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ORIGINAL ARTICLE

Neuraminidase (sialidase) from *Aeromonas* sp. strain 40/02 – isolation and partial purification

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Abstract Neuraminidase is a virulence factor in many viruses and pathogenic bacteria. There are few reports of the detection of neuraminidase in the *Aeromonas* genus, but they do not include biochemical studies on the enzyme. In this work the neuraminidase production of *Aeromonas* sp. 40/02 strain and some properties of the enzyme were investigated for the first time. Neuraminidase was found in the supernatant, as well as in the cytosolic and membrane fractions of disrupted cells. It was demonstrated that the enzyme production is induced by a number of compounds structurally related to sialic acid. New data on the presence of extracellular and intracellular N-acylneuraminate lyase were obtained, suggesting a nutritional role of sialometabolism in this strain. For the first time, *Aeromonas* neuraminidase was isolated and partially purified by ion-exchange chromatography. Different purification profiles were registered depending on the growth media. The preparations containing neuraminidase activity were analyzed by native PAGE, and one of them was associated with an active protein band with a molecular weight of about 130 kDa. Our results suggest the presence of neuraminidase isoforms in *Aeromonas* sp. 40/02.

Keywords *Aeromonas* · Neuraminidase · Aldolase · Inducers

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Introduction

The genus *Aeromonas* belongs to the *Aeromonadaceae* family and recently separated from *Vibrionaceae* on the basis of molecular and genetic data (Murray et al. 2005). *Aeromonas* is a genus of Gram-negative bacteria, which are hemoorganotrophic and widespread in fresh, marine and waste waters, and soil as well as in drinking water (Havelaar et al. 1992). In the environment, these organisms can be part of the autochthonous microflora or have a non-environmental origin including a anthropogenic one. They are causative agents of diseases in invertebrate animals, fish, amphibians, reptiles, farm animals, and in humans. Usually in humans, they are associated with gastroenteritis and wound infections and can be detected in the gastrointestinal tract both in the presence and the absence of disease (Altwegg et al. 1989). These microbes produce a large number of enzymes (cell-bounded or extracellular), which are considered to be virulence factors, such as amylase, protease, chitinase, lipase, nuclease, and xylanase (Pemberton et al. 1997).

Neuraminidase, also called sialidase (N-acylneuraminosyl glycohydrolase EC 3.2.1.18), is an exo-glucosidase that cleaves α-glycosidically bound terminal sialic acid residues from various substrates like oligosaccharides, mucins, glycoproteins, glycolipids, etc. Such compounds are usually widely distributed in animal tissues. Many viruses and pathogenic bacteria possess neuraminidases. The action of these enzymes results in decreasing the viscosity of mucin substances, thus, supporting the penetration of the microbe in the tissues facilitating its adhesion to the host cells. On the other hand, removing sialic acid residues by neuraminidase leads to exposure of various receptors on the surface of host cells, which facilitates the binding of the microbe to it. Due to these effects of neuraminidase action, it is considered to be a virulence factor (Corfield 1992). Besides, neuraminidase is involved also in bacterial metabolism, supplying the cell with free sialic

acids as an alternative carbon, nitrogen, and energy source (Muller 1974; Almagro-Moreno and Boyd 2009). It is suggested that the enzyme enhances bacterial survival in natural habitats (sediments, mud) containing sialic acid compounds (Muller 1974). The ability of bacteria to utilize sialic acids is an advantage in competition with other species both in the host and in natural environments.

The next stage in catabolism of sialoconjugates is the degradation of sialic acid by the enzyme acylneuraminate lyase (aldolase, EC 4.1.3.3) to N-acetylmannosamine and pyruvate. This enzyme is found in microorganisms that contain sialic acids or neuraminidases, which supply microbes with sialic acids from external sources (Traving and Schauer 1998).

