

**Biochemical Characterization of Tilapia (*Oreochromis niloticus*) Sialidases and their  
Significance in *Edwardsiella tarda* Infection**

ティラピアシアリダーゼの生化学的解析とエドワードジエラタルダ感染におけるその意義

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**Biochemical Characterization of Tilapia (*Oreochromis niloticus*) Sialidases and their  
Significance in *Edwardsiella tarda* Infection**

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## ABSTRACT

Various enzymes have been extensively explored in mammals and are now very useful physiological indicators. Sialidases, which cleave sialic acids from glycoconjugates, are among the important proteins in nature that show significant degree of homology, exhibiting a primary structure with highly conserved regions. In humans four sialidases, Neu1, Neu2, Neu3 and Neu4 have been cloned and are used as markers of various physiological aspects. Studies of sialidase in fish are limited and their diversity, evolution and roles are still unclear. Therefore, sialidase exploration was extended to tilapia (*Oreochromis niloticus*), a widely cultured and economically important fish species.

Eight putative tilapia sialidases, two *neu1*-like, designated *neu1a* and *neu1b*, five *neu3*-like designated *neu3a*, *neu3b*, *neu3c*, *neu3d* and *neu3e* and one *neu4*-like were predicted in tilapia genome. Tilapia *neu1* genes amplified from brain cDNA yielded 1164 bp and 1218 bp for *neu1a* and *neu1b* while only three *neu3* genes *neu3a*, *neu3d*, and *neu3e* yielded 1,227 bp, 1,194 bp and 1,155 bp, respectively. A single transcript of *neu4* genes was identified constituting 1,497 bp. All tilapia sialidase possessed conserved multiple Asp-boxes (SXDXGXTW), Y(V)RIP and VGPG motifs. Optimal sialidase activities were recorded at pH 4.5 and pH 4.2 for Neu1a and Neu1b, respectively. Neu1a showed preference towards 4-MU-NANA, 3-sialyllactose and colominic acid, while Neu1b showed narrow substrate specificity towards 4-methylumbelliferyl-N-acetyl- $\alpha$ -D-neuraminic acid (4-MU-NANA). Neu3a showed sialidase activity towards ganglioside mix optimally at pH 4.6. Neu3d and Neu3e showed no significant sialidase activities while transcripts for Neu3b

and Neu3c were not observed. Neu4 enzymatic profiles were partially conserved with mammalian and medaka Neu4.

To determine the roles of tilapia sialidase in bacterial infection, Neu1a, Neu3a and Neu4 overexpressing cells were infected with *Edwardsiella tarda*. Increase in *E. tarda* infection was observed in Neu1a and Neu4 overexpressing Goldfish scale fibroblast (GAKS) cells, while Neu3a overexpression showed attenuated *E. tarda* infection. To clarify the mechanism of tilapia sialidases involvement in bacterial infection, *E. tarda* and its endogenous sialidase NanA roles in infection were examined. NanA activity assay *in vitro* showed that NanA significantly desialylated 3-sialyllactose and fetuin. GAKS cell pretreated with recombinant NanA showed up-regulation of *E. tarda* infection, suggesting that NanA is critical in *E. tarda* infection. Moreover, sialidase inhibitor-treated *E. tarda* showed a significantly reduced ability to infect GAKS cells. These results indicated that NanA-induced desialylation of cell surface glycoconjugates is essential in the initial step of *E. tarda* infection. GAKS cell treatment with recombinant NanA showed alteration of glycoconjugates only in  $\alpha$ 2-3 sialo-linked glycoprotein, but not in glycolipids and  $\alpha$ 2-6 sialo-linked glycoproteins. The up-regulation of *E. tarda* infection by Neu1a and Neu4 was possibly due to desialylation of  $\alpha$ 2-3 sialo-glycoprotein similar to NanA. The reason for *E. tarda* attenuation due to Neu3a could not be established, however, GM3, a major substrate of Neu3a, promoted *E. tarda* infection. This suggested that GM3 could be one of the key molecules in *E. tarda* infection and that its desialylation by Neu3a could be responsible for reduced *E. tarda* infection. This study has revealed that sialidases are an important group of



enzymes, which if explored fully could help monitor and regulated important physiological functions in tilapia and other fish species.

## ABSTRACT (JAPANESE)

シアリダーゼは糖タンパクや糖脂質の非還元末端よりシアル酸を遊離させる糖鎖分解酵素である。最近、哺乳類シアリダーゼが種々の疾病や発生に深く関与していることが明らかとなってきたが、その一方で魚類におけるシアリダーゼの機能についてはよくわかっていない。本研究では、世界中で広く養殖されているティラピアを材料とし、魚類シアリダーゼの性状解析とその意義を明らかにすることを目的とした。ティラピアゲノムの解析により、シアリダーゼのホモログとして 2 つの *neu1* (*neu1a* および *neu1b*)、5 つの *neu3* (*neu3a*、*neu3b*、*neu3c*、*neu3d* および *neu3e*) および 1 つの *neu4* の計 8 遺伝子が同定された。実際にティラピア脳で発現が確認されたのはこのうち 6 遺伝子であり、残りの 2 つ (*neu3b* および *neu3c*) は偽遺伝子だと推察された。この 6 種類のシアリダーゼについて遺伝子クローニング、およびそのリコンビナントタンパクの性状解析を行った。その結果、それぞれに Asp-Box や RIP モチーフなどが保存されている一方で、その酵素学的性状は大きく異なっていることが明らかとなった。*Neu1a* はリソソームに局在し MU-NANA、3-シアロオリゴ糖およびコロミン酸を良い基質とするのに対し、*Neu1b* は MU-NANA のみを基質としていた。一方、*Neu3a* が形質膜に局在するガングリオシドシアリダーゼであったのに対し、*Neu3d* および *Neu3e* では優位な酵素活性は認められなかった。*Neu4* は 3-および 6-シアロオリゴ糖を良く切断するが、核にその局在が認められるなど哺乳類やメダカとは異なる特性を示した。

次にこれらシアリダーゼの魚類細胞における意義を明らかにするため、種々の解析を行ったところ、これらが魚病バクテリア *Edwardsiella tarda* 感染に関与していることを見出した。魚類 GAKS 細胞に対する *E. tarda* の感染実験を行ったところ、Neu1a および Neu4 過剰発現 GAKS 細胞において *E. tarda* の感染が上昇した。一方、Neu3a 過剰に細胞においてはその感染が逆に抑制されていた。その感染制御には宿主細胞のシアル酸脱離が関与していることが示唆されたため、シアリダーゼ阻害剤 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA) で *E. tarda* を前処理して感染実験を行ったところ、その感染が有意に抑制され、*E. tarda* の持つ脱シアリル化酵素 NanA が感染に重要であることが明らかとなった。そこで NanA のクローニングおよびリコンビナントタンパクの性状解析を行ったところ、NanA は 3-シアロオリゴ糖や糖タンパクを良く切断していた。この組換え NanA を GAKS 細胞に曝露すると *E. tarda* の感染が促進し、さらに GAKS 細胞表面の糖タンパクの Sia2-3 結合が良く切断されていた。Neu1 および Neu4 の過剰発現細胞においても同様の傾向が認められたことから、これら魚類シアリダーゼによる *E. tarda* 感染の助長機構はシアロ糖タンパクの脱シアリル化によるものだと考えられた。本研究ではティラピアにおける脱シアリル化機構、および脱シアリル化酵素の魚類における意義として *E. tarda* 感染制御について明らかにした。糖鎖は生理状態により構造が変化することが知られていることから、本研究の成果は魚類における生理状態の新規指標開発に繋がると期待される。

## **THESIS APPROVAL**

The Thesis

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Significance in *Edwardsiella tarda* Infection**

Submitted to

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By

**PETROS KINGSTONE CHIGWECHOKHA**

in partial fulfilment of the requirements for a degree of

Doctor of Philosophy (PhD)

is hereby approved in the recommendation of

**Professor Takao Itakuta**

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**Associate Professor Kazuhiro Shiozaki**

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**Professor Hirosuke Oku**

\_\_\_\_\_

## **DECLARATION**

I declare that the work presented in this thesis is entirely my own with all exceptions being clearly indicated or/and properly cited in the context.

**Petros Kingstone Chigwechokha**

## **DEDICATION**

I, specially dedicate this work to my son Cedric Chigwechokha, my Fiancé, Edith Jiah, my father, mother and all my sisters.

## PUBLISHED PAPERS

1. **Chigwechokha, P.** Tabata, M. Shinyoshi, S. Oishi, K. Araki, K. Komatsu, M. Itakura, T. Shiozaki, K. Recombinant sialidase NanA (rNanA) cleaves alpha 2-3-linked sialic acid of host cell surface N-linked glycoprotein to promote *Edwardsiella tarda* infection. Fish and Shellfish Immunology. 47 (2015) 34-45.
2. **Chigwechokha, P.** Komatsu, M. Itakura, T. Shiozaki, K. Nile Tilapia Neu3 sialidases: molecular cloning, functional characterization and expression in *Oreochromis niloticus*. Gene, 552 (2014) 155-64.

