Erysipelothrix rhusiopathiae Neuraminidase and its Role in Pathogenicity

Ignat Abrashev* and Petya Orozova

Bulgarian Academy of Sciences, Institute of Microbiology, Department of Microbial Biochemistry, 26 Acad. G. Bonchev str., 1113 Sofia, Bulgaria. Fax: ++ 3592870010 9. E-mail: abrashev@microbio.bas.bg

* Author for correspondence and reprint requests

Z. Naturforsch. **61c**, 434–438 (2006); received November 7/December 7, 2005

The role of the enzyme neuraminidase in pathogenicity of the bacillus *Erysipelothrix rhusiopathiae* was studied. Different substances with low and high molecular weight were tested as inducers of *E. rhusiopathiae* neuraminidase biosynthesis. It was found that macromolecular complexes induce the secretion of the enzyme. K_M values for different substrates showed that the affinity of the *E. rhusiopathiae* neuraminidase increases in parallel with the enlargement of the molecular weight of glycoproteins. Results from the rabbits skin test confirmed the role of *E. rhusiopathiae* neuraminidase as a factor of pathogenicity with spreading functions.

Key words: Neuraminidase, *Erysipelothrix rhusiopathiae*, Pathogenicity

Introduction

Erysipelothrix rhusiopathiae is a Gram-positive, non-spore-forming bacillus that causes a variety of diseases in many species of mammals, including erysipelas in swine, chickens, and farmed turkeys, and polyarthritis in sheep and lambs. Human infection is commonly seen in the form of erysipeloid (a superficial bacterial skin infection that characteristically extends into the cutaneous lymphatics) and, less frequently, endocarditis as a consequence of contact with the infected animals, their products or waste. Various virulence factors have been suggested for *E. rhusiopathiae.* One is neuraminidase, an enzyme responsible for the cleavage of sialic acid from sialo-glyco-conjugates such as glycoproteins, glycolipids, and oligo- and polysaccharides.

Until recently there was very limited information on the pathogenic mechanisms of *E. rhusiopathiae* infection due to the fact that toxins and enzymes related to the pathogenicity of this bacterium were unknown. However, extensive data exist in literature about the clinical symptoms as well as pathoanatomic changes caused by this infect. The discovery by Müller (1971) of the enzyme neuraminidase, produced by *E. rhusiopathiae,* was an important step towards the detailed study of the disease. During further investigations (Müller and Böhm, 1973; Nikolov *et al.*, 1978) neuraminidase production was discovered to be a permanent characteristic of this microbial species. Limbeck and Böhm (1974) for the first time isolated a toxin from the erythema causing bacterium, a fact that contributed to the study of the pathogenic mechanisms.

Toxin production in many pathogenic bacteria as well as toxin absence in saprophytic forms makes it possible to relate the characteristic pathogenicity to the ability to produce biologically active molecules (Ezepchuk, 1977). Therefore, the realization of the process of infection requires besides one of the attributes of pathogenicity – the toxic function – also macromolecules with a specific type of activity.

The role of toxins at the final stage of the infectious process does not allow the explanation of the interaction between stimulant and microorganism at the early stage of infection. At this stage macromolecules with enzymatic activity are acting as a means for influence of the microorganism upon the host. Biochemical activities of various macromolecules produced by pathogenic bacteria affecting host macroorganisms in different ways characterize them as factors of pathogenicity.

In view of the elucidation of some pathological mechanisms of infection and based on the already published data our objective was to elucidate the role of neuraminidase as one of the factors of pathogenicity which affect the physical properties of tissue matrices and intercellular spaces, thereby promoting the spread of the pathogen and its sub-

0939–5075/2006/0500–0434 $ 06.00

strate affinity towards low- and high-molecular weight substances.

Material and Methods

Strain

Research experiments were carried out with the strain *E. rhusiopathiae 401* serotype B from the collection of the Department of Microbial Biochemistry, Institute of Microbiology, Bulgarian Academy of Sciences. The initial strain was cultivated on Trypticase Soy Agar (TSA) (Scharlau Chemie S.A., Spain).

Cultivation conditions

A semi-synthetic culture medium with the following composition was used: 0.2% $(NH_4)_2HPO_4$, 0.3% NaCl, 0.1% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 0.05% yeast extract. To this medium the inductors *N*-acetyl-D-manosamine (Koch-Light, England), *N*-acetyl-neuramic acid (Sigma), χ-casein (Reanal, Hungary), casaminic acid (Difco, USA) and glucomacropeptide (GMP) (Bulg. Patent No 29145, 1981) were added, each to a different final content (0.25; 0.50; 1.0%), as single carbon sources. The pH value of the medium after sterilization was 7.6.