There are great amounts of data on production, isolation, and characterization of microbial neuraminidases in the literature, especially from pathogenic species like *Vibrio cholerae, Clostridium perfringens, Bacteroides fragilis*, and *Streptococcus pneumoniae* (Nakano et al. 2006; Xu et al. 2008; Almagro-Moreno and Boyd 2009; Li et al. 2011). To our knowledge, there are no publications concerning *Aeromonas* as a neuraminidase producer, except some publications, which report only the presence of the enzyme in a few *Aeromonas* strains (Vertiev et al. 1978; Kabir et al. 1984). Abrashev and Orozova (2004) and Orozova et al. (2007) investigated the distribution of some pathogenicity factors in *Aeromonas* spp., including neuraminidase. Recently, new evidence is emerging for the presence of enzymes, which are involved in sialometabolism in *Aeromonas*. For example, sialic acid synthase was found in *Aeromonas salmonicida* (Reith et al. 2008, NCBI reference sequence: YP_001140094.1). A neuraminidase gene was found and its corresponding amino acid sequence was determined recently in the strain *Aeromonas hydrophila* ML09-119 (Tekedar et al. 2013, reference sequence: AGM43219). Some studies on *Aeromonas* neuraminidase were carried out in 2010 (Engibarov et al. 2010). They were focused on screening for neuraminidase producers among different *Aeromonas* strains and specifying the proper conditions for enzyme production. The present work is a continuation of these studies. Its purpose is to acquire new data on the biochemical properties of neuraminidase from a strain of the genus *Aeromonas*.

Materials and Methods

Strain, media and cultivation conditions

The strain *Aeromonas* sp. 40/02 was isolated from the water environment National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria (N 8715) (web:www.nbimcc.org). It was cultivated in the following media: Brain Heart Infusion Broth (BHI), Nutrient Broth (NB), both from Difco, Detroit, MI, USA, pH 8.0, and Hottinger Broth (Bulbio, NCIPD, Sofia, Bulgaria, pH 7.6), at 30 °C, in aerobic conditions, statically. All the media mentioned above are used according to manufacturer's instructions, without addition of inducers.

The induction effect on neuraminidase secretion was studied as the producer strain was grown in a semisynthetic medium supplemented with each of the following inducers (in concentrations of 0.5 %): sialic acid (N-acetylneuraminic acid, Neu5Ac), N-acetylmannosamine (ManNAc), N-acetyl glucosamine (GlcNAc), transferrin, fetuin, casamino acids (all from Sigma chemical company), and glucomacropeptide (GMP) obtained by us in laboratory conditions (Abrashev et al. 1980). The GMP substrate was isolated from milk whey, a waste product from the milk manufacturing industry. It is rich in bound sialic acids (5–8 %), galactose, and galactosamine. The molar ratios of these compounds may vary between different batches of GMP due to the complex nature of the feedstock.

The composition of the semisynthetic medium was the following (in %): $(NH_4)_2HPO_4$ 0.2, NaCl 0.3, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.002, and yeast extract 0.05.

Enzyme and protein assays

The neuraminidase and aldolase activities were measured quantitatively by colorimetric determination of free sialic acid using the thiobarbituric acid method of Uchida et al. (1977). One unit of neuraminidase activity is defined as the amount that releases 1 μg of sialic acid (Neu5Ac) for 1 min under standard conditions using GMP as a substrate. One unit of N-acylneuraminic acid aldolase activity is defined as the amount that degrades 1 μg of Neu5Ac for 1 min under standard conditions.

Protein concentrations were measured by the Lowry procedure using bovine serum albumin as standard (Lowry et al. 1951).

Preparation of extracts for enzyme assays

The extracellular, cell-bound and the intracellular neuraminidase and aldolase activities were assayed as either the supernatant or the biomass was used. The strain was grown on BHI broth without addition of inducers. The culture liquid was centrifuged at 3,000 rpm for 20 min, and the cell-free supernatant was assayed for extracellular enzyme activities. The cell pellet was suspended in phosphate buffer saline (PBS), pH 7.2 and homogenized. After centrifuging at 5,000 rpm for 5 min, the supernatant from disrupted cells was collected and

subjected to further centrifugation at 14,500 rpm for 15 min. The supernatant (cytosolic fraction) and the pellet (membrane fraction) were assayed for intracellular and cell-bound neuraminidase and aldolase, respectively.

