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Biochemical and biological activity of arginine deiminase from *Streptococcus pyogenes* M22.

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Draft

Streptococcus pyogenes (group A Streptococcus – GAS) is an important gram-positive extracellular bacterial pathogen responsible for a number of suppurative infections. This microorganism developed complex virulence mechanisms to avoid host defense. We have previously revealed that Supernatant of Destroyed Streptococcal Cells (SDSC) from GAS type M22 causes endothelial cells' dysfunction, inhibits cellular adhesion, migration, metabolism and proliferation in a dose-dependent manner without affecting cells' viability. The present work is aimed at isolation and characterization of a component from GAS type M22 supernatant that suppresses proliferation of endothelial cells EA.hy926. Isolating a protein possessing antiproliferative activity allowed identifying arginine deiminase (AD). Further study showed that the enzyme is most active at pH 6.8. Calculating K_m and V_{max} gave the values of 0.67 mM and 42 s⁻¹, respectively. Distinctive feature of AD purified from GAS type M22 is the closest to neutral pH optimum of activity and the maximal rate of the catalytic process compared with such of the enzyme from other microorganisms. AD from GAS type M22 suppressed the proliferative activity of endothelial cells in a dose-dependent mode. At the same time in the presence of AD the share of cells in G₀/G₁ phase increased. When L-Arg was added at increasing concentrations to the culture medium containing 3 µg/ml AD, the enzyme's capacity to inhibit cell proliferation became partly depressed. The proportion of cells in phases S/G₂ increased concomitantly, though the cells did not fully recover their proliferation activity. All this allows regarding AD from GAS type M22 as a prospective pharmacy for suppression of excessive cell proliferation.

Key words: *Streptococcus pyogenes* M22, arginine deiminase, endothelial cell proliferation.

ABBREVIATIONS: AD – arginine deiminase, GAS – *Streptococcus pyogenes* or group A streptococcus, SDSC – supernatant of destroyed streptococcal cells, eIF2 - Eukaryotic Initiation Factor 2, GCN2 - general control nonderepressible 2, HAT - hypoxanthine-aminopterin-thymidine, PBS - phosphate-buffered saline, FITC - fluorescein isothiocyanate, HUVEC - human umbilical vein endothelial cells.

INTRODUCTION

L-Arg is conditionally essential amino acid involved in a number of physiological processes (immunity, growth and regeneration). It is a precursor for the synthesis of L-ornithine, L-proline, polyamines, agmatine, creatine and proteins. Uncombined L-Arg is produced in an organism due to the endogenous synthesis from nutrients. It is a substrate of four enzymes i.e. NO synthases, arginases, arginine: glycineamidinotransferases, and L-Arg decarboxylases (Morris 2002). During the development of a bacterial infection, arginine becomes a substrate for bacterial arginine deiminase (AD) (Das et al. 2010). This enzyme is likely to be used by bacteria to disrupt the function of host cells. Bacterial AD is known to inhibit proliferation of various eukaryotic cells (Altucci et al. 1966; Claesson et al. 1990; Degnan et al. 1998; Kuo et al. 2010; Miyazaki et al. 1990; Park et al. 2003; Takaku et al. 1992; Gong et al. 1999, 2000; Fiedler et al. 2015). By now AD from a number of streptococcus strains has been isolated and characterized (Yosida et al. 1985, 1987, 1989, 1991, 1994; Degnan et al. 1998).

Our previous studies showed that there was a dose-dependent decrease in the inclusion of 5-5-Bromo-2'-Deoxyuridine (synthetic analogue of thymidine) into the DNA of cells in the presence of Supernatant of destroyed streptococcal cells (SDSC) of *S. pyogenes* type M22. That was an indication of the decrease of the intensity of DNA synthesis and the intensity of cell proliferation. Analysis of cell cycle phases in the presence of SDSC revealed a significant increase of the proportion of cells in the resting phases G₀/G₁, while the proportion of cells in the phases of the synthesis S/G₂ was reduced. Further studies showed that culturing in the presence of SDSC inhibited the migration and adhesion of endothelial cells (Starikova et al. 2012). The study of the mechanisms underlying these effects showed that SDSC in the concentrations used did not exert cytotoxic and apoptogenic action on endothelial cells. A dose-dependent decrease of the activity of mitochondrial dehydrogenases was observed. The effect was reversible, i.e. removal of SDSC from the culture medium restored the cells' metabolism. In our experiments adding SDSC did not change the levels of phosphorylation of stress kinases NF- κ B, p38 and JNK, while phosphorylation of ERK1/2 and FAK became significantly reduced. The assumption was made that the active component responsible for the observed effect was AD.