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3. Ryuzono, S., Takase, R., Oishi, K., Ikeda, A., **Chigwechokha, P.**, Funahashi, A., Komatsu, M., Miyagi, T., Shiozaki, K., Lysosomal localization of Japanese medaka (*Oryzias latipes*) Neu1 sialidase and its highly conserved enzymatic profiles with human. Gene. 575 (2016) 513-523.
4. Ikema, S. Takumi, S. Maeda, Y. Kurimoto, T. Bohda, S. **Chigwechokha, P.** Sugiyama, Y. Shiozaki, K. Furukawa, T. Komatsu M. Okadaic acid is taken-up into the cells mediated by human hepatocytes transporter OATP1B3. Food and Chemical Toxicology. 83 (2015) 229-236.
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6. Shiozaki, K. Ryuzono, S. Matsushita, N. Ikeda, A. Takeshita, K. **Chigwechokha, P.** Komatsu, M. Miyagi, T. Molecular cloning and biochemical characterization of medaka (*Oryzias latipes*) lysosomal *neu4* sialidase. Fish Physiology and Biochemistry. 40 (2014) 1461-1472.
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## LIST OF ACRONYMS AND ABBREVIATIONS

<b>4MU-NANA</b>	NeuAc, 4-methylumbelliferyl-N-acetyl- $\alpha$ -D-neuraminic acid
<b>Bp</b>	base pairs
<b>BSA</b>	Bovine serum albumin
<b>cDNA</b>	Complementary deoxyribonucleic acid
<b>Cfu</b>	Colony forming units
<b>DAPI</b>	4',6-diamidino-2-phenylindole
<b>DMEM</b>	Dulbecco's modified Eagle's medium
<b>DNA</b>	Deoxyribonucleic acid
<b>DTT</b>	Dithiothreitol
<b>ECL</b>	Enhanced chemiluminescence
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>FA</b>	Formaldehyde
<b>FBS</b>	Fetal bovine serum
<b>GFP</b>	Green fluorescent protein
<b>HRP</b>	Horseradish peroxidase
<b>IPTG</b>	Isopropyl $\beta$ -D-thiogalactosidase
<b>LB</b>	Luria Broth
<b>MAA</b>	Maackia amurensis agglutinin
<b>MAM</b>	<i>Maackia amurensis</i>
<b>OD</b>	Optical density
<b>ORF</b>	Open reading frame

<b>PAGE</b>	Polyacrylamide gel electrophoresis
<b>PBS</b>	Phosphate buffered saline
<b>PCR</b>	Polymerase chain reaction
<b>PFA</b>	Paraformaldehyde
<b>PMSF</b>	Phenylmethylsulfonyl fluoride
<b>PVDF</b>	Polyvinylidene difluoride
<b>RT-PCR</b>	Real time-polymerase chain reaction
<b>SDS</b>	Sodium dodecyl sulphate
<b>SDS-PAGE</b>	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<b>SNA</b>	<i>Sambucus nigra</i> agglutinin
<b>SNP</b>	Single nucleotide polymorphism
<b>SSA</b>	<i>Sambucus sieboldiana</i>
<b>SV40</b>	Simian virus 40 polyadenylation

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## TABLE OF CONTENTS

ABSTRACT .....	I
ABSTRACT (JAPANESE) .....	IV
THESIS APPROVAL .....	VI
DECLARATION .....	VII
DEDICATION .....	VIII
PUBLISHED PAPERS .....	IX
OTHER PAPERS .....	IX
LIST OF ACRONYMS AND ABBREVIATIONS .....	XI
AKNOWLEDGEMENT .....	XIII
TABLE OF CONTENTS .....	XV
LIST OF FIGURES .....	XXII
LIST OF TABLES .....	XXV
CHAPTER 1: GENERAL INTRODUCTION .....	1
1.1. Glycoconjugates and cellular functions .....	1
1.2. Sialidases and their significance .....	3
1.2.1. Sialidases (Neuraminidase).....	3
1.3. Focus of this study.....	6
1.4. Objectives.....	8

1.4.1.	Overall objective .....	8
1.4.2.	Specific objectives .....	8
CHAPTER 2: Tilapia sialidases: molecular cloning, functional characterisation and		
	expression in <i>Oreochromis niloticus</i> .....	9
2.1.	Abstract .....	9
2.2.	Introduction .....	11
2.3.	General materials and methods .....	16
2.3.1.	Preparation of chemically competent cells .....	16
2.3.2.	Animals and tissue preparation.....	17
2.3.3.	Determination of putative tilapia sialidase genes and post-cloning sequence analysis .....	17
2.3.4.	RNA extraction .....	18
2.3.5.	First strand cDNA synthesis .....	18
2.3.6.	Cloning Primer design .....	19
2.4.	Cloning process and gene characterization of tilapia <i>neuIs</i> .....	21
2.4.1.	cDNA amplification of tilapia <i>neuIs</i> .....	21
2.4.2.	Gel electrophoresis and DNA purification .....	22
2.4.3.	DNA restriction, electrophoresis, gel extraction.....	23
2.4.4.	DNA ligation and transformation in <i>E. coli</i> .....	23

2.4.5.	Sequencing.....	25
2.4.6.	Post sequencing analysis.....	26
2.4.7.	Tilapia sialidase genes cloning into pcDNA3.1 expression vector.....	27
2.4.8.	Cell culture and culture conditions .....	28
2.4.9.	Transfection for protein expression in mammalian cells .....	29
2.4.10.	Biochemical characterisation of tilapia sialidase polypeptides .....	30
2.4.11.	Protein harvest and sialidase activity assay .....	30
2.4.12.	Determination of optimal pH and substrates for sialidase activity. ....	31
2.4.13.	Preparation of HA-tagged expression plasmids.....	32
2.4.14.	Transfection for Biochemical characterisation .....	35
2.4.15.	Cell harvest and fractionation .....	36
2.4.16.	Immunoblotting analysis.....	38
2.4.17.	Indirect immunofluorescence.....	39
2.4.18.	Tissue expression of tilapia sialidase genes.....	40
2.5.	Results .....	41
2.5.1.	Tilapia <i>neu1</i> genes cloning and Neu1 polypeptide characterisation.....	41
2.5.1.1.	Identification of tilapia <i>neu1</i> genes .....	41
2.5.1.2.	Actual nucleotide and amino acid properties .....	43
2.5.1.3.	Identification and cloning of Cathepsin A gene ( <i>csta</i> ) .....	53

2.5.1.4.	Cathepsin A activity .....	54
2.5.1.5.	Cathepsin A activation of tilapia Neu1a and Neu1b .....	55
2.5.1.6.	Tilapia Neu1s sialidase activity .....	57
2.5.1.7.	Immunoblotting analysis .....	61
2.5.1.8.	Tilapia <i>neu1</i> tissue expression .....	62
2.5.2.	Neu3 gene cloning .....	63
2.5.2.1.	Identification of sialidase genes in <i>O. niloticus</i> .....	63
2.5.2.2.	Molecular cloning of tilapia <i>neu3</i> genes and sequence analysis .....	66
2.5.2.3.	Enzymatic characterisation of tilapia neu3 sialidases .....	75
2.5.2.4.	Tilapia <i>neu3</i> sialidase subcellular localization and fractionation .....	78
2.5.2.5.	Tilapia <i>neu3</i> tissue expression .....	80
2.5.3.	Tilapia neu4 cloning and characterisation .....	81
2.5.3.1.	Tilapia <i>neu4</i> gene bioinformatic analysis .....	81
2.5.3.2.	Tilapia <i>neu4</i> gene cloning .....	81
2.5.3.3.	Neu4 sialidase activity .....	87
2.5.3.4.	Neu4 subcellular localization and tissue expression .....	89
2.6.	Discussion .....	91



## CHAPTER 3: Functional sialidase roles in modulating cell surface glycoconjugates:

Roles in bacterial infection using sialidase NanA (rNanA) as a model in <i>Edwardsiella tarda</i> infection.....	106
3.1. Abstract .....	106
3.2. Introduction .....	109
3.3. Materials and Methods .....	112
3.3.1. Materials .....	112
3.3.1.1. Growth medium.....	112
3.3.1.2. Antibiotics .....	113
3.3.1.3. Inducer .....	113
3.3.2. Buffer and solutions .....	113
3.3.2.1. Buffer and solutions for SDS-PAGE .....	113
3.3.2.2. Buffers and solution for gel staining .....	114
3.3.3. Solutions for western blotting .....	114
3.3.4. Bacterial strains and plasmids.....	114
3.3.5. Bacteria strain and culture conditions.....	114
3.3.6. Cell culture.....	115
3.3.7. Selection of suitable cell line for <i>E. tarda</i> infection studies .....	115
3.3.8. Effect of tilapia sialidase overexpression on <i>E. tarda</i> infection .....	116

3.3.9.	Cloning of <i>E. tarda</i> sialidase <i>nanA</i> .....	116
3.3.10.	Expression of recombinant NanA protein in mammalian cells .....	118
3.3.11.	Preparation and expression of recombinant NanA polypeptide .....	119
3.3.12.	GST tagged <i>nanA</i> expression.....	120
3.3.13.	Purification of GST-NanA from inclusion bodies .....	121
3.3.14.	NanA sialidase activity assay .....	122
3.3.15.	Effect of rNanA exposure to <i>E. tarda</i> infection.....	124
3.3.16.	Effect of sialidase inhibitor on <i>E. tarda</i> infection .....	124
3.3.17.	Lectin blotting.....	125
3.3.18.	Thin layer chromatography.....	127
3.3.19.	Flourescence lectin staining for cell surface glycoprotein.....	127
3.3.20.	Sugar-mediated inhibition of <i>E. tarda</i> adherence to GAKS cells.....	128
3.4.	Data analysis .....	129
3.5.	Results .....	129
3.5.1.	Effect of tilapia sialidase overexpression on <i>E. tarda</i> infection.....	129
3.5.2.	Sequence analysis of <i>nanA</i> sialidase gene from <i>E. tarda</i> FPC498 strain .	130
3.5.3.	Endogenous NanAand mammalian cell NanA expressed sialidase activity .....	133
3.5.4.	Expression of recombinant NanA sialidase. ....	134