A 1.5 ml liquid sample was taken from the statically grown culture every 30 min, and its optical density (OD) and neuraminidase activity were estimated to observe the correlation between growth phases and enzyme production. Growth was estimated turbidimetrically and expressed as OD at 650 nm with a spectrophotometer (UV/VIS Spectrophotometer HElIOS b, UNICOM).

Isolation and partial purification of the enzyme

Aeromonas sp. 40/02 neuraminidase was isolated from stationary phase cultures in BHI, NB (pH 8.0), and Hottinger broth (pH 7.6). The culture liquid was harvested at 3,000 rpm for 20 min and the supernatant was gradually mixed with $(NH_4)_2SO_4$ to 100 % saturation. The precipitate formed after 18 h was collected by centrifugation at 3,000 rpm for 30 min. The pellet was dissolved in saline solution and dialyzed against distilled water for three days. The dialyzed solution was lyophilized.

Part of the dried substance was resuspended in 0.05 M PBS (10 mg/ml) and loaded onto an ion-exchange chromatography column with DEAE cellulose (Whatman; GE Healthcare, UK). The column resin was equilibrated with 0.05 M PBS, pH 7.5. Elution was performed in three steps with the following buffers: 0.05, 0.2, and 0.2 M PBS supplemented with 0.6 M NaCl, pH 7.5. Protein concentrations of column effluents were estimated by measuring absorbance at 280 nm (A280). Fractions of 2 ml were collected at a flow rate of 20 ml/h (LKB column chromatography).

All concentrations were carried out on a Amicon device with an Diaflo PM 30 membrane (Amicon Corporation, Lexington, MA).

The results were presented as the arithmetic mean of three independent experiments. The statistical error was approximately 2 %.

Native PAGE analysis

The crude extract and the active peaks eluted from the ion-exchange column were analyzed by native PAGE in 7.5 % separating and 4 % stacking gels. Prior to staining with Coomassie Brilliant Blue R250, neuraminidase was visualized in the gel according to Berg et al. (1985), using 2′-(4-methylumbelliferyl) α-D-N-acetyl neuraminic acid (4MU-Neu5Ac; Sigma-Aldrich, Buchs, Switzerland). The protein band containing neuraminidase emits bluish light when irradiated with UV light of a wavelength of 366 nm (UV-Box DESAGA Sarstedt-Gruppe, Germany). The molecular weight of the active band was assessed using a PAGE ruler Plus Prestained Protein Ladder 10–250 kDa, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA.

Results and Discussion

Dynamics of enzyme accumulation in growth phases

In our previous study on neuraminidase in *Aeromonas*, we selected the producer strain 40/02 and optimized the conditions for its maximum neuraminidase production (Engibarov et al. 2010). In the present work, aiming to observe the relation of enzyme production to the growth phases, we cultivated the producer strain in two types of nutrient media—BHI and Hottinger Broth. The bacterial growth in these media and the accumulation of the enzyme in the culture liquid are presented in Fig. 1. The growth phases in both media were of similar duration, but the optical density in BHI reached higher levels. The exponential phase was between the third and the ninth hour of cultivation, and neuraminidase became detectable in low levels during these hours. Significant enzyme accumulation was observed in the late stationary phase and the death phase with maximum values about the 48th hour, which, in the case of cultivation in BHI, was associated with reduction of optical density.