The bacterial growth was defined during experimentation through measurement of the absorbance at 540 nm.

Assay of neuraminidase activity

Neuraminidase activity was established quantitatively according to Aminoff (1961). One unit of neuraminidase activity is defined as the amount that releases 1 μg of *N*-acetyl-neuraminic acid for 1 min under standard condition using glucomacropeptide as a substrate.

Constant of K_M

K_M values were determined by the method of Lineweaver and Burk (1934).

Irritation skin test

The *E. rhusiopathiae* neuraminidase was assessed as pathogenic factor with spreading functions using the test method of McClean (1943) with slight modification. Six healthy albino New Zealand rabbits, weighed (3.080.05) kg, were selected for the study. Each rabbit was caged individually and there was no restriction on food and water supply during the test. 2 d before each study the hair was cut from a 12 cm × 12 cm area on the back of each rabbit using a hair clipper. 1 d before each study, a depilatory (Carter-Wallace, Mississanga, ON, Canada) was applied for 15 min to the 12 cm × 12 cm area on the back and then removed. During the preparation of the rabbits backs 1 d before the study commenced, a veterinarian inspected the rabbits carefully. No visual skin irritations were observed. Rabbits were divided into three groups. Rabbits from the first group were injected intradermally with 500 μl of an solution containing equal parts of pure *E. rhusiopathiae* enzyme (40 U/ml) and 0.5% (w/v) solution of trypan blue in 0.9% saline. Rabbits from the second group were injected intradermally with 0.5 ml of an solution containing equal parts of pure *E. rhusiopathiae* enzyme (20 U/ml) and 0.5% (w/v) solution of trypan blue in 0.9% saline. Rabbits from the third (control) group were injected intradermally with 500 μl of an solution containing equal parts of pure inactivated *E. rhusiopathiae* enzyme (40 U/ml) and 0.5% (w/v) solution of trypan blue in 0.9% saline.

The test skin areas were observed for erythema and edema at 24 and 48 h after injection of the solutions. The used enzyme was isolated and purified neuraminidase from *Erysipelothrix rhusiopathiae* strain B_{401} according to Abrashev (1988).

Results and Discussion

After a comparative study of the stimulating effect of several different substances upon biosynthesis of extracellular neuraminidase in *E. rhusiopathiae* we found that *N*-acetyl-D-manosamine and *N*-acetyl-neuraminic acid, considered as universal inducers by some authors, did not show an inducing effect in this case. However, the macromolecular complexes used in our study – χ-casein, casaminic acid and glucomacropeptide (GMP) – proved to be very effective enzyme inducers. The highest stimulating effect was found by application of GMP (Fig. 1). Data by Pardoe (1974) showed a more prominent stimulating effect of the macromolecular substances upon induction of neuraminidase in pathogenic microorganisms. Analogical results were obtained by Abrashev *et al.* (1977) by studying the influence of blood serum on biosynthesis of the enzyme in *E. rhusiopathiae. N*-Acetyl-D-manosamine and *N*-acetyl-neuraminic acid were

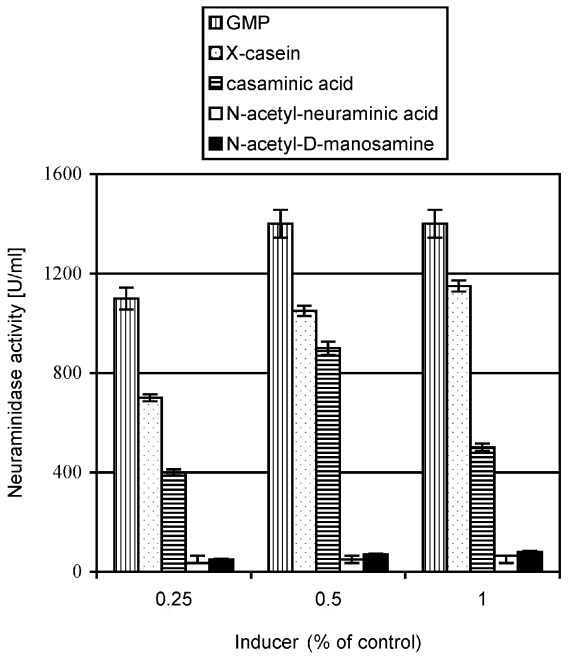

Fig. 1. Effect of different contents of some inducers on *Erysipelothrix rhusiopathiae* neuraminidase production.