3.5.5.	Exposure of GAKS cells to rNanA increases <i>E. tarda</i> invasion and infection	136
3.5.6.	Enzymatic properties of NanA	138
3.5.7.	Sialidase NanA predominantly desialylates $\alpha$ 2-3 linked glycoconjugates on GAKS cell surface.	141
3.5.8.	<i>E. tarda</i> preferentially infects cells well decorated with $\alpha$ 2-3 linked sialoglycoproteins	144
3.5.9.	Potential mechanism for effect of tilapia sialidase on <i>E. tarda</i> infection.	145
3.5.10.	Effects of monosaccharides on <i>E. tarda</i> invasion of GAKS cells	147
3.6.	Discussion	148
CHAPTER 4: GENERAL DISCUSSION		161
REFERENCES		173

## LIST OF FIGURES

<b>Figure 1-1.</b> Chemical structure of N-acetylneuraminic acid (Neu5Ac). .....	2
<b>Figure 2-1.</b> Tilapia Neu1 sialidase gene organizations and evolutionary relationships. ....	42
<b>Figure 2-2.</b> Syntenic analysis around <i>neu1s</i> . .....	43
<b>Figure 2-3.</b> Tilapia <i>neu1</i> genes electrophoresis. ....	44
<b>Figure 2-4.</b> Insert checking for <i>neu1a</i> and <i>neu1b</i> . .....	45
<b>Figure 2-5.</b> Exon analysis. ....	46
<b>Figure 2-6.</b> Putative and actual Neu1 amino acid sequences.....	47
<b>Figure 2-7.</b> Nucleotide and deduced amino acid sequence for Neu1a. ....	49
<b>Figure 2-8.</b> Nucleotide and deduced amino acid sequence for Neu1b. ....	51
<b>Figure 2-9.</b> Multiple alignment of amino acid sequences from tilapia Neu1s, human NEU1 and medaka Neu1. ....	52
<b>Figure 2-10.</b> Electrophoresis of the ctsa PCR product. ....	53
<b>Figure 2-11.</b> Cathepsin A activity.....	55
<b>Figure 2-12.</b> Tilapia Neu1 activation by Cathepsin A. ....	56
<b>Figure 2-13.</b> Optimal pH and substrate specificity of tilapia Neu1a sialidase. ....	58
<b>Figure 2-14.</b> Optimal pH and substrate specificity of tilapia Neu1b sialidase. ....	60
<b>Figure 2-15.</b> Neu1a localization. ....	61
<b>Figure 2-16.</b> Tissue expressions of tilapia <i>neu1</i> sialidases. ....	62
<b>Figure 2-17.</b> Syntenic analysis and exon intron organisation for <i>neu3</i> genes. ....	65
<b>Figure 2-18.</b> Nucleotide and deduced amino acid sequence for <i>neu3a</i> . ....	67
<b>Figure 2-19.</b> Nucleotide and deduced amino acid sequence for <i>neu3d</i> . ....	68

<b>Figure 2-20.</b> Nucleotide and deduced amino acid sequence for <i>neu3e</i> . .....	69
<b>Figure 2-21.</b> PCR amplification of tilapia <i>neu3</i> genes. ....	70
<b>Figure 2-22.</b> Multiple alignment of amino acid sequences from tilapia Neu3a, Neu3b, Neu3c, Neu3d and Neu3e, human NEU3, medaka Neu3a and Neu3b. ....	74
<b>Figure 2-23.</b> Optimam pH and substrate specificity of tilapia Neu3a sialidase. ....	76
<b>Figure 2-24.</b> Neu3s sialidase activity comparison with mock. ....	77
<b>Figure 2-25.</b> Subcellular localization of tilapia Neu3s. ....	79
<b>Figure 2-26.</b> Tissue expressions of tilapia <i>neu3</i> sialidases. ....	80
<b>Figure 2-27.</b> Sequence alignment, syntenic analysis and exon organisation. ....	83
<b>Figure 2-28.</b> Nucleotide and deduced amino acid sequence for <i>neu4</i> . ....	84
<b>Figure 2-29.</b> Multiple alignment of amino acid sequences from tilapia Neu4, medaka Neu4, zebrafish Neu4 and human NEU4. ....	86
<b>Figure 2-30.</b> Tilapia Neu4 optimal activity pH and substrate specificity. ....	88
<b>Figure 2-31.</b> For subcellular localization of Neu4. ....	90
<b>Figure 2-32.</b> Tissue expressions of tilapia <i>neu4</i> sialidases. ....	91
<b>Figure 3-1.</b> Infection of tilapia sialidase overexpressing GAKS cells. ....	130
<b>Figure 3-2.</b> Electrophoresis of the nanA PCR product. ....	131
<b>Figure 3-3.</b> NanA nucleotide and deduced amino acid sequences. ....	132
<b>Figure 3-4.</b> Endogenous and mammalian-cell-expressed NanA sialidase activity. ....	134
<b>Figure 3-5.</b> Recombinant NanA properties. ....	136
<b>Figure 3-6.</b> <i>E. tarda</i> Infection and invasion rates to GAKS monolayers. ....	137
<b>Figure 3-7.</b> Effect of pH and temperature on <i>E. tarda</i> NanA sialidase activity. ....	139

<b>Figure 3-8.</b> Substrate specificity of NanA sialidase. ....	140
<b>Figure 3-9.</b> GAKS cell surface glycoconjugates alterations after exposure to rNanA. (A-C) .....	142
<b>Figure 3-10.</b> Lectin binding analysis of GAKS cell surface.....	143
<b>Figure 3-11.</b> Comparison of <i>E. tarda</i> invasion in HeLa and GAKS cells. ....	145
<b>Figure 3-12.</b> Glycoprotein profiles by lectin analysis. ....	146
<b>Figure 3-13.</b> Schematic diagram for the proposed mechanism of sialidase NanA involvement in <i>E. tarda</i> infection. ....	159

## LIST OF TABLES

<b>Table 1-1.</b> General properties of mammalian sialidases .....	4
<b>Table 2-1.</b> The primers used for gene cloning of tilapia sialidase genes .....	20
<b>Table 2-2.</b> KOD plus Neo polymerase reaction mixture .....	21
<b>Table 2-3.</b> Components for sialidase activity reaction mixture .....	30
<b>Table 2-4.</b> C-terminal HA – tagging primers for each tilapia sialidase genes. ....	33
<b>Table 2-5.</b> C-terminal HA – tagging PCR reaction mixtures.....	34
<b>Table 2-6.</b> Ligation reaction mixture. ....	35
<b>Table 2-7.</b> Sonication buffer for homogenate preparation.....	37
<b>Table 2-8.</b> Ingredients composition of acrylamide gel for SDS-PAGE .....	38
<b>Table 2-9.</b> Observed single nucleotide polymorphisms (SNPs). ....	71
<b>Table 3-1.</b> <i>E. tarda</i> nanA cloning and GST-tagging primers .....	117
<b>Table 3-2.</b> The effects of monosaccharide pretreatment on <i>E. tarda</i> infection to GAKS monolayers.....	148

## **CHAPTER 1:**

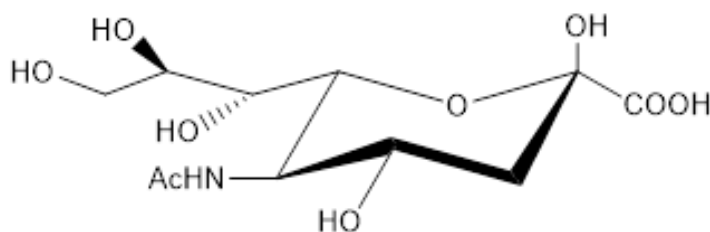
### **GENERAL INTRODUCTION**

#### **1.1. Glycoconjugates and cellular functions**

Understanding the molecular architecture of cell membranes is a central goal for cell biology, as structure is central to function. An overwhelming amount of data has implicated the cell surface as the primary site for control of cell growth, division, development, communication, differentiation and infection. The carbohydrates present inside and at the surface of cells mediate many biological processes that are fundamentally important for both the healthy and diseased states of living organisms. Usually carbohydrates are covalently linked to other biomolecules such as proteins or lipids forming glycoconjugates (Varki et al., 2009). Glycoconjugates play roles in a wide array of physiological processes including serving as modulators of signaling receptors, regulation of membrane fluidity, cellular recognition, adhesion, proliferation, differentiation, apoptosis, and signal transduction, neurotransmission, and interaction with regulatory proteins in the nervous system (Anderson, 1998; Lloyd and Furukawa, 1998; Rampersaud et al., 1999; Sorice et al., 2004). It has recently been revealed that oligosaccharides in glycoproteins participate in the phenomena of life such as fertilization and differentiation, signal transmission, canceration, intracellular transport of proteins and control of physiological activity (Tulsiani et al., 1997; Wassarman, 1992). Glycoconjugate structures are altered by syltransferases and sialidase, which regulate the amount and construction of their sugar chains. Most glycoconjugates possess sialic acids at their terminal non-reducing positions (Miyagi et al., 2008), a family of nine-carbon acidic monosaccharides. Sialic acids are electro-negatively charged



monosaccharides present in higher animals and limited microorganisms (Varki et al., 1992; Schauer and Kamerling, 1997). It is believed that the appearance of sialic acid has facilitated evolution in higher organisms. The sialic acid family of monosaccharides consists of about 50 members all of which are derived from neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid) (Miyagi and Yamaguchi, 2007) (Figure 1-1). The carboxyl group at position one renders a negative charge to the molecule due to the proton dissociation under physiological conditions and it is characterized as a strong organic acid.