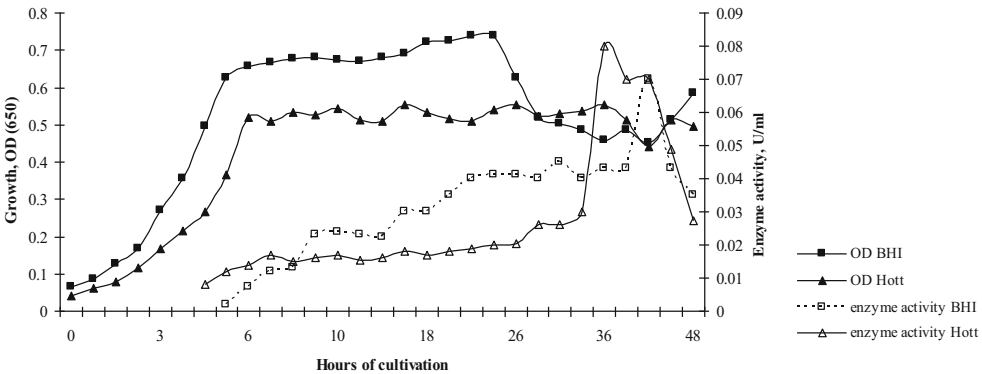

Fig. 1 Growth and neuraminidase activity of *Aeromonas* sp. 40/02 in BHI and Hottinger Broth

Table 1 Neuraminidase and aldolase activity of *Aeromonas* sp. 40/02

Enzyme	Extracellular Supernatant	Cell-bound Membrane	Intracellular Cytosol
Neuraminidase, U/ml	6.25	62	82
Aldolase, U/ml	98	–	114

Usually, the entrance into the stationary phase is accompanied by a decrease of enzyme activity that may be a consequence of protease secretion. Such a feature of neuraminidase production is shared by some streptococci from group A and B, *Streptococcus oralis*, and *Pseudomonas aeruginosa* (Milligan et al. 1977; Davis et al. 1979; Straus and Portnoy-Duran 1983; Byers et al. 2000; Ghazaei et al. 2010a, b). Although quite rare, in some *Arthrobacter* strains, maximum neuraminidase production occurs during the lag-phase with a significant reduction of enzyme activity as bacteria enter the exponential phase (Flashner et al. 1977). To some extent, our results resemble those obtained with *Erysipelothrix rhusiopathiae* where maximum enzyme accumulation has been achieved in the middle of the stationary phase (Wang et al. 2005). Similar data were reported also for *Pasteurella multocida* in which maximum enzyme production was observed between the 50th and the 70th hours (late stationary phase). It was accompanied by a decrease of the optical density (Drzeniek et al. 1972).

Location of neuraminidase and aldolase in the cell

Increased enzyme accumulation in liquid culture of strain 40/02 during the late stationary phase may be explained by release of an intracellular neuraminidase due to cell lysis. In this regard, we studied the location of neuraminidase in the cell. Our results indicated the presence of both extracellular and cell-bound activity in the strain 40/02 (Table 1). The latter was concentrated predominantly in the soluble fraction (82 U/ml), which we consider as cytosol, and to a lesser extent in the membrane fraction (62 U/ml). Other studies in this respect have shown that neuraminidase can have a variety of locations, probably depending on its function. Most bacterial neuraminidases are secreted enzymes, but there are also cell-bound forms. Some of the latter are associated with the membrane or with the cell wall (*Haemophilus parasuis, Arcanobacterium pyogenes*, and *P. aeruginosa*), while others are cytosolic (*C. perfringens, P. aeruginosa*, etc.) (Jost et al. 2002; Lichtensteiger and Vimr 2003; Chiarezza et al. 2009; Ghazaei et al. 2010a, 2010b). In many cases a bacterial species can produce two or more neuraminidases, which are encoded by the same or by different genes and can have different locations (*C. perfringens, S. pneumoniae*, and *Vibrio cholerae*) (Pettigrew et al. 2006; Chiarezza et al. 2009; Eneva et al. 2011).

Another intriguing result was the detection of aldolase activity in the supernatant and in the cytosolic fraction (Table 1). Typically, aldolase is an intracellular enzyme, with some exceptions. For example, extracellular aldolase has been discovered in culture supernatants of *P. multocida* and *S. pneumoniae* strains (Hughes and Jeanloz 1964; Drzeniek et al. 1972). Moreover, the activity of the *P. multocida* extracellular aldolase is higher than it is in cell extracts (Drzeniek et al. 1972).

