

Original article

Characterization of tilapia sialidase and its significance in bacterial infection

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Keywords: *Edwardsiella tarda*; Sialidase; Tilapia.

Received: 17 July 2017 / Accepted: 10 September 2017

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Introduction

Sialic acids have been known to play important roles in biological processes. Their significances are divided into two ways; one renders glycoconjugates physicochemical effects such as hydrophilicity, acidic and electronegative nature, and the other acts as receptor or antirecognition agent of molecules and cells.

Sialidase is a family of glycosidase removing sialic acid from the terminal end of sugar chain on glycoproteins and gangliosides. Sialidase exists in a wide variety of organism from microorganism to vertebrates [1].

Edwardsiella tarda is causative pathogen of Edwardsiellosis which is one of serious bacterial diseases in both freshwater and marine fish. Edwardsiellosis brings about high mortality in both farmed and wild populations while there are not therapeutic approaches and vaccines. Our previous report revealed that NanA sialidase, endogenously expressed in *E. tarda*, is helpful for invading into host cells [2]. Indeed, *E. tarda* infection was downregulated by the inhibition of NanA sialidase [3]. Taken together, we focused on the effect of “host” sialidases on *E. tarda* infection, endogenous sialidase could regulate bacterial invasion through desialylation. However, fish sialidases were not fully analyzed. Therefore, we focused on tilapia, which is one of most targeted species of *E. tarda* infection and useful to be utilized for molecular biological study because their genome sequence was disclosed.

Materials and methods

Animals and tissue preparation

Wild tilapias (*Oreochromis niloticus*) were sampled from Ibusuki River (Ibusuki, Japan) for using in this study. Tissues excised from each fish were immediately frozen by dry ice and stocked at -80°C until usage.

Molecular cloning of tilapia sialidase gene

Total RNA was prepared from the tilapia brain using Sepasol-RNA I Super G (Nacalai Tesque, Japan). Synthesis of cDNA was done by ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). PCR was carried out using the sequence specific primers based on putative tilapia sialidase sequences from bioinformatics analysis. Gene amplifications were carried out using cDNAs from a few individual tilapias. The amplified products were digested with specific restriction enzymes, sub-cloned into pBluescript vector and analyzed by ABI3130xl Genetic Analyzer (Applied Biosystems, USA). After confirming their sequences, the cDNAs were transferred to expression vector pcDNA for the transfection.

Cell culture and temporary gene expression

HEK293T (human embryonic kidney cells), Hela (human cervical cancer cells), GAKS (goldfish scale fibroblast) and Hepa-T1 (tilapia hepatic cells) were obtained from RIKEN CELL BANK (Osaka, Japan). HEK293T, Hela and GAKS were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) with 5% CO₂ at 37°C and Hepa-T1 were cultured in E-RDF medium containing 15% FBS without CO₂ supply at 28°C. Expression vectors were introduced into mammalian cells by using a calcium phosphate method and to Hepa-T1 by using X-treme Gene HP DNA (Roche, Swiss).

Sialidase activity

Transfected cells were rinsed with Dulbecco's phosphate-buffered saline (PBS), curreted and made into pellet. The pellet were sonicated with PBS included 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml leupeptin, and centrifuged at 600 g for 5 min. Supernatant was used

for sialidase activity measurement. The sialidase activity was examined with various substrates and several pH. Cleaved sialic acids were determined by HPLC with malononitrile derivatization as described elsewhere.

Subcellular localization

To clarify the subcellular localization of tilapia sialidases, C-terminal hemagglutinin (HA) tagging of Neu4 was performed using PCR with specific primers. The amplified products were sequenced, and then subcloned into expression vector as mentioned above. Immunofluorescent staining was carried out with Hepa-T1 cultured on glass coverslips and tilapia sialidase transfection-treated. After 48 h at the transfection, cells were fixed with 4% paraformaldehyde/PBS for 15 min, permeabilized with 0.1% Triton X-100/PBS for 10 min and blocked with 1% bovine serum albumin (BSA) at 37°C for 30 min. Cells were kept with primary antibody for HA at 37°C for 1 h, incubated with secondary antibody included fluorochrome Alexa488 at 37°C for 45 min and followed by held with 4',6-diamidino-2-phenylindole (DAPI) for staining nuclear at 37°C for 10 min. The coverslips mounted onto glass slide and observed using sectioning fluorescence microscopy system (Apotome, Carl-zeiss, Germany). Some organelle marker was used following each manufacturer's protocol.