**Figure 1-1.** Chemical structure of N-acetylneuraminic acid (Neu5Ac). (Adopted from Miyagi and Yamaguchi, 2007)

N-Acetylneuraminic acid (Neu5Ac) (Figure 1-1), N-glycolylneuraminic acid (Neu5Gc), N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac<sub>2</sub>) and deaminoneuraminic acid (Kdn) are the most frequently occurring members of the Sialic acid family (Schauer, 2004). Sialic acids are known to serve significant roles in physiological and pathological functionality of bearing sugar chains. This family of monosaccharides has been reported to influence organ development (Wang and Brand-miller, 2003), immune regulation (Varki, 1993), malignancy (Miyagi et al., 2008), regulate infection by masking binding epitopes on

glycoconjugates (Honma et al., 2011; Tong et al., 2000) and acting as binding sites as well as providing nourishment for the bacteria (Varki, 1993; King et al., 2006). Indeed, the regulation of sialic acid content in the cells has been a focus recently to understand and reveal more of their functions and that of other sugar chains, especially by sialidase (EC 3.2.1.18). Sialidases are the key enzymes in sialic acid catabolism, thereby facilitating a wide range of physiological functions. Hence, significant attention in recent times has focused on sialidases to reveal any important information about their roles and evolution while enriching literature on the significance glycobiology in nature.

## **1.2. Sialidases and their significance**

Sialidases are glycosidases catalyzing the removal of  $\alpha$ -glycosidically-linked sialic acid residues from carbohydrate groups such as glycoproteins and glycolipids. They catalyze the hydrolytic cleavage of non-reducing sialic acid residues ketositically linked to mono- or oligosaccharide chains of glycoconjugates (Miyagi and Yamaguchi, 2007). They have been reported to show a wide distribution, present in vertebrates and also in microorganisms (Rosenberg and Schengrund, 1976; Corfield et al., 1981; Corfield and Schauer, 1982).

### **1.2.1. Sialidases (Neuraminidase)**

Several reports demonstrated the presence of sialidase activity in a wide variety of mammalian cells and tissue after sialidase activity in higher organisms was first detected in bovine and human glycoproteins in Cohn Fraction VI (Warren and Spearing, 1960). Carubelli *et al.* (1962) detected activity in soluble fractions isolated from different tissues

of rats, and later study described their lysosomal occurrence (Mahadevan et al., 1967). With time, sialidases activity was detected in plasma membranes (Schengrund and Rosenberg, 1970) and Golgi fractions (Kishore et al., 1975). Sialidase activities towards various glycoconjugates are key in the regulation of the functions of glycoconjugates and have been reported to be involved in various physiological functions and diseases of mammals. Several aspects of mammalian sialidases have been reported (Table 1-1).

**Table 1-1.\*** General properties of mammalian sialidases

	<b>NEU1</b>	<b>NEU2</b>	<b>NEU3</b>	<b>NEU4</b>
Major subcellular localization	Lysosome	Cytosol	Plasma membrane	Lysosome Mitochondria and ER
Good Substrates	Oligosaccharides Glycopeptides	Oligosaccharides Glycoproteins Glycolipids	Gangliosides	Oligosaccharides Glycoproteins Glycolipids
Optimal PH	4.4-4.6	6.0-6.5	4.5-4.7	4.5-4.7
Proposed functions	Degradation in lysosomes Immune functions Phagocytosis Elastic fiber assembly	Myoblast differentiation Neuronal differentiation.	Neuronal differentiation Apoptosis Adhesion	Neuronal differentiation Apoptosis Adhesion

\*Adopted from Miyagi et al., 2012

On the other hand, in fish, the enzymatic properties and functions of sialidase have not been fully understood, with the exception of zebrafish (Manzoni et al., 2007) and medaka (Shiozaki et al., 2013; Shiozaki et al., 2014; Ryuzono et al., 2016). No information is available on sialidases of tilapia despite several authors reporting the importance of sialidases in other vertebrates and the strong link between sialidase effects on glycoconjugates and modulation of physiological processes.

Sialidases have also been reported and extensively investigated in a variety of microorganisms, including viruses, bacteria and protozoa. They have been implicated in the pathogenesis of diseases caused by microorganisms and parasites. Among the most well characterized microbial sialidases is the influenza virus sialidase, which plays a role in processing progeny viral particles and contributes to the efficient virus release from infected cells and host spread (von Itzstein et al., 1996). In bacteria, sialidases have been reported to enhance the pathogenic potential of various species. Bacterial sialidases are classified based on their sizes (Vimr, 1994) with smaller sialidases constituting about 40 kDa reported in bacterial species such as *Clostridium perfringens* and large sialidases with molecular weights of about 80 kDa (Crenell et al., 1994). The small and large bacterial sialidase groups have also been considered on their requirements for calcium (Vimr et al., 1988; Galen et al., 1992). Evolutional analyses of bacterial sialidases have yielded unsatisfactory results suggesting that sialidase occurrence is not in accordance with the phylogenetic groupings in bacteria. Frequently, closely related bacterial species have shown different sialidase production (Roggentin et al, 1985; Hoyer et al., 1992), which has led to suggestions that sialidases might have evolved through other mechanism(s) than ancestors in the bacterial kingdom. Nonetheless, bacterial sialidase play various roles in pathogenicity and nutrition, which include providing pathogenic advantages to bacteria by counteracting the host's innate and adaptive immune responses (Corfield, 1992), promoting biofilm formation (Soong et al., 2006), inducing chemokine release from epithelial cells (Kuroiwa et al., 2009), exposing cryptitopes by unmasking sialic acid-masked epitopes for adhesion (Corfield, 1992; Tong et al., 2000), and host glycoproteins degradation for

nutrition (Bradshaw et al., 1994; King et al., 2006). Sialidases are indeed indispensable in pathogenesis of microbial diseases and hence have attracted significance attention in the recent times. Notably, sialidases have been implicated in the pathogenicity of some bacteria, including *Clostridium perfringens* (Rood, 1998), *Streptococcus pneumoniae* (Tong et al., 2005), *Pasteurella multocida* (Steenbergen et al., 2005), and *Pseudomonas aeruginosa* (Soong et al., 2006). Sialidases can also facilitate colonization by exposing cryptic receptors for bacterial adhesion (Corfield, 1992). In addition, sialidases have been reported to modify the host's ability to respond to bacterial infection by increasing the susceptibility of immunoglobulin molecules to proteolytic degradation (Reinholdt et al., 1990). They may also provide bacteria with a nutritional carbon source (Corfield, 1992).

### **1.3. Focus of this study**

It is clear from the initial facts presented here-in that glycoconjugates sialylation and desialylation by syltransferases and glycosialidase, regulate many biological events in vertebrates. The cleavage of sialic acids from glycoconjugates has been reported to change conformation of glycoconjugates, thereby masking the biological sites of these molecules and their binding sites to cells consequently, altering their associated roles (Miyagi et al., 2008). The desialylation process achieved by sialidases, has been significantly explored in mammals. Various groups of sialidases have been identified and are designated Neu1, Neu2, Neu3 and Neu4 in mammals and NEU1, NEU2, NEU3 and NEU4 in humans. The roles of glycoconjugates desialylation and its patterns are still not well understood in fish because of lack of adequate studies. Limited sialidase studies are only present for zebrafish

(Manzoni et al., 2007) and medaka (Shiozaki et al., 2013; Shiozaki et al., 2014; Ryuzono et al., 2016). To provide insights on the desialylation patterns and their roles in fish physiological functions, this study focused on the cloning and characterization of sialidase genes in tilapia (*O. niloticus*) and assessed their roles in regulating bacterial infection, one of the major desialylation roles reported in mammals. The present study determined the actual sialidase genes in tilapia, enzymatic properties, substrate specificity, physiological functions and subcellular localization. Tilapia has for longtime been used as a valuable experimental model in the study of various gene regulations and functionality in fishes. Furthermore, the species is one of the most important aquaculture species in the world, ranking among the three important cultured species and only surpassed by carp and catfish in terms of production (FAO, 2012). Intensive tilapia production has often resulted in increased diseases due to poor water quality and high stock densities. Tilapias are susceptible to a number of infectious agents including bacteria (Shoemaker et al., 2006). Therefore, exploration of sialidase protein family and its roles in tilapia, a good model for fish diseases, could be key in the development of regulatory mechanisms for diseases in fish. Previously the roles of glycoconjugates in pathogens and viruses adherence was reported in mammals and fish (Tsai et al., 2003; Low et al., 2006; Matsunaga et al., 2011; Chisada et al., 2013). Hence, sialidase genes cloning in tilapia has potential to reveal possible linkages between glycoconjugates and infection in fish. While enriching the scientific literature on the genome understanding of tilapia (*O. niloticus*), the study was also the first step in understanding the potential use of sialidase as marker candidates for patho- and physiology processes in fish.

## **1.4. Objectives**

### **1.4.1. Overall objective**

The work outlined here-in focused on biochemical characterisation and molecular cloning of sialidases in tilapia (*O. niloticus*) and their significance in *E. tarda* infection through glycoconjugates desialylation.

### **1.4.2. Specific objectives**

The specific objectives of the current study were:

1. To investigate the presence sialidase genes *in silico* in tilapia (*O. niloticus*) genome.
2. To comprehensively carry-out molecular cloning of sialidases (*neu1s*, *neu3s* and *neu4*) in tilapia.
3. To determine the enzymatic properties and characteristics of sialidase polypeptides in tilapia.
4. To determine the significance of tilapia sialidases on *E. tarda* infection and their mechanisms. .
5. To perform a molecular cloning of *E. tarda* sialidase NanA and characterize its enzymatic properties.
6. To determine the effect of sialidase NanA on *E. tarda*'s infection rates *in vitro* using fish cell lines.
7. To determine the mechanism of sialidase NanA's involvement in the *E. tarda* infection towards fish cell lines.