Our results established the presence of the enzyme both in culture supernatants and in the cell extracts of the A40/02

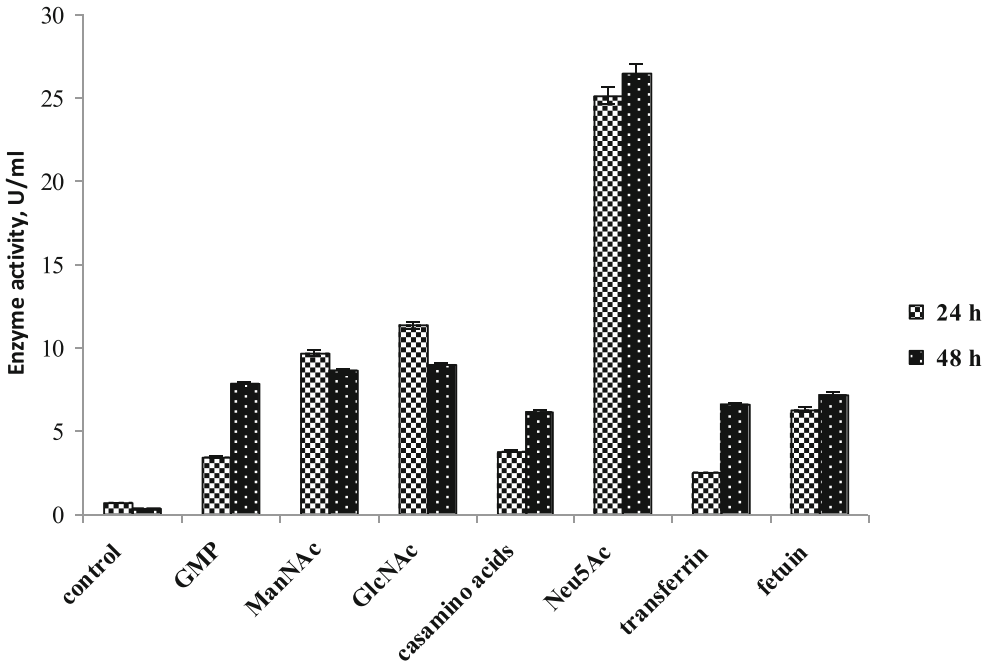

Fig. 2 Inductive effect of various compounds on *Aeromonas* sp. 40/02 neuraminidase production