The present work was aimed at isolation and characterization of a component of GAS type M22 supernatant that suppresses proliferation of endothelial cells EA.hy926.

MATERIALS AND METHODS

Cell culture

Endothelial cells EA.hy 926 were a generous gift of Dr. Cora-Jean S. Edgell (University of North Carolina, USA). This cell line displays major phenotypic and functional features of

endothelial cells from human large vessels (Edgell et al. 1983). Cells were cultured in DMEM (Sigma, USA) containing 10% embryonic calf serum (Sigma, USA), HAT (Sigma, USA), 4 mM L-glutamine and 100 µg/ml gentamycin (Biolot, Russia). Subculturing was done twice a week. Cellular monolayer disintegration was induced by incubation in 0.02% solution of ethylene diamine tetra-acetic acid (EDTA) (Biolot, Russia).

Obtaining supernatant of ultrasonic treated streptococci

Supernatant of destroyed streptococcal cells (SDSC) by ultrasonic treatment containing active intracellular components was prepared using GAS type M22, strain AL168, generously provided by Dr. Lindahl (Department of Laboratory Medicine, University of Lund, Sweden). Strain was grown aerobically in Todd-Hewitt broth (Difco, USA) overnight at 37°C. The cells then were centrifuged, and the pellet was twice washed with lipopolysaccharide-free phosphate-buffered saline (PBS).

Concentration of bacterial suspension was standardized by optical density to achieve 2.5×10^8 CFU/ml. Portions of 5ml of suspension in PBS, pH 7.0, were sonicated at 22 kHz and 0.6-0.8 mA output in ultrasound disintegrator (MSE, U.K.). Completeness of disintegration of bacterial cells was controlled under microscope, after which the suspension was centrifuged at 16 000xg for 30 min. The supernatant was filtered through 0.45 µm Filtropure S (Sarstedt, Germany) and stored at -20°C. Supernatant of bacterial components used in dilutions did not show toxic effect on cell cultures.

Cells proliferation assay

Proliferation activity of cells was assayed according to Cheong J.K. et al. 2011 with some modifications. Cells were seeded in 96-well tissue culture plates (Greiner, Austria) so that each well contained 5×10^3 cells in 100 µl of complete culture medium, and incubated at 37°C in the presence of substances under study for 72 h in humid ambience containing 5% CO₂. Cells were then fixed and stained with 0.2% crystal violet dye (Sigma-Aldrich, USA) solution in 10% methanol for 10 min. Excess crystal violet dye was removed by five washings with deionized water and the culture plates were dried overnight. Then cells were incubated in 10% acetic acid to release the dye, after which optical density of solutions was measured at 570 nm in an automatic spectrophotometer (Bio-Rad Laboratories).

Flow cytometric analysis of the cell cycle

Cells were seeded in the wells of flat-bottom 6-well plate (Nunc, USA). The amount of cells in complete culture medium was 5×10^5 per well. After that substances under study were introduced into the wells. 24 h later cells were transferred into microtubes and fixed/permeabilized for 5 min with 96% ice-cold methanol. This was followed by staining the cell suspension with FITC-conjugated anti-cyclin A2 antibodies (Beckman Coulter, USA).

Staining was performed in accordance with the manufacturer's instructions. 2 µg/ml propidium iodide (Sigma, USA) and 100 µg/ml Ribonuclease A (Sigma, USA) were added to each sample. Samples were incubated for 20 minutes at room temperature in the dark. After incubation cell samples were analyzed using flow cytometer Navios (Beckman Coulter, USA).

Isolation and identification of the protein from SDSC capable of suppressing cell proliferation