## CHAPTER 2:

### **Tilapia sialidases: molecular cloning, functional characterisation and expression in *Oreochromis niloticus***

#### **2.1. Abstract**

Sialidases cleave sialic acids from the terminal ends of various sialo-derivates. Various mammalian sialidases have been cloned and their roles significantly described. Mammalian sialidases show significant degree of homology, exhibiting a primary structure with highly conserved regions. On other hand, studies of fish sialidases are limited and the diversity of sialidase genes and roles are still unclear in majority of fish genera. To gain more insights of fish sialidases, molecular cloning and characterisation was carried out in tilapia (*Oreochromis niloticus*). A tilapia genome-wide search for orthologues of human *NEU1*, *NEU2*, *NEU3* and *NEU4* yielded eight putative tilapia sialidases, two *neu1*-like, designated *neu1a* and *neu1b*, five *neu3*-like designated *neu3a*, *neu3b*, *neu3c*, *neu3d* and *neu3e* and one *neu4*-like. The two *neu1* genes were present on the same chromosome neighboring each other spanning 19 kbp and were flanked by conserved genes when compared to medaka and zebrafish *neu1* genes. Amplification of the *neu1* genes from brain cDNA yielded 1164 bp and 1218 bp for *neu1a* and *neu1b*, respectively. Among five *neu3* genes, *neu3a* and *neu3b* were located in different chromosomes while *neu3c*, *neu3d* and *neu3e* formed a cluster on a different chromosome. Transcripts of *neu3a*, *neu3d* and *neu3e* were amplified by PCR from adult fish brain cDNA with consensus sequences of 1,227 bp, 1,194 bp and 1,155 bp, respectively. A single transcript of *neu4* genes was identified in tilapia genome with conserved flanking genes similar to medaka and zebrafish, with a molecular weight of



1,497 bp. Multiple alignments for each groups of tilapia sialidases revealed the presence of conserved multiple Asp-boxes (SXDXGXTW), Y(V)RIP and VGPG motifs. The predicted molecular weights for Neu1a, Neu1b, Neu3a, Neu3d, Neu3e and Neu4 were 42.4 kDa, 44.7 kDa, 45.9 kDa, 44.4 kDa, 43.6 kDa and 55.7 kDa, respectively. Neu1a and Neu1b sialidase were found to be activated by Cathepsin A, which was encoded by *ctsa* gene consisting 1422 bp nucleotide. Cell homogenates prepared from HEK293T co-transfected *neu1a/ctsa* and *neu1b/ctsa* showed significantly higher sialidase activity compared to only single transfected *neu1a* and *neu1b* cells homogenate. Optimal sialidase activity were recorded at pH 4.5 and pH4.2 for Neu1a and Neu1b, respectively. Neu1a showed preference towards 4-MU-NANA, 3-sialyllactose and colominic acid as major substrates, while Neu1b showed narrow substrate specificity towards 4-MU-NANA. Neu3a transfected HEK293T cells showed sialidase activity in Neu3a towards ganglioside mix optimally at pH 4.6. Using pure gangliosides as substrates, highest sialidase activity for Neu3a was observed towards GD3 followed by GD1a and GM3, but not GM1. On the other hand, sialidase activities were not observed in Neu3d and Neu3e towards various sialoglycoconjugates. Indirect immunofluorescence showed that tilapia Neu1a was localized in the lysosome, Neu3a and Neu3d were present at the plasma membrane, while most Neu3e and Neu4 were co-localized with the cytosol and nucleus, respectively. Neu4 showed optimal activity pH of 4.0 with significant sialidase activity towards 3-sialyllactose, 6-sialyllactose and 4-MU-NANA. These findings provide a fundamental and profound step towards a comprehensive understanding of sialidases in fish.

## **2.2. Introduction**

In nature, sialidases are among important modulators of gangliosides. Sialidases are glycohydrolytic proteins that catalyze the cleavage of sialic acids from sialoglycoconjugates (Miyagi and Yamaguchi, 2007). They are found widely distributed in nature ranging from viruses, bacteria and higher animals (Saito and Yu, 1995, Rosenberg and Schengrund, 1976) where they modulate and influence various physiological functions by their sialoglycohydrolytic activity (Miyagi, 2008; Valaperta et al., 2006; Yamaguchi et al., 2006). Various groups of mammalian sialidases have been identified and extensively characterized. They are designated as Neu1, Neu2, Neu3 and Neu4 (NEU1, NEU2, NEU3 and NEU4 in human). Each group of mammalian sialidase has been reported to possess unique properties including subcellular localization and substrate specificity.

Of the four mammalian sialidases identified to-date, Neu1 is one of the most extensively explored sialidase. In humans, NEU1 sialidase have been investigated extensively as a target gene for sialidosis, an autosomal-recessive lysosomal-storage disease caused by mutations in the sialidase NEU1 gene (Bonten et al., 1996). NEU1 has been localized to lysosomes where it initiates the degradation of sialoglycoconjugates by removing terminal sialic acids (d'Azzo and Bonten, 2010). Mammalian Neu1 sialidase forms a complex with protective protein (Cathepsin A) and  $\beta$ -galactosidase (Bonten et al., 2009). Several studies have reported the significance of Cathepsin A, which is for optimal targeting and correct compartmentalization in the lysosomes as well as subsequent activation of the sialidase enzyme (Bonten et al., 1995; Bonten and d'Azzo, 2000). Other reports have demonstrated

the presence of Neu1 at the plasma membrane in various cell types, although the mechanism through which NEU1 might be translocated to the plasma membrane is poorly understood. Recently the significance of plasma membrane localization has been demonstrated through NEU1 clear desialylate of cell surface structures such as EGFR, MUC1 and other plasma membrane sialoglycoproteins (Lillehoj et al., 2012). In addition, NEU1 forms a complex with both EGFR and MUC1 (Lillehoj et al., 2012). Apart from being implicated sialidosis and galactosialidosis, Neu1 is known to play various roles including cellular signaling for immune responses and inflammation (Pshezhetsky and Hinek, 2011). NEU1 contributes to the regulation of phagocytosis in macrophages and dendritic cells through desialylation of cell surface receptors (Seyrantepe et al., 2010). Neu1 shows narrow substrate specificity mainly towards glycoproteins and oligosaccharides as optimal substrates (Miyagi et al., 1984). Work on sialidase Neu1 is very limited in fish. Recently, molecular cloning and biochemical characterization of sialidase Neu1 has been carried out in zebrafish (Manzoni et al., 2007) and medaka (Ryuzono et al., 2016). The partial investigation in zebrafish revealed one orthologue of human NEU1, which did not show significant sialidase activity due to lack of Cathepsin A. A much more detailed Neu1 investigation was carried in medaka (Ryuzono et al., 2016). The results revealed that just like mammalian Neu1, medaka Neu1 required activation and correct compartmentalization by Cathepsin A. Medaka *neu1* co-transfected with medaka *ctsa* showed significantly higher sialidase activity, when compared to the wild and *neu1* transfection. Despite these studies, the roles of sialidase Neu1 are still unclear in fish. If mammalian studies are anything to go by, Neu1 could play very crucial roles especially

associated with the lysosome and the plasma membrane. To understand further the properties of Neu1 sialidases in fish, it is imperative to expand sialidase exploration to other fish species.

Predictions for orthologues of the second mammalian sialidase Neu2, have not yielded any *neu2*-like genes in the fish species so far reported (Manzoni et al., 2007; Shiozaki et al., 2013). These studies have suggested the lack of *neu2* genes in fish and it has further been suggested that other paralogous genes within sialidase family that have duplicated genes may play the roles of Neu2. For instance, in medaka, Neu3b showed properties superimposable with that of Neu2 and could possibly be responsible for Neu2 roles in medaka (Shiozaki et al., 2013).

Among mammalian sialidases, Neu3, a plasma membrane and ganglioside specific sialidase, holds a strategic role in glycoconjugates modulation; consequently, affecting cell surface events and cellular physiology such as apoptosis (Valaperta et al., 2006), cell proliferation and myoblast differentiation (Anastasia et al., 2008), neurite formation (Hasegawa et al., 2000 and Proshin et al., 2002), regulation and regeneration of neurons (Rodriguez et al., 2001; Da Silva et al., 2005). Fish Neu3 sialidases have not been extensively characterized. Molecular cloning and characterization of *neu3* genes have so far been reported only in zebrafish (*Danio rerio*) with five *neu3* sialidases genes identified (*neu3.1*, *neu3.2*, *neu3.3*, *neu3.4* and *neu3.5*) (Manzoni et al., 2007) and medaka (*Oryzias latipes*) with *neu3a* and *neu3b* (Shiozaki et al., 2013). Medaka and zebrafish Neu3 sialidases possess well conserved YRIP, VGPG and Asp-box motifs. Interestingly,

variations that exist in mammalian sialidase properties have also been observed in fish Neu3 sialidases. For instance, medaka Neu3a is localized at plasma membrane (Shiozaki et al., 2013), while its zebrafish orthologue, Neu3.1, is present at endoplasmic reticulum (Manzoni et al., 2007). These results suggest the existence of evolutionary diversified sialidase properties among fish species. Therefore, expansion of sialidase studies to other fish species could reveal more striking property patterns of these enzymes.