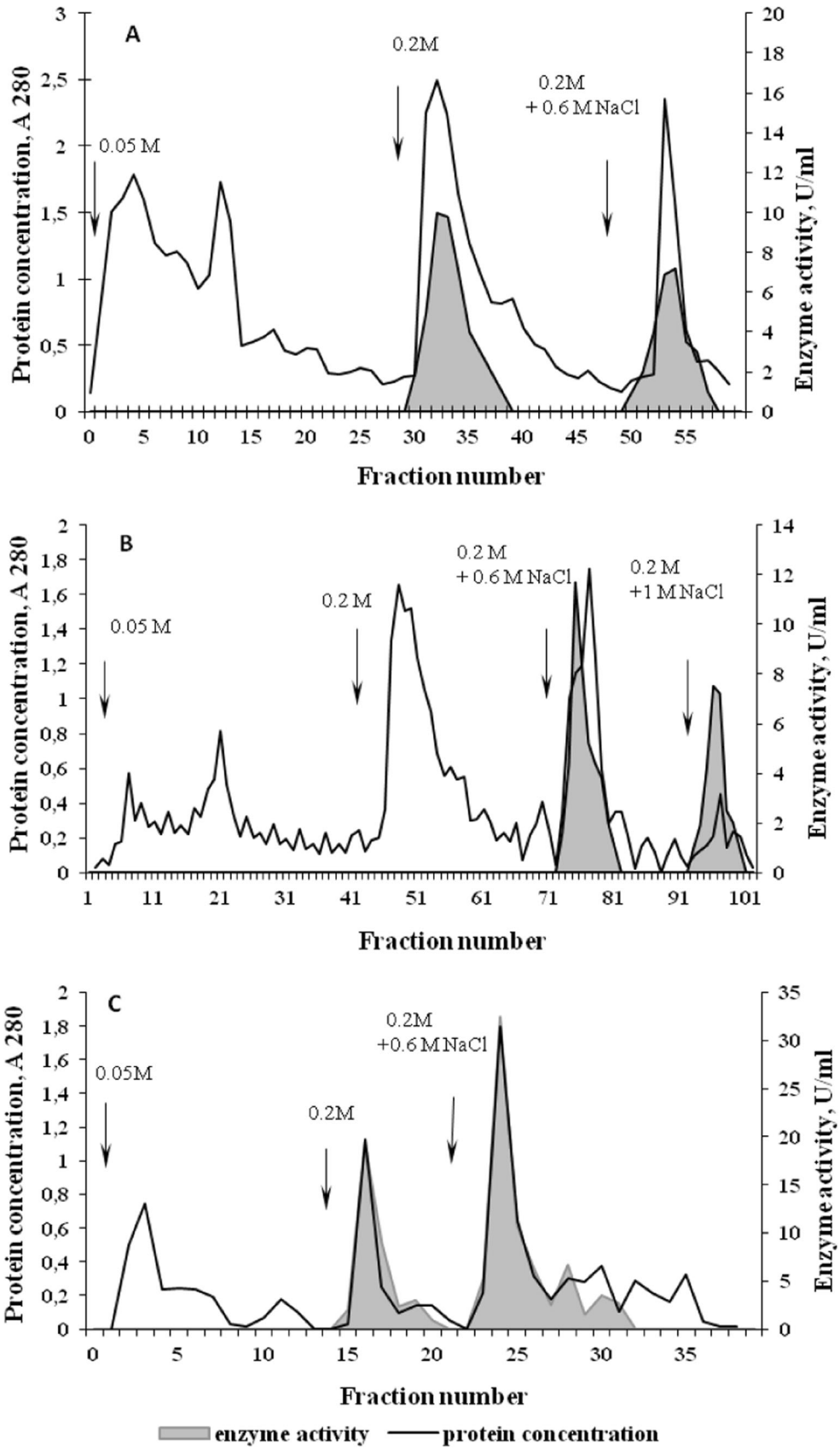

Fig. 3 Ion-exchange chromatography on a DEAE cellulose column. Different elution profiles resulting from cultures grown on BHI (**a**), NB (**b**), and Hottinger broth (**c**). Enzyme activity is represented by gray areas

strain. In the latter, aldolase was located in the cytosol and was not detected in the membrane fraction. Independently of its origin (culture supernatants or cell extracts), the enzyme demonstrated relatively high activity (98 and 114 U/ml, respectively) after 1 h incubation. By comparison, detectable extracellular aldolase activity in *C. perfringens* strains has been achieved only after 22 h incubation, while the intracellular aldolase activity in these strains is in accordance with our results (Fraser and Collee 1975). When the thiobarbituric assay is used for the detection of neuraminidase, the presence of aldolase can give misleading reduced or negative results. The reason is that aldolase degrades the product of the neuraminidase reaction. Therefore, it is possible that the real neuraminidase activity in the strain A40/02 was to a lesser or greater extent "obscured" by the presence of aldolase activity.

Aldolase was not detected in culture supernatant after 100 % saturation by ammonium sulfate. Some researchers have assumed for a similar purification scheme that at this stage of purification, the enzyme was partially or completely removed (Vertiev et al. 1975).

The inductivity of *Aeromonas* sp. 40/02 neuraminidase

Most studies determine bacterial neuraminidases as inductive enzymes. Rarely, some bacteria synthesize them in a constitutive manner, as some streptococci (*S. anginosus*, *S. constellatus*, and group B streptococci) (Milligan et al. 1977; Beighton and Whiley 1990). The constitutive synthesis of neuraminidases is usually typical for nonpathogenic representatives of intestinal microflora (Corfield et al. 1992).