To purify from SDSC the protein that suppresses cell proliferation, the supernatant was fractionated by ion-exchange and affinity chromatography. Fractions obtained at each stage were flown through 0.22 µm filter, and their capacity to suppress cell proliferation was assayed. Only fractions that, upon dilution 1:25, induced a drop of proliferation at least to 50% were selected for further study. SDSC was diluted 1:5 with water and loaded on an UNO-Sphere S column (20×2.5 cm) equilibrated with PBS diluted with water 1:5 (PBS/5). Ballast proteins were removed by washing the column with PBS/5, which was followed by elution with two linear gradients, namely 40 ml of PBS/5 → PBS and 50 ml of PBS → 1M NaCl. Antiproliferation activity was detected in fractions eluted by gradients, but partly also in the fraction of ballast proteins washed out by PBS/5. All fractions were pooled and loaded on an UNO-Sphere S column (18×2.5 cm) equilibrated with PBS/5. The column was washed again with PBS/5, after which the same two linear gradients were used for protein elution (see above). Antiproliferation activity was found in the fractions eluted with 150-200 mM NaCl. Those were diluted with water 1:5 and subjected to chromatography according to the same protocol. Once more the antiproliferation activity was found in the fractions eluted with 150-200 mM NaCl. The fractions were pooled and concentrated to the volume of 0.2 ml in VivaSpin cells (retaining proteins above 30 kDa). This volume was loaded on a Sephacryl S-200 HR column (115×1 cm) equilibrated with PBS. Antiproliferation activity was eluted by PBS and detected in fractions with proteins having approximate M 150-160 kDa. Again the fractions were pooled, concentrated to the volume of 0.2 ml in VivaSpin cells (retaining proteins above 30 kDa) and subjected to electrophoresis in SDS-containing PAAG (10% acrylamide). A protein with M ~ 45 kDa was revealed, treated with trypsin, and its fragments were analyzed in a mass spectrometer.

To prepare a sample for mass spectrometry a piece of protein-containing gel (1 mm³) was cut out. That piece was destained by repeating twice its 30-min incubation at 37°C in 100 µl of 40% acetonitrile in 0.1 M NH₄HCO₃. Then PAAG was transferred into 100 µl of acetonitrile for dehydration, after which it was taken out and dried. 4 µl of modified trypsin in 0.05 M NH₄HCO₃ (15 µg/µl) were added to the gel. Hydrolysis lasted for 18 h at 37°C, after which 8 µl of 0.5% TFA in 10% water solution of acetonitrile were added and the content was thoroughly mixed. Gel particles were let precipitate and the supernatant was used to obtain MALDI mass

spectra. 1 μ l of a sample obtained as described was mixed with 0.3 μ l of 2,5-dihydroxybenzoic acid (10 ml/ml in 20% acetonitrile/water with 0.5% TFA), and the mixture obtained was dried in the air. Mass spectra were obtained in a tandem MALDI TOF-TOF spectrometer Ultraflex II BRUKER (Germany) supplemented with a UV laser (Nd). Mass spectra were registered using reflectron in a positive ion mode. Precision of the masses determined upon refinement on account of trypsin autolysis was 0.005%. Peptide fingerprints thus obtained were analyzed on-line using MASCOT program (<http://www.matrixscience.com>). The search based on a peptide fingerprint was done with precision mentioned among the bacterial proteins using NCBI database. Possible oxidation of methionines in the open air and modification of cysteines by acrylamide were accounted for.

Assaying the activity of arginine deiminase (AD)

Our protocol of assaying the activity of arginine deiminase (AD) was based on the modified reaction of Sukaguchi that allows measuring the yellow-colored derivative of arginine upon its interaction with thymol and sodium hypobromite in basic medium (Sastry and Tummuru. 1984). Ammonium produced in that reaction, instead of the yellow color typical of arginine, gave blue staining (indophenol reaction).

To carry out the assay 100 μ l of 5 mM arginine hydrochloride in PBS (150 mM NaCl, 10 mM Na-phosphate buffer, pH 7.4) were taken, to which a sample under study (2.5, 5 or 10 μ l) was added and incubated for 30 min at 37°C. The reaction was stopped by adding 50 μ l of 100 mM thymol and 100 μ l of 40 mM sodium hypobromite diluted in 2M NaOH. The reaction was run in 96-well plates, and the optical density was registered in a plate spectrometer at 450 nm. Calibration curve reflecting the dependence of absorption at 450 nm on arginine concentration (0.25 – 5.0 mM) was plotted for each separate experiment. Enzymatic activity was expressed as μ M of arginine utilized by the enzyme with a correction for an added sample volume. Specific activity was related to the bulk protein (mg).

To determine K_m the amount of arginine utilized by AD was measured within the range of concentrations 1-3 mM in sodium-phosphate buffer, pH 6.8 containing 1 μ g/ml AD during 30 min. A graph in Hanes-Woolf coordinates $[Arg]$; $[Arg]/V$ was plotted, V_{max} and K_m were calculated.