Neu4 is the final group of sialidase cloned and described to date. It was first cloned in human and was determined to be encoded by the *NEU4* gene, which shares the typical sequence features of the mammalian sialidase enzymes. NEU4 possesses two isoforms, the long and short form differing in the N-terminal region (Yamaguchi et al., 2005). The long isoform possesses an extra 12 amino acids at the N-terminal region, which was predicted to be a mitochondrial targeting sequence (Yamaguchi et al., 2014) and absent in the short isoform. Both forms catalyze the removal of sialic acid from oligosaccharides and glycoproteins as well as gangliosides (Monti et al., 2004). The two NEU4 isoforms (NEU4L and NEU4S) show very similar enzymatic properties, are localized in different places in the cell. NEU4L was found to localize with the mitochondria, as predicted by the presence of the N-terminal 12 amino acids (Yamaguchi et al., 2005; Bigi et al., 2010). On the other hand, the short form, lacking the mitochondrial signal sequence, is not localized in mitochondria. In terms of roles, the long form of NEU4 (NEU4L) may be involved in apoptosis with hydrolysis of ganglioside GD3 in mitochondria, while short form of NEU4 (NEU4S) suppresses malignancy in colon cancer cells, mainly through desialylation of

some glycoproteins (Hasegawa et al., 2007). Neu4 is unique among all sialidases in its tissue expression pattern and enzymatic properties. Medaka Neu4 has recently been investigated (Shiozaki et al., 2014). This study and partial exploration of zebrafish Neu4 (Manzoni et al., 2012) are the only studies on Neu4 in fish. Several properties of Neu4 such as subcellular localization are still controversial, which suggest that roles of Neu4 are still unconfirmed and some remain unknown.

In general, medaka and zebrafish, with significant sialidase exploration, are smaller in size and not aquaculture candidates. This potentially limits other types of investigations such as the application of *in vitro* biochemical results to a full-scale aquaculture operation. For example, an aquaculture species could not only be studied with regard to its economical interest but also as a model for more fundamental research in immunology, physiology, toxicology and others. Hence, a widely cultured fish species would be a suitable model animal for understanding the biological consequences of sialoglycoconjugate modulation by various sialidases. Intensification of fish farming has led to increased outbreaks of infectious disease (FAO, 2012), which have caused huge losses to the industry. Therefore, exploration of sialidases and understanding their target glycoconjugates substrates could provide an ideal focal point towards further elucidation of the regulatory mechanisms in cultured fish species. For instance, glycoconjugates are receptors of various pathogens in mammals (Kanda and Watanabe, 2001; Tsai et al., 2003; Low et al., 2006; Markwell et al., 1981; Oda et al., 2013) and in avian (Ferreira et al., 2004). Despite several reports on the significance of glycoconjugates in pathogenesis in mammals and avian, similar information

is still obscure in fish. Only recently, *Vibrio trachuri* has been reported to adhere to glycolipids in red sea bream (*Pagrus major*) (Matsunaga et al., 2011 and Chisada et al., 2013). Based on these studies, enzymatic modulator of glycoconjugates could be indispensable in regulating infection and invasion of various pathogens. Against this background, the present study focused on molecular cloning and characterisation of sialidase genes in Nile tilapia, *O. niloticus*, one of the most important aquaculture species in the world ([http://www.fao.org/fishery/culturedspecies/Oreochromis\\_niloticus/en](http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en)). The positive attributes of the species in aquaculture such as its wide environmental tolerance, high growth rates and large harvest body weights make it valuable resource as an aquaculture experimental animal, especially for *in situ* gene and protein regulation and functionality. Therefore, the present study enhanced and expanded the understanding of the structure and evolution of sialidase genes in teleosts and other vertebrates.

## **2.3. General materials and methods**

### **2.3.1. Preparation of chemically competent cells**

*E. coli* HST08 bacterial cells were spread on an antibiotic-free LB agar plate and incubated overnight at 37°C. A single colony was picked and sub-cultured in 5 mL LB medium followed by an overnight incubation at 37°C in the shaking incubator. To generate a large volume of bacterial culture, 3 mL of the overnight culture was added to 200 ml of LB medium and incubated for another 2-3 hours at 37°C in the shaking incubator. The optical density (OD) of the culture was tested every 30 minutes until it reached 0.6-1.0. The culture medium was spun down at 21,450 xg for 15 minutes at 4°C to precipitate the bacterial

pellet. The pellet was then fully re-suspended in 80 ml of 0.1 M MgSO<sub>4</sub> by pipetting up and down several times on ice. To remove the MgSO<sub>4</sub> solution, the solution was centrifuged at 2,000 xg for 15 minutes at 4°C. Lastly the bacterial pellet was re-suspended in 2ml of ice-chilled 0.1M CaCl<sub>2</sub>. Aliquots of 50 µl of the competent HST08 were stored at – 80°C in cryo-protective tubes.

### **2.3.2. Animals and tissue preparation**

Wild *Oreochromis niloticus* were used in this study. The fish were collected from Ibusuki River (Ibusuki, Japan). Tilapia tissues were excised from each fish, immediately frozen by dry ice and kept at -80°C until use.

### **2.3.3. Determination of putative tilapia sialidase genes and post-cloning sequence analysis**

Putative tilapia sialidase nucleotide sequences were identified from genomic sequence available in central repositories including National Center of Biotechnological Information (NCBI) (<http://www.ncbi.nlm.nih.gov/pubmed>), Ensembl (<http://asia.ensembl.org/index.html>) and University of California Santa Cruz (UCSC) genome browser (<http://genome.ucsc.edu/>). TBLASTN and BLAST searches were conducted in tilapia genome for putative orthologs of human *NEU1*, *NEU2*, *NEU3* and *NEU4* sialidase genes. Using visual inspection of the 5' and 3' ends, candidates for the first ATGs and stop codons were identified to determine the putative open reading frame (ORF).



#### **2.3.4. RNA extraction**

Total RNA was extracted from adult tilapia brain, eye, heart, liver, muscle and spleen using Sepazol-RNA I Super G (Nacalai Tesque, Japan). Using this protocol, about 20 mg of tissues was homogenized in 1000  $\mu$ l of Sepazol, incubated for 5 minutes at room temperature and the resulting homogenates were mixed with 200 ml of chloroform, mixed by inverting 4-6 times and incubated at room temperature for 3 minutes. Upon adding the chloroform, the RNA remains in the aqueous phase, while proteins and high molecular weight DNA enter the organic phase and the interphase, respectively and the aqueous was subsequently recovered by centrifugation at 13,200 xg for 15 minutes. Total RNA was recovered by precipitation of the aqueous, upper phase with 500  $\mu$ l isopropanol-alcohol, vortexed and incubation at room temperature for 10 minutes. The mixture was centrifuged at 13,200 xg for 10 minutes to collect the precipitate. The pellet was washed once with 75% ethanol, centrifuged again for 5 minutes, air-dried and dissolved in 30  $\mu$ l RNA water. The concentration of RNA was determined by measuring the absorbance at 260 nm ( $A_{260}$ ) in a spectrophotometer. RNA samples were diluted in RNase-free water. Cuvettes were cleaned with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water. Total RNA was stored at -80°C until use.

#### **2.3.5. First strand cDNA synthesis**

First strand cDNA was synthesized from mRNA using ReverTra Ace qPCR RT Master Mix with genomic DNA remover (TOYOBO, Japan). A 0.5  $\mu$ g of total RNA was mixed

with DEPC-treated water up to 6 µl volume incubated at 65°C and immediately transferred on ice for 5 minutes. After 5-minutes ice incubation, 2 µl of 4x DNA Master mix was added to the samples, incubated at 37°C for 5 minutes and mixed with 5 x RT Master mix containing reverse transcriptase ReverTra Ace, RNase inhibitor, oligo dT primer, random primers and dNTPs. For cDNA synthesis, contents of the tube were mixed by pipetting and incubated sequentially as follows; 37°C for 15 minutes, 50°C for 5 minutes and 98°C for another 5 minutes.

#### **2.3.6. Cloning Primer design**

Primers were designed using predicted sialidases sequences. The forward primers for each sequence were designed by incorporating a restriction enzyme site, Kozak sequence, the start codon and the rest of the sequence up to 27-30 base pairs, while maintaining a GC content of about 50% (Table 2-1). On the other hand, the antisense sequence was designed by including the restriction enzyme sequence, stop codon and the rest of the predicted sequence up to 30 base pairs (Table 2-1).

**Table 2-1.** The primers used for gene cloning of tilapia sialidase genes

Gene	Sense	Antisense
<i>neu1a</i>	5'-GT <u>AAGCTT</u> CCACCATGGCAGCAGGAA-3'	5'-GTGAATTCTTAACGACCACCATACAGGTGG-3'
<i>neu1b</i>	5'-GTGAATTCCACCATGGCAGCATGGCGGCTC-3'	5'-GTGAATTCTTAATGGCCACTATACAGGTGG-3'
<i>Ctsa</i>	5'-GTGAATTCCACCATGCAGCTGCTGCTGTTG-3'	5'-GTGAATTCTCAGTAGGGCAGCTTCTTGATG-3'
<i>neu3a</i>	5'-GTGAATTCCACCATGGGAAACACGTCGTCG-3'	5'-GTGAATTCTCAACGCTTTTCTTTCCTGCCG-3'
<i>neu3b</i>	5'-GTGAATTCCACCATGGGCAACAAACCTTCA-3'	5'-GTGAATTCTTACTCTTCAGTACCCTTCTTG-3'
<i>neu3c</i>	5'-GTGAATTCCACCAATGGTCGTTTTGCATGC-3'	5'-GTCTCGAGCTAAAATTCAGTCCCTTCCTTG-3'
<i>neu3d</i>	5'-GTGGATCCACCATGGGCTGTGGACATTCAG-3'	5'-GTGAATTCTTATTTCTTGGTGATGTCCTCA-3'
<i>neu3e</i>	5'-GTGAATTCCACCATGGGACTGTGGAT-3'	5'-GTCTCGAGTTATTTCTTGGTGATGTCCTC-3'
<i>neu4</i>	5'-GTGAATTCCACCATGAGGTCGCCCTATTTC-3'	5'-GTGAATTCTTAAGACACAGAGCACATCTCA-3'

*\*The underlined sequence represents the restriction enzyme sequence used in the cloning process*

## 2.4. Cloning process and gene characterization of tilapia *neu1s*

### 2.4.1. cDNA amplification of tilapia *neu1s*

To amplify tilapia *neu1* genes 1  $\mu$ l of reverse transcribed cDNA was used for PCR amplification with *neu1s* specific primer pairs using KOD neo plus in a reaction mixture of up 25 $\mu$ l. The reaction mixture was prepared as shown in Table 2-2.