A number of sialoglycoconjugates, aminosugars, free sialic acid, etc., are described in the literature as effective neuraminidase inducers (Corfield 1992). Our experimental data revealed that the addition of such compounds leads to stimulation of neuraminidase synthesis in the A40/02 strain (Fig. 2). According to our study, the effect of some of them is more pronounced at the 48th hour. For example, when glucomacropeptide (GMP) or casamino acids were added to the semisynthetic medium, the enzyme activity at 48 h was almost twice as high as the one at 24 h. When *Aeromonas* sp. 40/02 was grown in semisynthetic medium supplemented with sialoconjugates as fetuin or transferrin, there was a negligible inducing effect at the 24th hour for fetuin, and at the 48th hour for transferrin.

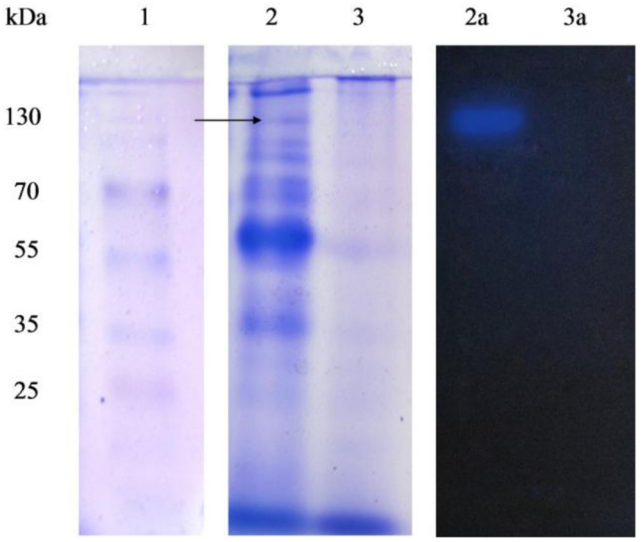

Fig. 4 Native PAGE of *Aeromonas* sp. 40/02 neuraminidase preparations obtained from BHI cultures by ion-exchange chromatography: *1* – protein marker; *2* – AN1 (peak II); *3* – AN2 (peak III); *2***a**, *3***a** – lanes 2 and 3 treated with 4MU-Neu5Ac

N-acetylmannosamine and N-acetylglucosamine, which are structurally related to sialic acid, exhibited a positive effect on neuraminidase production in *Aeromonas* sp. 40/02 at the 24th hour of cultivation. The significance of the first compound has been well-documented for *Gardnerella vaginalis, S. pneumoniae*, and especially for the *P. multocida* representatives (Drzeniek et al. 1972; von Nicolai et al. 1984; Mizan et al. 2000; Gualdi et al. 2012). The influence of these aminosugars is negligible in other microorganisms (*Arthrobacter* spp., *E. rhusiopathiae*) (Wang et al. 1978; Abrashev and Orozova 2006).

The neuraminidase production in A40/02 was induced to the highest extent when sialic acid was added to the medium. Such results are in accordance with a number of investigations that establish the positive influence of this compound on neuraminidase synthesis in *Arthrobacter sialophilus*, *C. perfringens*, *Micromonospora viridifaciens*, *Tannerella forsythia*, *S. oralis*, *P. aeruginosa*, and *S. pneumoniae* (Wang et al. 1978; Bouwstra et al. 1987; Aisaka et al. 1991;

Table 2 Purification steps for *Aeromonas* sp. 40/02 neuraminidase preparations AN1 and AN2

Purification steps		Volume (ml)	Activity (U/ml)	Total activity ($U \cdot 10^3$)	Protein (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Culture liquid		1,600	7.8	12.48	12	0.65	100	1
Saturation with 100 % $(NH_4)_2SO_4$		130	38.4	4.992	3.5	11	40	17
DEAE cellulose column	AN1	6.3	57.5	0.362	2	28.75	2.9	2.6
	AN2	7.4	23.75	0.175	0.68	34.9	1.4	3.2

Byers et al. 2000; Thompson et al. 2009; Ghazaei et al. 2010a, 2010b; Gualdi et al. 2012). However, there are microorganisms, in which the inductive effect of sialic acid is negligible (some *Arthrobacter* representatives) (Flashner et al. 1977).