demonstrated to have an inducing effect on the enzyme's secretion in apathogenic bacteria as *Arthrobacter nicotianae* (Abrashev *et al.,* 1998). This allows the assumption that most probably the inducing effect has different mechanisms in *E. rhusiopathiae* and *Arthrobacter nicotianae.*

The ability of macromolecular complexes to induce the synthesis of the enzyme neuraminidase in pathogenic microorganisms may be regarded as a means for their adaptation and attacking the host. This hypothesis is corroborated by the specificity of bacterial neuraminidases towards the one or other type of bonds in the substrate, while the rate of hydrolysis varies for the different substrates depending on their molecular size, hydrophobic or hydrophilic character.

Enzyme-substrate interactions of neuraminidase from *E. rhusiopathiae* were characterized using several substrates such as GMP, χ-casein and casaminic acid (Fig. 1). After comparison of the values of the Michaelis constant for the different substrates an increase of enzyme's affinity with the increase in molecular weight of glycoproteins has been found (Table I). In this respect the neuraminidase from *E. rhusiopathiae* is a typical model of neuraminidases produced by bacteria with high

Table I. K_M values of purified neuraminidase from *E. rhusiopathiae* B_{401} strain for different substrates.

Substrate	M_r	K_M [M]
Sialil lactose (Fluka AG, Buchs SG, Switzerland)	633	8.6×10^{-4}
GMP (Bulg. Patent No 29145, 1981)	7000	2.4×10^{-4}
Colominic acid (Fluka AG, Buchs SG, Switzerland)	30000	1.9×10^{-5}
Fetuin (Koch-Light Lab. Ltd, England)	45548	1.3×10^{-5}
χ-Casein (Reanal, Budapest, Hungary)	60000	4×10^{-5}
Transferrin (Sigma-Aldrich, Spain)	90000	9×10^{-6}

pathogenicity, which have higher affinity towards large substrates, present in all tissues and macroorganism fluids. These data could explain some molecular mechanisms of neuraminidase participation in *E. rhusiopathiae* pathogenicity.

Independently of lack of specificity of neuraminidases towards the substrate glycoside bonds to be cleaved some preferences still exist. The neuraminidase isolated from *E. rhusiopathiae* cleaves the α-2,3 bonds with higher speed than the α-2,8 bonds. A similar finding was reported by von Nicolai *et al.* (1978) for neuraminidase from the same species.

Experiments were conducted to establish the role of the enzyme as a factor of pathogenicity with propagation function. A diffusion check was done according to McClean (1943) with rabbits through application of the purified enzyme from *E. rhusiopathiae* strain B_{401}. The data showed that surface diffusion of trypan blue in intradermal samples depended on the concentration of the injected enzyme (Table II). The difference between test and control animals showed up already a few hours after injection. At the skin area where the enzyme was administered an inflammation reaction with local edema was observed followed by infiltrate formation. In control animals injected with trypan blue, physiological solution and inactivated enzyme only a small swelling was observed which dispersed in a few hours. The largest area of diffusion of the colorant was obtained at the enzyme concentration of 40 U/ml 48 h after the infiltrate slowly disappeared. Ezepchuk *et al.*

Group	Number of rabbits (*n*)	Concentration of neuraminidase [U/ml]	Skin diffusion area ± S.D. 24 h [cm^2]	Skin diffusion area ± S.D. 48 h [cm^2]
I	2	40	2.0 ± 1.7	2.4 ± 1.56
II	2	20	1.3 ± 0.48	1.8 ± 0.12
III	2	Inactivated enzyme	0.2 ± 0.10	0.1 ± 0.09

Table II. Correlation between diffusion area of injected trypan blue and concentration of *Erysipelothrix rhusiopathiae* neuraminidase.

(1973) reported similar results for the neuraminidase from *Corynebacterium diphteriae*.

A protective effect of an antineuraminidase serum was established previously by us during the experimental infection of white mice with *E. rhusiopathiae*. The antineuraminidase serum specifically blocked the enzyme and protected a high percentage of the animals (80%) from septicemia and death, while from control animals not a single mouse survived. Müller (1971) obtained similar results from similar experiments. Ifeanyi and Baitil (1992) demonstrated the passive protection of mice with antiserum against neuraminidase from *Pasteurella multocida*. These studies show that pathogenicity depends to a high extent on neuraminidase production.