**Table 2-2.** KOD plus Neo polymerase reaction mixture

<b>KOD plus Neo Polymerase</b>	
<b>Reagent</b>	<b>Volume</b>
10x Buffer for KOD -Plus- Neo	2.5 $\mu$ l
2mM dNTPs	2.5 $\mu$ l
25mM MgSO <sub>4</sub>	1.5 $\mu$ l
10 pmol/ $\mu$ l Primer (sense)	1 $\mu$ l
10 pmol/ $\mu$ l Primer (antisense)	1 $\mu$ l
Template DNA	1 $\mu$ l
PCR grade water	
KOD-Plus- (1.0 U/ $\mu$ l)	0.5 $\mu$ l
<b>Total volume</b>	<b>25 <math>\mu</math>l</b>

On the other hand, tilapia *neu3* sialidase genes were amplified by PCR using each sequence specific primers shown in Table 2-1. The coding regions of tilapia *neu3* genes were amplified using PrimeStar Max DNA polymerase (TAKARA, Japan) with the following conditions; an initial denaturation of 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 5 seconds and extension for 1 minute at 68°C. Tilapia *neu3* genes amplification was carried out using cDNAs from brain tissues of three individual adult fish to confirm their sequences. The PCR products were digested with appropriate restriction enzymes, cloned into pBluescript SK (+) vector (TAKARA) and sequenced using ABI3130xl Genetic Analyzer (Applied Biosystems). After sequencing, the cDNAs were later subcloned into an expression vector pcDNA3.1 (+) (Invitrogen). As a positive control for sialidase genes amplification, PCR was done using genomic DNA extracted by Fast Tissue-to-PCR kit (Fermentas, Japan) following manufacturer's protocol.

#### **2.4.2. Gel electrophoresis and DNA purification**

The PCR products were applied to a 1% agarose gel for electrophoresis and the gels were visualized using ethidium bromide (Nacalai Tesque). The bands with the expected size were gel extracted and purified using Fast Gene Gel/PCR Extraction kit (Genetics, Japan). For purification, 500 µl of GP1 from the kit was added to the tube and incubated at 55°C for 15 minutes. Then, the samples were loaded onto a column and vortexed at 13,200 xg for 30 seconds, washed with GP2 and eluted using 30-50 µl of GP3.

#### **2.4.3. DNA restriction, electrophoresis, gel extraction**

Restriction enzyme treatment was used for the cloning process using pBluescript SK (+) cloning vector. Restriction digestion was done using about 1 µg of vector plasmid DNA and about  $\approx 1$  µg of PCR product with appropriate endonucleases and corresponding buffers (TAKARA). A double endonuclease digestion was employed for *neu1a* (HindIII and EcoRI), *neu3d* (BamHI and EcoRI), *neu3e* (EcoRI and PstI) and a single endonuclease digestion with EcoRI for *neu1b*, *neu3a*, *neu4* and *ctsa*. At the same time, pBluescript vector was also treated with appropriate endonucleases corresponding to the endonucleases used for each sialidase gene. The samples were incubated at 37°C for 1 hour. For the single endonuclease digestion of pBluescript vector, the samples were treated with Thermosensitive Alkaline Phosphatase (TSAP) to catalyze the removal of 5' phosphate groups from DNA, thus preventing the recircularization and re-ligation of linearized cloning vector DNA during ligation and incubated at 37°C for 15 minutes. The TSAP was heat deactivated by incubating the sample at 70°C for 15 minutes. To analyze the DNA size and re-purify the DNA, the samples were gel electrophoresed on 1% agarose gel. DNA extraction after gel electrophoresis was performed in accordance with Fast Gene Gel/PCR Extraction kit (Genetics, Japan) protocol.

#### **2.4.4. DNA ligation and transformation in *E. coli***

DNA/vector ligation was done using Ligation-Convenience Kit containing 2x Ligation Mix (Nippon Gene). For ligation, vector DNA and cDNA fragment were mixed in a molar ratio

of 1:1 and subsequently mixed with equal volume of 2x Ligation mixture. The ligation reaction was performed for 30 minutes at 16°C. The ligated product (10 ng DNA) was added to 100 µl of ice-thawed chemo-competent *E. coli* HSTO8 competent cells (TAKARA) and incubated on ice for 30 minutes. The mixture was heat-shocked by incubating at 42°C for 45 seconds and immediately transferred into ice for another 2 minutes. The transformed cells were mixed with Lysogeny Broth (LB) up to 1000 µl, incubated for 45 minutes with shaking at 37°C and then plated on LB agar plates containing 50 µg/ml ampicillin using 100 µl and 500 µl for the selection of the clones. The plates were incubated for 16 hours at 37°C. The plates were checked after 16 hours for growth of bacterial colonies, where each colony represented growth from a single clone.

After an overnight incubation, bacterial colonies were selected, picked up transferred to 4 ml LB medium containing 50 µg/mL ampicillin and cultured overnight with rigorous shaking. The following morning, miniprep plasmid preparation was done using High Pure Plasmid Isolation kit (Roche), a miniprep procedure based on alkaline lysis of *E. coli* followed by adsorption of DNA onto a special matrix. For this procedure, the overnight culture samples were centrifuged at 2,000 xg for 10 minutes and the resultant bacterial pellet was re-suspended with 250 µl Suspension buffer containing RNase followed by the addition of 250 µl Lysis buffer and mixed gently by inverting. The suspension was mixed with 350 µl cold Binding buffer and the mixture was chilled on ice for 10 minutes and centrifuged at 13,200 xg for 10 minutes. The supernatant was loaded onto a column, and eluted with buffer to a final volume of 50 µl. To confirm the presence of the insert in the

isolated plasmid, the samples and the intact pBluescript vector were electrophoresed using 1% agarose gel. Furthermore, to confirm that correct insert was present in the plasmid from the positive clones, endonuclease digestion was used, employing the restriction enzymes used in the cloning process. The endonuclease treated samples were checked for band sizes using agarose gel electrophoresis.

#### **2.4.5. Sequencing**

The sequencing reactions were carried out using the BigDye Terminator. The Terminator Ready Reaction Mix contained the four dNTPs with different fluorescence labels (BigDye<sup>TM</sup> Terminators), unlabeled dNTPs, AmpliTaq DNA Polymerase FS, MgCl<sub>2</sub>, Tris-HCl buffer (pH 9.0). Template DNA (100 ng), 3.2 pmol of the sequencing primer (M13 forward and M13 reverse primers for sequencing of cDNA inserted in pBluescript vector) and 8 µl of the Terminator Ready Reaction Mix were used in a 20 µl sequencing reaction. The sequencing reaction mixes were subjected to 30 amplification cycles. Each cycle consisted of 10 seconds at 96°C, 5 second at 50°C and 4 minutes at 60°C preceded with initial denaturation at 96°C for 3 minutes. The amplified probes were then precipitated using ethanol. For precipitation, the PCR products were mixed with 4 µl 125 mM EDTA, 4 µl 3M sodium acetate buffer (pH 5.2), 100 µl ethanol and 20 µl PCR grade water vortexed and incubated on ice for 20 minutes without light exposure. The mixture was centrifuged at 13,200 xg for 15 minutes and the supernatant was discarded. The pellet, which was not visible at this stage, was carefully re-suspended with 100 µl of cold 70% ethanol and centrifuged for 5 minutes at the 13,200 xg. The sample was then dried and re-suspended



with 20 µl formamide, boiled for 3 minutes and immediately chilled on ice for 3 minutes. The samples were subjected to DNA capillary electrophoresis on the ABI3130xl Genetic Analyzer (Applied Biosystems). Sequence analysis and alignment with reference genomic DNA were performed using sequence scanner (Applied Biosystems) and Genetyx version 1 softwares.

#### **2.4.6. Post sequencing analysis**

The deduced nucleotide and amino acid sequences assembly, further editing, determination of putative molecular masses and sequence alignments were performed using GENETYX software (GENETYX, Japan). Phylogenetic analysis (neighbor-joining method) and percentage sequence identities of tilapia sialidase sequences to that of other species (medaka, zebrafish and human) were performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). Phosphorylation sites were predicted using NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>). Glycosylation prediction was performed by NetNGlyc 1.0 server (N-glycosylation, <http://www.cbs.dtu.dk/services/NetNGlyc/>) and NetOGlyc 3.1 server (O-GalNAc attachment site, <http://www.cbs.dtu.dk/services/NetOGlyc/>). The signal peptide cleavage sites were analyzed using SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). Intron-exon organization and syntenic analysis were determined using Ensembl genome browser. Key residues in catalytic sites of tilapia sialidases were predicted according to Giacomuzzi et al. (2012).

#### **2.4.7. Tilapia sialidase genes cloning into pcDNA3.1 expression vector**

The successfully sequenced *neu1s* and *neu3s* and *neu4* cDNAs were cloned in pcDNA3.1 (Invitrogen) expression vector. The pcDNA3.1 vector contains a cytomegalovirus immediate-early (CMV) promoter that provides high expression of the recombinant protein in a wide range of mammalian cells. The pBluescript cloned tilapia sialidase genes were isolated by endonuclease digestion and ligated into pcDNA3.1 treated with the corresponding endonucleases. The ligated pcDNA3.1-*neu1s/neu3s/neu4* were each transformed in *E. coli* HST08 competent cells by heat-shocking at 42°C for 1 minute and immediately transferring on ice for 2 minutes. Plasmids were extracted from positive clones using High Pure Plasmid Isolation kit (Roche). The plasmids were checked for insert by 1% agarose gel electrophoresis by comparison with empty pcDNA 3.1 vector. All positive plasmid samples were digested with endonucleases to confirm the sizes and the orientation of the insert in pcDNA 3.1.

