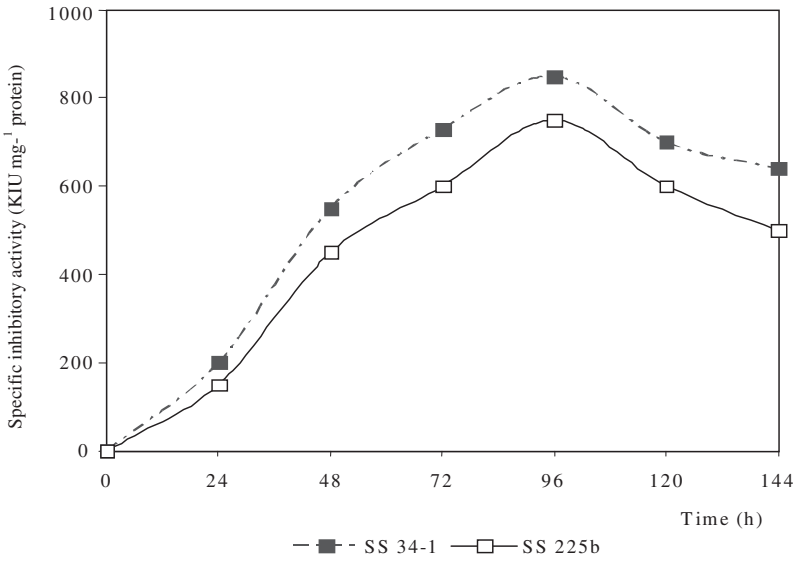

Fig. 1
Dynamics of the proteinase inhibitory activity during the submerged cultivation of *Streptomyces chromofuscus* 34-1 and *Streptomyces* sp. 225b with glucose as carbon source
SS 34-1, *Streptomyces chromofuscus* 34-1; SS 225b, *Streptomyces* sp. 225b

11.2 kDa; the amino acid analysis (Table 3) shows that the protein was very rich in proline (9 %) and in hydrophobic amino acid residues: Ala (16.9%), Gly (10.8%), Val (10%), and Leu (9.5 %). SS 225b was very rich in Ala (21%) and Val (10%) residues and very poor in Ile (Table 3).

SS 225b and SS 34-1 were classified on the basis of their properties, the similarity of their structures and protease inhibitory specificities as members of the *Streptomyces* subtilisin inhibitor (SSI) family (ANGELOVA *et al*. 2005). SSI share several common physico-chemical properties: they have small molecular masses and are stable at low pH and high temperatures (LASKOWSKI and KATO 1980, TAGUCHI *et al*. 1993).

Antiviral effects of proteinase inhibitors from Streptomyces

We studied the antiviral effects of 23 partially purified cell-free extracts from 18 microorganisms, 3 morphological variants and 2 variants active and inactive with respect to antibiotic synthesis. Firstly the toxicity to CEF, MDCK and MDBK cells was tested. This was necessary for evaluating the specificity of the virus-inhibitory effect. The results are presented in Tables 1, 2. TC_{50}-s varied from 0 to 0.24 mg ml^{-1} for CEF, from 0 to 0.18 mg ml^{-1} for MDCK and from 0 to 0.4 mg ml^{-1} for MDBK. The cell-toxic effect was cell-specific, CEF was more sensitive than MDCK; MDBK was the least susceptible to the toxic effects of the preparations.

The samples were screened for a specific influenza virus-inhibitory effect in a model system. The results are presented in Table 1. 52.2% of the samples inhibited significantly the replication of influenza virus A/Rostock in CEF cells with $SI \geq 4$ (Table 1). Furthermore the specificity of the virus-inhibitory effect of the active samples was tested on the growth of human influenza virus A/Aichi in MDCK cells and on the replication of HSV-1 in MDBK cells (Table 2.). Nine of the inhibitors suppressed significantly A/Aichi and four – HSV-1. The inhibitory effect of the samples was virus-specific: influenza viruses A/Rostock

Table 2
Inhibitory effect of fermentation extracts from *Streptomyces* strains on the replication of A/Aichi in MDCK cells and of HSV-1 in MDBK cells