Infection of cultured cells with *E. tarda*

Tilapia sialidase transfected-GAKS cells were seeded on 12 well plate. The number of cells was measured using hemocytometer. The cells were washed with PBS, followed by exposure to *E. tarda* at MOI 100. After an hour of incubation at 28°C, *E. tarda* existing without the cells were eliminated by antibiotics. To collect *E. tarda* which infected into the cells, monolayers were lysed with 0.5% Triton X-100 treatment, and then the plates were shaken for 5 min. The lysate was serially diluted in PBS, plated on TSA and cultured for 48 h at 28°C to allow bacterial growth and colony counting.

Results and discussion

The enzymatic properties of tilapia sialidase and subcellular are shown in Table 1. All sialidases worked at acidic pH, but some sialidases sustained their activity in broad pH range. Substrate specificity of Neu1a is similar to mammalian Neu1 and medaka sialidase, while Neu1b showed the activity toward narrow substrates [4]. Their localizations are at lysosome. According to mRNA expression in several tissues, tilapia *neu1a* expressed exclusively. The activity of Neu3a is toward only gangliosides and localized at plasma membrane correspond to mammalian Neu3 and medaka Neu3a [5]. The high activity of tilapia Neu4 toward various substrates resembles mammalian Neu4 and medaka [6]. On the other hand, subcellular

localization of tilapia Neu4 completely differed from other species Neu4s.

Table 1. The enzymatic properties of tilapia sialidases and subcellular localization

Sialidase	Optimal pH	Substrate specificity	Subcellular localization
Neu1a	4.5	Sialyllactose	Lysosome
Neu1b	4.0	MU-NANA	Lysosome
Neu3a	4.6	Gangliosides	Plasma membrane
Neu4	4.0	Sialyllactose	Nucleus

The effects of tilapia sialidase on *E. tarda* infection were indicated in Fig1. Neu3 transfected cells showed the downregulation of *E. tarda* infection, whereas the increased infection of *E. tarda* in Neu1 and Neu4 transfected cells. The mechanism that influences the tilapia sialidase on *E. tarda* remains to be revealed. Further investigation is required to understand this mechanism.

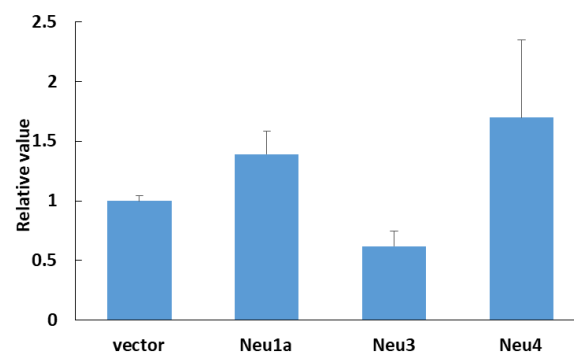


Fig. 1. Comparison of *E. tarda* invasion in each tilapia sialidase transfected GAKS cells.

Conclusions

To know the tilapia sialidase functions, influences of tilapia sialidases on *E. tarda* infection was examined since *E. tarda* utilizes sugar chains on host cell surface at cell attachment. *E. tarda* was infected for the sialidase-transfected cells. As a result, Neu1a and Neu4 sialidase induced the increment of bacterial infection into the cultured cells, while Neu3 significantly suppressed the infections. These results suggest that tilapia sialidases could be useful as an effective bio-marker in bacterial infection and as a tool to develop the novel inhibitor for *E. tarda*.

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