Isolation of AD from SDSC

To isolate AD several sequential chromatographies were used. First, SDSC diluted with water 1:5 was loaded on an UNO-Sphere Q column (18 $\times$ 2.5 cm) equilibrated with PBS/5 and the ballast proteins were washed out with PBS/5. This was followed by elution with two linear gradients i.e. 40 ml of PBS/5 $\rightarrow$ PBS and 50 ml of PBS $\rightarrow$ 1M NaCl. The fractions displaying AD activity were concentrated to 0.7 ml in a VivaSpin20 cell (retaining proteins from 30 kDa).

This sample was loaded on a Sephacryl S-200 HR column (115×1 cm) equilibrated with PBS. The fractions with AD activity were diluted with water 1:5 and applied on a column (5×1 cm) with arginine-Sepharose equilibrated with PBS/5. Proteins were eluted with stepwise gradient, each step consisting of 5 ml of PBS/5, PBS/4, PBS/3, PBS/2 and PBS. AD activity was found in the PBS/2 fraction that was concentrated in a VivaSpin20 cell to 1.2 ml. An aliquot of pure enzyme (10 µg) was used for another analysis on mass spectrometer. 100 mg of bovine serum albumin were added to the remaining 140 µg of AD to stabilize the enzyme.

Statistical analysis

Experiments were repeated three times ($n=3$) and the mean values were calculated as $X_m=(1/n)\sum X_i$, where X_i is a value of each successive sample. The standard error was expressed as S^*/n , where $S^*=\sqrt{\frac{\sum (X_i - X_m)^2}{(n-1)}}$, and the confidence interval was calculated as $X_m \pm (S^*/n^{1/2})t_{n-1,1-\alpha/2}$, for which t was found in the table of values on condition that in our experiments $\alpha=0.05$.

RESULTS

Data on isolation from SDSC the protein possessing antiproliferative activity are summarized in Table 1. Analysis of mass spectra obtained for tryptic fragments of the 45-kDa band in PAAG revealed 9-10 peptides attributed to AD from various strains of GAS, M-types: 1, 2, 3, 4, 5, 6, 12, 18, 28 and 49 (Fig. 1, Table 2).

In the course of isolation, the protein demonstrated features (molecular mass, anionic character) that suggested its relation to an AD from some GAS strains, as judged by the data presented Degnan et al. 1998.

To reduce protein losses at purification we elaborated an arginine utilization-based assay of AD activity, done concurrently with the protein's isolation. The assay provided rapid detection of the AD-containing fractions. It is worth noting that, in contrast to arginine, ammonium produced in AD-catalyzed reaction gave blue staining (indophenol reaction) upon mixing with alkaline solutions of thymol and hypobromite. The yellow/blue shift of the solution color was a handy and reliable indication that a sample contained AD, but no other arginine-utilizing enzyme. Calibration plots reflecting the changes of absorption at A_{630} and A_{450} upon reaction of arginine, citrulline and ammonium with thymol and hypobromite, show that utilization of arginine allows revealing AD activity in fractions with the most precision. Absorption of the arginine derivative at 450 nm was ten times stronger than absorption of the ammonium derivative at 630 nm (Fig. 2, *a, b*).

Ion-exchange chromatography on UNO-Sphere Q allowed eluting AD with 150-200 mM NaCl. In the course of gel filtration its elution volume corresponded to that of proteins with M 150-160 kDa. When subjected to affinity chromatography, AD was eluted from arginine-Sepharose with 75 mM NaCl. Data on the step-by-step purification of AD are summarized in Table 3. The yield of AD was 53% as judged by the activity assay. Finally obtained 150 µg of AD amounted to 0.026% of the whole protein, which means 2030-fold purification.

Electrophoresis in SDS-containing PAAG showed that AD obtained is a homogeneous protein with M ~ 45 kDa (Fig. 3).

A study of the pH optimum for revealing the AD activity showed that the enzyme is most active at pH 6.8. At this pH its activity is equal to 83%, 88% and 86% of the maximum AD activity at pH 6.4, 7.2 and 7.8, respectively. Allowing for M ~ 45 kDa, the turnover number (k_{cat}) for AD obtained was calculated as 35 s^{-1} (Fig. 2, c).

Calculating K_m and V_{max} according to the Hanes-Woolf plot (Fig 2, d) gave the values of 0.67 mM and 42 s^{-1} , respectively. Table 4 contains the comparison of characteristics of AD purified from *S. pyogenes* M22 with such of the enzyme from other microorganisms. It is seen that a distinctive feature of the enzyme obtained in our study is the closest to neutral pH optimum of activity and the maximal rate of the catalytic process.