Sample	A/Aichi in MDCK cells			HSV-1 in MDBK cells		
	TC_{50}[b] (μg ml^{-1})	EC_{50}[c] (μg ml^{-1})	SI[d]	TC_{50}[b] (μg ml^{-1})	EC_{50}[c] (μg ml^{-1})	SI[d]
Streptomyces fradiae	40.0	= TC_{50}	1	200.0	> TC_{50}	
Streptomyces lincolnensis	13.4	= TC_{50}	1	67.0	1.6	40
Streptomyces sp. 7–12[i]	183.0	11.4	16	365.0	182.0	2
Streptomyces sp. 19-B[i]	67.0	5.0	12.8	84.0	> TC_{50}	
Streptomyces sp. 32	34.0	2.1	16	86.0	> TC_{50}	
Streptomyces chromofuscus 34-1[i]	>1 600[e]	23.0	70	400.0	50.0	8
Streptomyces hygroscopicus 155+	6.0	= TC_{50}	1	9.5	> TC_{50}	
Streptomyces hygroscopicus 155o	7.7	2.0	4	77.0	> TC_{50}	
Streptomyces sp. 225	5.0	0.16	32	25.0	> TC_{50}	
Streptomyces sp. 225b[f]	>1360[e]	2.2	617	340.0	34.0	10
Streptomyces albogriseolus 444o	34.0	0.54	64	86.0	> TC_{50}	
Streptomyces sp. A-12[i]	>1400[e]	n.t.		>1 660[e]	417.0	4
Streptomyces sp. DSB-3[i]	26.0	8.0	3.2	257.0	> TC_{50}	
Streptomyces sp. DSB-3b[h,i]	>1 024[e]	32.0	32.0	256.0	> TC_{50}	
ε-ACA	50[j]	2[j]	25	n.t.	n.t.	
Rimantadine	>32.0	0.2	>160			
Acyclovir				>50	1.0	>50

b, c, d, e, f, h, i as in Table 1 j, mg ml^{-1}, n.t. not tested

Table 3
Amino acid composition* of the protease inhibitors, produced by *Streptomyces chromofuscus* 34-1 and *Streptomyces* sp. 225b

Amino acids	Residues (%)	
	SS 34-1	SS 225b
Asx	5.6	3.7
Glx	5.9	6.9
Ser	7.2	7.1
Gly	10.8	8.0
His	2.4	2.3
Arg	4.7	6.2
Thr	7.5	8.5
Ala	16.9	21.3
Pro	9.1	6.9
Tyr	1.4	2.7
Val	8.8	10.3
Met	0.8	0.8
Ile	1.2	2.0
Leu	9.5	0.7
Phe	5.1	7.6
Lys	3.0	3.0

*the results are provided by Ms. MICHELLE DALGALARRONDO, Institut National de la Recherche Agronomique-BIA-FIPL, Nantes, France

and A/Aichi were more susceptible to inhibition than HSV-1. It should be noted that the replication of A/Aichi in MDCK cells could take place only in the presence of trypsin; presumably the inhibitory effect of the fermentation extracts would be diminished.

A separate experiment was set up in order to verify this possibility. A/Aichi was pretreated for 1 h with trypsin (1 mg ml^{-1}), the effect of trypsin was stopped by the addition of lime bean inhibitor (1 mg ml^{-1}) (A/Aichi-pr); further A/Aichi-pr was cultivated in a medium without trypsin containing a purified preparation of SS 34-1. The inhibitory effect of SS 34-1 was tested in parallel on A/Aichi (cultivated in a medium supplemented with 2 μg ml^{-1} trypsin) and A/Aichi-pr. The virus-inhibitory effect towards A/Aichi-pr was stronger: EC_{90}–s was 100 and 33 μg ml^{-1}, respectively. Thus the presence of trypsin in the culture medium reduced the antiviral effect of SS 34-1. An analogous result was obtained with SS 225b.

Influenza virus replication was inhibited most effectively by SS 225b and SS 34-1. The selectivity indices for A/Rostock reached the values of 256 and 128 respectively and for A/Aichi – 617 and 70 respectively. The marked antiviral effect corresponded to the high value of their specific protease inhibitory activity – 653.9 and 625.0 KIU mg^{-1} respectively (Table 1). Virus inhibition seemed specific as HSV-1 was far less susceptible to inhibition (SI = 10 and 8, respectively).