Our results suggest that A40/02 neuraminidase is an inductive enzyme. Taking into account that the inductivity of these enzymes is often related to pathogenicity and nutrition of the producer. It can be assumed that neuraminidase in the strain A40/02 is related to the possible pathogenic potential of this bacterium and has also a nutritional role (Corfield 1992).

Isolation and partial purification of the enzyme

Three types of nutrient media were used to isolate the extracellular neuraminidase of *Aeromonas* sp. 40/02—BHI, NB, and Hottinger Broth. An interesting relation between cultivation media and enzyme activity of the eluted peaks from the ion-exchange chromatography column was observed. When the first buffer (0.05 M PBS) was applied, the obtained protein peak always lacked neuraminidase activity, regardless of the nutrient source. The overall elution profiles with BHI and Hottinger Broth were similar. There were two active peaks – peak II and peak III. Peak II was eluted with 0.2 M PBS and peak III was eluted with 0.2 M PBS with 0.6 M NaCl. In the case of BHI, the activity of peak II was higher than that of peak III, and the opposite results occurred when Hottinger Broth was used (Fig. 3). When the strain was grown in NB, the first (eluted with 0.05 M PBS) and the second (eluted with 0.2 M PBS) peaks were neuraminidase negative. The fractions, which were eluted by 0.2 M PBS with 0.6 M NaCl (peak III) and by the same buffer with 1 M NaCl (peak IV), were neuraminidase positive. The majority of enzyme activity was related to peak III.

The results from the purification steps when the producer strain was grown in BHI are listed in Table 2. The dialyzed crude extract obtained after 100 % $(NH_4)_2SO_4$ saturation and the active peaks II and III, eluted from the ion-exchange column, which we designated as *Aeromonas* neuraminidase 1 and 2, respectively, (AN1, AN2) were analyzed via native PAGE. All samples were derived from an *Aeromonas* sp. 40/02 culture grown in BHI. The result revealed a significant degree of purification, but still not enough to obtain single protein bands. Treating the gel with the fluorogenic substrate 4MU-Neu5Ac, we could visualize only the active band of AN1, and its molecular weight was, thus, estimated to be about 130 kDa. Although containing relatively high enzyme activity (23.75 U/ml), AN2 failed to demonstrate the characteristic bluish light (Fig. 4). This may be a result of low or no affinity of AN2 towards 4MU-Neu5Ac. We can speculate that the different active preparations obtained by ion-exchange chromatography contain neuraminidase isoforms, for which production depends on the media content. Probably, the different nutrient sources influence the synthesis of particular isoforms with defined biochemical properties. It is also possible that the culture liquid subjected to purification contains both extracellular and intracellular neuraminidases that may be encoded by different genes. The presence of isoforms of a different gene origin and/or different cellular location may explain, for example, the different behavior of neuraminidases AN1 and AN2 towards the 4MU-Neu5Ac substrate. These hypotheses are to be clarified by further experiments.

Our results implicate the presence of neuraminidase and aldolase in a representative of the *Aeromonas* genus. Both enzymes are expressed as exogenous and endogenous forms. We suggest that *Aeromonas* sp. 40/02 may possess neuraminidase isoforms. It is possible that this enzyme plays a dual role in pathogenicity and nutrition of the studied bacterium. Further purification of *Aeromonas* sp. 40/02 neuraminidase is needed to determine its biochemical properties. Studying sialometabolism in the genus *Aeromonas* would reveal new aspects of its biology and probably some mechanisms of its pathogenicity.

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