Our preliminary study showed that SDSC taken at the latest non-toxic dilution 1:50 significantly decreased cell proliferation to 60% of that in the control culture (Fig. 4, grey columns). Adding L-Arg to cell culture evened the inhibitory effect of SDSC (Fig. 5, grey columns). L-Arg at concentration 4 mM had the optimum effect. At increasing concentrations its capacity to restore cell proliferation went down.

AD isolated from SDSC suppressed the proliferative activity of endothelial cells in dose-dependent mode (Fig. 4, black columns). In view that the initial SDSC contained 30 µg/ml AD and that purified AD at concentrations 0.75 µg/ml and 0.375 µg/ml corresponded to SDSC diluted, respectively, to 1:400 and 1:200, it can be concluded that it is properly the activity of AD that underlies the antiproliferation effect of the SDSC. When L-Arg was added at increasing concentrations to the culture medium containing 3 µg/ml AD, the enzyme's capacity to inhibit cell proliferation became partly depressed, but the cells did not fully recover their proliferation activity (Fig. 5, black columns). On the one hand, this can be explained by the fact that L-Arg is not an inhibitor, but a substrate of AD. On the other hand, L-Arg itself improves cell proliferation, though at concentrations below 8mM. This seems to be due to the toxicity of arginine or its metabolites (ammonia and citrulline), since an excess of L-Arg resulted in a drop of cell proliferation to the level observed in L-Arg-free samples.

Studying the distribution of cells according to their cycling state showed that under standard culturing conditions, as well as when 4 mM L-Arg was introduced into the medium, about 60% cells remained in G₀/G₁ state, while some 30% were in the state of synthesis S/G₂ (Fig. 6).

In the presence of SDSC the portion of cells in G₀/G₁ significantly increased to 80% with concomitant diminution of the share of cells in S/G₂ to 10%. Similar, though less pronounced, changes (increased portion of G₀/G₁ cells and reduced portion of S/G₂ cells) were observed upon adding 3 µg/ml AD to the culture medium.

DISCUSSION

The arginine deiminase system is widely spread among prokaryotes. It contains three principal enzymes i.e. AD, ornithine-carbamoyltransferase and carbamate kinase. AD is a hydrolase converting L-Arg to NH₃ and L-citrulline (Cunin et al. 1986). NH₃ generated by L-Arg degradation increases pH in the environment, therefore, protects bacteria in conditions of high acidity (Fenske et al. 1976; Casiano-Colon and Marquis 1988; Gruening et al. 2006). Enzymes purified from several bacteria inhibit proliferation of different types of eukaryotic cells, malignant in that number. (Altucci et al. 1966; Claesson et al. 1990; Miyazaki et al. 1990; Takaku et al. 1992; Takaku et al. 1995; Komada et al. 1997; Degnan et al. 1998; Gong et al. 1999, 2000; Park et al. 2003; Kuo et al. 2010; Fiedler et al., 2015).

Like many prokaryotes, *S. pyogenes* expresses AD and antiproliferative activity of the enzyme expressed towards eukaryotic cells was demonstrated. The extract of *S. pyogenes* M5 (Manfredo strain) cells inhibited in dose-dependent manner the peripheral blood mononuclear leukocytes' proliferation, induced under the influence of superantigens and mitogens. Isolation and purification of the component responsible for the antiproliferative effect from the extract of *S. pyogenes* cells was performed. It was shown that the inhibitor had a molecular weight of about 100 kDa, and consisted of two identical subunits with a molecular mass of 47 kDa. NH₂-terminal sequence of the protein showed up to 39.0% amino acid homology with AD from *Mycoplasma hominis*, *Mycoplasma arginini*, *Pseudomonas putida* and *Pseudomonas aeruginosa* (Degnan et al. 1998). AD isolated from *S. pyogenes* (Su strain) at concentration 3 µg/ml decreased to 50% inclusion of labeled thymidine (Yosida et al. 1987), which indicated the inhibition of DNA synthesis in BALB/3T3 cells. AD from *S. pyogenes* M49 strain 591 heterologously expressed in *E. coli* efficiently reduced glioblastoma cell lines' proliferation (Fiedler et al. 2015). The impact of AD on the endothelial cells' functions was studied only with the enzyme isolated from *Mycoplasma arginini*. Beloussow et al. showed that recombinant AD (rAD) from *M.*

arginini, expressed in the *E. coli* inhibited the proliferation, migration, tube-like structure formation by human umbilical vein endothelial cells (HUVEC) *in vitro* (Beloussow et al. 2002). These data were confirmed by observations of Park et al. that rAD suppressed the vascular growth *in vivo* in the model of chicken chorioallantoic membrane and in the model of Matrigel plug assays in mouse (Park et al. 2003). It was shown that active rAD destroyed actin filaments in the endothelial tip cells, and thus deregulated and reduced their migration ability. The enzyme caused enhancement of reactive oxygen species' production and induced caspase 8-dependent apoptosis of endothelial cells (Zhou et al. 2011).