Further studies concerning the mode of the anti-influenza virus effect are in progress and are carried out with HPLC-purified preparations of the inhibitors. Some preliminary results show that the preparations do not inactivate the virus but are able to interfere with the penetration of viral particles into susceptible cells as well as with the late stages of viral replication (post-transnational 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 HA2 (ZHIRNOV *et al.* 1984). Thus, the antiviral effect of SS 225b and SS 34-1 is probably due to inhibition of this cleavage through inhibition of the host cells serine type proteases.

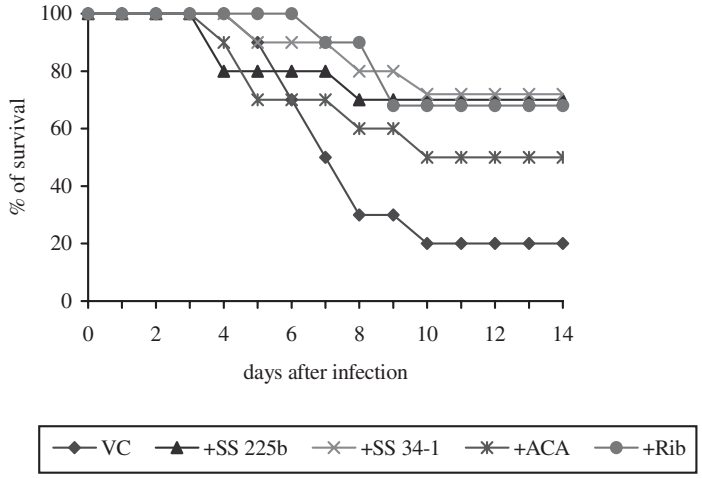

Fig. 2
Effect of the fermentation extracts from *Streptomyces* sp. 225b and *Streptomyces chromofuscus* 34-1 on the survival rate of mice in the experimental influenza A/Aichi-a infection
VC, virus control; SS 225b, cell-free filtrate from *Streptomyces* sp. 225b, 5 × 0.14 mg/mouse/day; SS 34-1, cell-free filtrate from *Streptomyces chromofuscus* 34-1, 5 × 0.16 mg/mouse/day; ACA, ε-amino caproic acid, 5 × 50.0 mg/mouse; Rib, ribavirin, 5 × 0.2 mg/mouse

Table 4
Effect of the fermentation extracts from *Streptomyces* sp. 225b and *Streptomyces chromofuscus* 34-1 on the lung parameters* of mice in the experimental influenza A/Aichi-a infection

Experimental group	Score	Weight (g)	Infectious titre ($log_{10}TCID_{50}/ml^{-1}$)
VC	3.5	0.29	5.1
VC + SS 225b	1.0	0.24	1.5
VC + 34-1	0.5	0.21	1.0
VC + ACA	1.5	0.25	2.3
VC + Rib	0.5	0.21	1.0

* evaluated on 6th day after infection
VC, SS 225b, SS 34-1, ACA, Rib, as in Fig. 2

SS 225b and SS 34-1 were tested in the murine model of experimental influenza A/Aichi-a infection. Applied according to a prophylactic-therapeutic schedule, the extracts protected mice from mortality; this protection reached 60.0% and 64.9%, respectively. The preparations were not toxic to the experimental animals and the protection was comparable to that shown by the known antiviral agent Ribavirin (PI = 62.4%) and surpassed that, exhibited by the known proteolytic inhibitor ACA (LOZITSKY *et al*. 1987) PI = 56.1%, both inoculated according to the same treatment schedule (Fig. 2). Both the rate of survival and MST were increased (); the effect was dose-related. Lung parameters (infectious virus titres, consolidation and weights) were all significantly reduced in the SS 225b and SS 34-1-treated mice (Table 4).

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 2001), cytomegalovirus (SHU *et al*. 1997) and influenza virus (LOZITSKY *et al*. 2002) were all efficiently inhibited by proteinase inhibitors, produced by *Streptomyces* species.

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