As a result of another study of ours we proved in genetically related strains of *E. rhusiopathiae* that the neuraminidase acitivity was lower in avirulent chloramphenicol resistant strains compared to startin strains. Nikolov *et al.* (1978) found that *E. rhusiopathiae* substrains with higher virulence demonstrate higher neuraminidase activity as well. According to Wang *et al.* (2005) there was no neuraminidase activity among apathogenic *Erysipelothrix* spp. Influence of pure neuraminidase on cell cultures shows that the cell damages are in consequence of the enzyme activity while contributed bacterial invasion (Abrashev *et al.,* 1988).

The current investigation confirms the important role of the neuraminidase in *E. rhusiopathiae* infection.

Acknowledgements

This work was supported by a grant from Bulgarian Ministry of Sciences and Education (B-1313). The authors thank the Bulgarian Ministry of Sciences and Education for supplying a grant to I. Abrashev.

Abrashev I. (1988), Isolation and purification of neuraminidase from *Erysipelothrix rhusioathiae*. Acta Microbiol. Bulg. **22**, 33–38.

Abrashev I., Nikolov P., Todorov D., and Avramova T. (1977), Effect of blood serum on neuraminidase biosynthesis in the case of *Erysipelothrix rhusiopathiae*. Acta Microbiol. Bulg. **3**, 73–76.

Abrashev I., Velcheva P., Andreeva S., and Bozhkov S. (1988), The action of neuraminidase from *Erysipelothrix rhusiopathiae* on cell cultures. Acta Microbiol. Bulg. **22**, 3–8.

Abrashev I., Genova V., Sotirova A., and Ilieva K. (1998), Purification and partial characterization of neuraminidase from non-pathogenic *Arthrobacter nicotianae*. Enzyme Microb. Technol. **22**, 142–146.

Aminoff D. (1961), Methods for quantitive estimation of *N*-acetylneuraminic acids and their application to hydrolysates of sialocompoids. Biochem. J. **81**, 384–392.

Ezepchuk Y. (1977), Biomolecular bases of bacterial pathogenicity. Moscow Russ. Acad. Sci., 28–31.

Ezepchuk Y., Vertiev Y., and Kostoyukova N. (1973), Neuraminidase of *Corynebacterium diphteriae* as factor of pathogenicity with spreading function. Bull. Exp. Biol. Med. **75**, 63–65.

Ifeanyi F. L. and Baitil W. E. (1992), Passive protection of mice with antiserum to neuraminidase from *Pasteurella multocida*. Vet. Res. Commun. **16**, 97–105.

Limbeck R. and Böhm K. H. (1974), Über ein Toxin bei Rotlaufbakterien. In: Vortrag 5, Arb. Tag. Dtsch. Ges. Hyg. Microbiol. Mainz, Germany. **10**, 3–5.

Lineweaver S. and Burk D. (1934), The determination of enzyme dissociation constant. J. Am. Soc. **56**, 658–666.

McClean D. (1943), Studies of diffusings factors. III. Methods of assay of hyaluronidase and their correlation with skin diffusing activity. Biochem J. **37**, 169–171.

Müller H. E. (1971), Über das Vorkommen von Neuraminidase bei *Erysipelothrix insidiosa*. Path. Microbiol. **37**, 241–248.

Müller H. E. and Böhm K. H. (1973), Untersuchungen *in vitro* über die Neuraminidase bei *Erysipelothrix insidiosa*. Zentralbl. Bacteriol. Orig. [A] **233**, 220–227.

Nikolov P., Abrashev I., Ilieva K., and Avramova T. (1978), Virulence and neuraminidase activity of *Erysipelothrix rhusiopathiae*. Acta Microbiol. Bulg. **2**, 62–65.

Pardoe G. (1974), The inducible neuraminidases of pathogenic microorganisms. Behring Inst. Mitt. **55**, 103–112.

von Nicolai H., Müller H. E., and Zilliken F. (1978), Substrate specificity of neuraminidase from *Erysipelothrix rhusiopathiae*. Hoppe-Seylers Z. Physiol. Chem. **359**, 393–398.

Wang Q., Chang B., Mee B., and Riley T. (2005), Neuraminidase production by *Erysipelothrix rhusiopathiae*. Vet. Microbiol. **107**, 265–272.