Our own studies have revealed that the SDSC of *S. pyogenes* type M22 reduced adhesion, proliferation, migration and metabolism of endothelial cell line EA.hy926 (Starikova *et al.* 2012). In this work isolation and characterization of the component responsible for the antiproliferative activity of SDSC were accomplished. Our study showed that suppression of proliferation of endothelial cells observed upon adding SDSC from *S. pyogenes* M22 occurred due to the activity of AD.

L-Arg depletion from the medium is likely to be a major factor of suppression of eukaryotic cells' proliferation in the presence of AD. An additional argument in favor of this statement is that the recovery of cell proliferation upon introducing exogenous L-Arg in the culture medium was observed. Deprivation of amino acids is known to entail the general nutritional starvation which, in turn, activates translation-inhibiting mechanisms in eukaryotic cells (Ye *et al.* 2010). Bulky evidence of the causal role of L-Arg deprivation in hampering cell proliferation has been accumulated (Saha and Kashina, 2011; Lee M. J., 2012). L-Arg deficiency caused by the activity of arginases results in activation of cytoplasmic GCN2 (general control nonderepressible 2) kinase in the presence of uncharged tRNA. It phosphorylates Ser51 in eIF2 α , which is followed by the overall inhibition of translation and cell cycle arrest in G₀/G₁ (Wek *et al.* 1990, 1995; Ramirez *et al.* 1992; Graller and Boye 2007; Hamanaka *et al.* 2005). Cyclin-dependent cdk4 and cdk6 kinases in mammalian cells are associated with D-type cyclins (D1, D2 and D3) and regulate the transition from G₁ to S phase of cell cycle. In case of L-Arg deficiency transcription of cyclin D3 mRNA goes down as well as stability of mRNA, and translocation of cyclin D3 to the nucleus becomes blocked (Raber *et al.* 2012; Morris 2012). These data can explain the results of our experiments in which adding L-Arg together with AD abrogated the anti-proliferative effect of the enzyme.

Despite the fact that adding exogenous arginine to the culture medium containing AD resulted in significant augmentation of endothelial cells' proliferation, the initial level was never recovered. The intensity of endothelial cells' proliferation in the presence of AD and L-Arg was significantly lower than in the presence of L-Arg alone, in all concentrations studied from 2 to 16

mM (Fig. 5), which is consistent with the data obtained by Park et al., 2003. In our experiments high concentrations (8 – 16 mM) of L-Arg added to the medium containing AD, unlike the lower ones, did not help cells to restore their proliferation, in contrast to the cells cultured in the absence of exogenous L-Arg. This indicates that cell proliferation went down affected by the L-Arg metabolites produced in reaction with AD. An important metabolite accumulated in culture medium as the result of L-Arg degradation by AD, is NH_3 (Komada et al. 1997; Miyazaki et al. 1990; Park et al. 2003). It seems likely that due to the high catalytic turnover of AD such concentrations of added L-Arg caused an increase of NH_3 in the medium. Ammonia induces the production of reactive oxygen and nitrogen species that intoxicate cells in the medium (Norenberg et al. 2003, Bobermin et al. 2012). It can be suggested that NH_3 can have similar toxic effect on the cells used in our study.

On the other hand the level of endothelial cells' proliferation after adding exogenous L-Arg in presence of AD did not reach the initial level due to the deficiency of L-Arg metabolites i.e. polyamines that should be produced upon its hydrolysis by eukaryotic arginase (Morrison et al. 1995, Morris 2009). Polyamines (small organic cations) seem to be essential for endothelial cell proliferation, even though the mechanism of their effect is not fully clear. They are supposed to play a role of importance for such fundamental cellular processes as signaling, replication and translation (Morrison et al. 1995; Li et al. 2002; Park et al. 2003; Rato et al. 2011; Landau et al. 2010). Thus, AD can affect protein synthesis not only by provoking amino acid starvation, but by inhibiting translation as well.

The AD from *S. pyogenes* type M22 purified in our study has high turnover (35 s^{-1}) and the pH optimum (6.8) closest to that of human blood plasma (7.4). Its specific activity is no less than the activity of other AD microorganisms (Table 4). Lower activity of AD obtained from strain M49 expressed in *E. coli* DH5 α (Hering et al. 2013) can be explained by the differences in protein glycosylation or by incomplete oligomerization of the recombinant protein. Indeed, the attempts to obtain a recombinant SAGP resulted in the reduced activity of such protein (Kanaoka et al., 1991).

To our knowledge this study is the first to describe the isolation of AD from *S. pyogenes* type M22 and to present its characteristics. Besides, the enzyme obtained had a pronounced antiproliferative activity. Concentration of L-Arg in human plasma is 80-120 $\mu\text{mol/L}$, which is of the same order that was maintained in the cultural medium during our experiments. In inflammation the need of L-Arg grows substantially (Morris 2009) and the conditions in our experiments (duration, AD content, L-Arg concentration), mimicking a case of infection, resulted in complete depletion of L-Arg in the medium. AD can be the pathogenic factor leading to L-Arg deficiency and endothelial dysfunction during streptococcal infection. At the same

time the ability of this enzyme to inhibit the proliferation of endothelial cells allows regarding it as a likely antiangiogenic factor. Studying streptococcal AD is topical not only for understanding the L-Arg metabolism deregulation during bacterial infection, but also for obtaining efficient enzymes degrading L-Arg, with the aim of their use in treatment of pathological processes associated with enhanced angiogenesis (Hering et al., 2013).

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LEGENDS TO FIGURES

Fig. 1. Mass spectrum of trypsin-digested 45-kDa protein possessing antiproliferative activity isolated from SDSC (Table 1).

Fig. 2. Calibration plot of the changes of A_{450} (a) and A_{630} (b) for arginine, citrulline and ammonium occurring upon their reaction with thymol and sodium hypobromite. Plot [Arg]; V (c) and Hanes-Woolf plot [Arg]; [Arg]/V (d) within the range of Arg concentrations 1-3 mM in sodium-phosphate buffer, pH 6.8 containing 1 µg/ml AD.

Fig. 3. Results of electrophoresis in SDS-containing PAAG. 1 – molecular mass markers, 2 – SDSC, 3 – a fraction displaying AD activity, eluted from UNO-Sphere Q, 4 – AD-containing fraction eluted from Sephacryl S-200 HR, 5 – AD obtained by affinity chromatography on arginine-Sepharose. Arrows mark M, kDa.

Fig. 4. Proliferative activity of the cell line EA.hy 926 in the presence of SDSC (grey columns) and in the presence of AD (black columns). Asterisk marks the statistically significant difference between the proliferative activity of the cell line EA.hy 926 in control group and the proliferative activity of the cell line EA.hy 926 in the presence of SDSC, $p < 0.05$. Grid marks the significant difference between the proliferative activity of the cell line EA.hy 926 in control group and the proliferative activity of the cell line EA.hy 926 in the presence of AD $p < 0.05$.

Fig. 5. Proliferative activity of the cell line EA.hy 926 in the presence of L-Arg (white columns), in the presence of both L-Arg and SDSC 1:50 (grey columns), and in the presence of L-Arg and 3 µg/ml AD (black columns). Asterisk marks the significantly higher cell proliferation in the presence of L-Arg than in the presence of both L-Arg and SDSC. Grid marks the significantly higher cell proliferation in the presence of L-Arg than in the presence of both L-Arg and AD.

Fig. 6. Distribution of EA.hy 926 cells according to their cycling state upon 24-hr culturing in: a) culture medium, isotype control; b) culture medium (control); c) culture medium with SLS 1:50; d) culture medium with 3 µg/ml AD; e) culture medium with 4 mM L-Arg; f)

culture medium with SLS 1:50 and 4 mM L-Arg; g) culture medium with 3 $\mu\text{g/ml}$ AD and 4 mM L-Arg. Asterisk marks the statistically significant difference from the control, $p < 0.05$.

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Table 1. Stepwise purification from SDSC of a protein with anti-proliferative activity

Step	Volume, ml	Protein, mg
1. SDSC	10	274
2. Chromatography on UNO-Sphere S	80	187
2. Chromatography on UNO-Sphere Q	15	8.6
3. Chromatography on UNO-Sphere Q	15	1.8
4. Concentration in VivaSpin20	0.2	1.2
5. Gel filtration on Sephacryl S-200 HR	5	0.05
6. Concentration in VivaSpin20	0.2	0.04

Draft

Table 2. Peptide fingerprint of the purified protein with anti-proliferative activity. Search results using MASCOT <http://www.matrixscience.com>. Start-end amino acids correspond to the sequence Arginine deiminase OS=Streptococcus pyogenes type M2

Start-End	Observed	Mr(expt)	Mr(calc)	Delta	Peptide
39-52	1663.8486	1662.8413	1662.8505	-0.0092	R.LLFDDIPFLEDAQK.E
194-206	1489.7577	1488.7504	1488.7514	-0.0010	K.YIFTHHPIYGGGK.V
230-239	1057.5761	1056.5688	1056.5928	-0.0239	K.DVLAVGISQR.T
248-254	846.5348	845.5275	845.5375	-0.0099	K.LLVNIFK.Q
255-260	706.3895	705.3822	705.3810	0.0013	K.QNLGFK.K
262-271	1180.6059	1179.5986	1179.6036	-0.0050	K.VLAFEFANNR.K
300-316	2081.0156	2080.0083	2080.0000	0.0083	R.VYSVTYDNEELHIIEEK.G
332-336	615.3856	614.3783	614.3752	0.0032	K.VDLIR.C
387-394	910.5106	909.5033	909.5032	0.0001	K.IHGSELVR.G
401-408	1000.4135	999.4062	999.3976	0.0086	R.CMSMPFER.E

Draft

Table 3. Stepwise purification of AD from SDSC

Step	Volume, ml	Protein, mg	Overall activity, $\mu\text{mol Arg/min}$	Specific activity, $\mu\text{mol Arg/min/mg protein}$	Purification, fold	Yield, %
1. SDSC	20	578	13.3	0.023	-	100
2. Chromatography on UNO-Sphere Q	30	15.7	10.6	0.68	30	80
3. Gel filtration on Sephacryl S-200 HR	15	3.2	8.4	2.63	114	63
4. Affinity Chromatography on arginine-Sepharose	5	0.16	7.2	45	1956	54
5. Concentration in VivaSpin20	1.2	0.15	7	46.7	2030	53

Draft

Table 4. Parameter of AD obtained as compared to those from the literature. <http://www.brenda-enzymes.org/enzyme.php?ecno=3.5.3.6> and Hering et al. 2013

AD from	M , kDa	k_{cat} , s^{-1}	Specific activity, μmol Arg/min/mg protein	Optimum pH	K_m , mM
GAS type M22	45	35	46.7	6.8	0.67
GAS type M49	47	-	1.5	6.5	1.33
<i>Bacillus cereus</i>	-	3.2	-	7.0	0.06
<i>Mycoplasma arthritidis</i>	80	29	27	6.5	10
<i>Pseudomonas plecoglossicida</i>	47	5.6	4.76	6	2.9
<i>Mycoplasma arginini</i>	90	-	44.5	6	0.2

Draft

Fig. 1. Mass spectrum of trypsin-digested 45-kDa protein possessing antiproliferative activity isolated from SDSC (Table 1).
130x71mm (300 x 300 DPI)

Fig. 2. Calibration plot of the changes of A450 (a) and A630 (b) for arginine, citrulline and ammonium occurring upon their reaction with thymol and sodium hypobromite. Plot [Arg]; V (c) and Hanes-Woolf plot [Arg]; [Arg]/V (d) within the range of Arg concentrations 1-3 mM in sodium-phosphate buffer, pH 6.8 containing 1 μ g/ml AD.
738x483mm (72 x 72 DPI)

Fig. 3. Results of electrophoresis in SDS-containing PAAG. 1 – molecular mass markers, 2 – SDSC, 3 – a fraction displaying AD activity, eluted from UNO-Sphere Q, 4 – AD-containing fraction eluted from Sephacryl S-200 HR, 5 – AD obtained by affinity chromatography on arginine-Sepharose. Arrows mark M, kDa.
74x85mm (300 x 300 DPI)

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161x100mm (300 x 300 DPI)

Fig. 5. Proliferative activity of the cell line EA.hy 926 in the presence of L-Arg (white columns), in the presence of both L-Arg and SDSC 1:50 (grey columns), and in the presence of L-Arg and 3 µg/ml AD (black columns). Asterisk marks the significantly higher cell proliferation in the presence of L-Arg than in the presence of both L-Arg and SDSC. Grid marks the significantly higher cell proliferation in the presence of L-Arg than in the presence of both L-Arg and AD.
161x106mm (300 x 300 DPI)

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162x121mm (300 x 300 DPI)