For Midiprep, 100 mL LB medium containing 50 µg/mL ampicillin was prepared in 500 mL flask and the positive clone from the master plate was picked and cultured overnight at 37°C with vigorous shaking. The following morning, the culture was transferred in 50 mL falcon tubes and centrifuged at 2,000 xg for 10 minutes at 4°C to harvest the bacterial cells. The supernatant was discarded and the Midprep was done using Genopure Plasmid Midi kit (Roche). For this procedure, the bacterial pellet re-suspended using 4mL suspension until no cell clumps remained. Then 4 mL of Lysis buffer was added, mixed immediately by inverting and incubated at room temperature for 5 minutes. Subsequently, 4 mL of chilled

Neutralization buffer was added, gently mixed by inverting and incubated on ice for 10 minutes. During the incubation, the column was equilibrated by adding 2.5 mL Equilibration buffer and let it flow-through. The mixture was applied to the column by passing it through a filter to sieve out all unwanted cell debris. The column was washed twice with wash buffer and the DNA was eluted with 5 mL pre-warmed elution buffer. The DNA was precipitated by adding 3.6 mL isopropanol to the eluted DNA, vortexed and centrifuged at 2,580 xg for 1 hour. The DNA pellet was re-suspended with 400 µl TE buffer to which 1 mL ethanol and 40 µl 3M CH<sub>3</sub>COONa (pH 5.2) were added. The mixture was incubated on ice for 20 minutes before being centrifuged at 15,590 xg for 30 minutes at 4°C. The resulting DNA pellet was washed by re-suspending with cold 70% ethanol and centrifuging at 15,500 xg for 5 minutes. From here, all procedures were carried out in the clean bench to avoid any DNA contamination. DNA pellet was dried and subsequently, suspended with filtered TE buffer.

#### **2.4.8. Cell culture and culture conditions**

Human Embryonic Kidney (HEK293) and its derivative HEK293T cells (RIKEN cell bank, Japan) were used to express tilapia sialidase proteins. HEK293 cells lines were cultured under standard conditions. To revitalize the cell lines, the cryogenic vials were taken from the -80°C freezer and quickly thawed by incubating in a water bath at 37°C. The cells were transferred to a 15 ml falcon tube with 5 ml of fresh medium and centrifuged at 600 xg for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 5 ml of fresh medium, transferred to 60 mm dish and culture in for 48 hours at 37°C in a 5% CO<sub>2</sub>

incubator. The cells were maintained in Dulbecco modified eagle medium (DMEM) containing 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> incubator. Cultures at ~90% confluence were routinely seeded as follows; after removing the growth medium, cells were washed once with 1 X PBS followed by the addition of 1 ml PBS containing EDTA and 200 µl trypsin (Nacalai Tesque). The plates were incubated at 37°C for 3 minutes to facilitate cell detachment and 5 ml pre-warmed culture medium was added. The cells were split into new plates with appropriate dilution ratios.

#### **2.4.9. Transfection for protein expression in mammalian cells**

Expression vectors were transiently transfected into HEK293 or HEK293T cells using a calcium phosphate method (Chen and Okayama, 1987). The pcDNA 3.1 cloned sialidase genes were introduced into HEK293 cells using calcium phosphate transfection method. The calcium phosphate transfection method is based on the formation of a calcium phosphate-DNA precipitate. The calcium phosphate facilitates the binding of the DNA to the cell surface and the DNA enters the cell by endocytosis. Cells were seeded one day before transfection as described above and the medium was changed 4 hours before transfection. Transfection mixture was prepared in 1.5 mL tube by mixing 225 µl ddH<sub>2</sub>O, 25 µl of 2.5M CaCl<sub>2</sub> and 10 µg of plasmid DNA and mixed gently by pipetting. This mixture facilitated the formation of DNA-CaCl<sub>2</sub> complex. Then, 225 µl of 2 X BES buffered saline was added drop wise to the CaCl<sub>2</sub>/DNA mixture while gently shaking. The mixture was vortexed and incubated at room temperature for 20 minutes. The DNA mixture was dropwise added to the cells and incubated at 37°C in a CO<sub>2</sub> incubator for 48 hours.

#### 2.4.10. Biochemical characterisation of tilapia sialidase polypeptides

Biochemical characterizations were conducted *in vitro* using total cell lysates prepared from the each sialidase gene transfected cells.

#### 2.4.11. Protein harvest and sialidase activity assay

After 48 hours, the transfected cells were washed in Dulbecco's phosphate buffered saline (PBS), scraped and harvested using centrifuging at 2,580 xg for 5 minutes. The cell pellets were lysed in PBS containing 1mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin by incubating on ice for 15 minutes with occasional vortexing. The total lysate containing each tilapia sialidase polypeptide were isolated by centrifugation at 13,200 xg for 30 minutes as the supernatant. The resulting supernatant was used for sialidase activity assay using 50 µl reaction mixture towards 4-methylumbelliferyl-N-acetyl- $\alpha$ -D-neuraminic acid (4-MU-NANA) (Table 2-3) and its protein concentration was determined by the Dye binding assay (Nacalai, Japan).

**Table 2-3.** Components for sialidase activity reaction mixture

Reagent	Volume
4MU-NANA	5 µl
2% Triton X-100	2.5 µl
Buffer (pH3.5 – 7.0)	5 µl
Lysate	10 µl
Water	37.5 µl
<b>Final Volume</b>	<b>50 µl</b>

#### **2.4.12. Determination of optimal pH and substrates for sialidase activity.**

For *neu1a* and *neu1b* and *neu4* genes, the optimal pH was determined against 4-MU-NANA. The pH effect on sialidase activity was determined by 2.5 mM sodium acetate buffer (pH 3.5 to 5.5) and sodium phosphate buffer (pH 5.5 to 7) using ganglioside mix as substrate. Neu3s optimal pH was determined under the same conditions using ganglioside as a substrate.

Substrate specificities for tilapia Neu1, Neu3 and Neu4 polypeptides were assessed at the determined optimal pH for each sialidase towards various substrates; ganglioside mix (IsoSep, Sweden), 3-siallyllactose, 6-siallyllactose and 4-methylumbelliferyl-N-acetyl- $\alpha$ -D-neuraminic acid (4-MU-NANA) (Nacalai Tesque), bovine fetuin (*N*-and *O*-glycosylated protein with  $\alpha$ 2-3 and  $\alpha$ 2-6 sialosyl linkages) (Bovogen, Australia), bovine submaxillary gland mucin (*O*-glycosylated protein with  $\alpha$ 2-3 and  $\alpha$ 2-6 sialosyl linkages) (Worthington, NJ) as previously described (Shiozaki et al., 2013).

The activity was determined from a reaction mixture (Table 2-3) after incubation at 37°C for 1 hour. The reaction mixtures were incubated at 37°C for 1 hr. To determine the amount of free sialic acid, thiobarbituric acid assay was used as describe previously (Warren, 1959). Briefly, sialidase enzyme reaction was terminated by 50  $\mu$ l of 0.2 M metaperiodate solution, followed by 0.5 ml of 10% arsenite solution. After the addition of 1.5 ml of 0.6% TBA solution, reaction mixture was boiled for 15 minutes and then mixed with 1.5 ml of

cyclohexanone. Absorbance at 549 and 534 nm of the upper phase was determined using spectrophotometer. Released sialic acid was calculated as follows: micro moles of NeuAc =  $0.084 \times OD_{549} - 0.031 \times OD_{532}$ . One unit (U) of sialidase activity was defined as the desialylation of 1 nmol of sialic acid/hr from each substrate.

#### **2.4.13. Preparation of HA-tagged expression plasmids**

The discovery of PCR greatly facilitated creation of tagged proteins by making it possible to fuse the desired epitope to the target by designing PCR primers encoding the epitope. An epitope is a portion of a molecule to which an antibody binds. To investigate the subcellular localization for tilapia Neu3 sialidase proteins, C-terminal hemagglutinin (HA) tagging of each tilapia sialidase gene was done using PCR primers shown in Table 2.4. HA- tag is derived from the binding domain of the Influenza hemagglutinin protein and it contains a high proportion of charged residues (YPYDVPDYA) so it is likely to form a strong antibody recognition site. HA- tagging was carried using PCR with pBluescript cloned sialidase genes as templates. Except for *neu3* genes, PCR was carried using KOD-neo-plus (TAKARA) with the following PCR 25 µl reaction mixture (Table 2-5).

**Table 2-4.** C-terminal HA – tagging primers for each tilapia sialidase genes.

<b>Gene</b>	<b>Sense primer</b>	<b>Anti-sense primer</b>
<i>neu1a</i> -HA	5'-TCCAGATTACGCTTAAGAATTCCTGCAGCC-3'	5'-ACATCGTATGGGTAACGACCACCATACAGG-3'
<i>neu1b</i> -HA	5'-TCCAGATTACGCTTAAGAATTCCTGCAGCC-3'	5'-ACATCGTATGGGTAATGGCCACTATACAGG-3'
<i>neu3a</i> -Ha	5'-ACGTCGTATGGGTAACGCTTTTCTTTCCTG-3'	5'-GCCTGACTACGCCTGAGAATTCCTGCAGCC-3'
<i>neu3d</i> -HA	5'-GCCTGACTACGCCTAAGAATTCGATATCAA-3'	5'-ACGTCGTATGGGTATTTCTTGGTGATGTCC-3'
<i>neu3e</i> -HA	5'-GCCTGACTACGCCTAAGAATTCCTGCAGCC-3'	5'-ACGTCGTATGGGTATTTCTTGGTGATGTCC-3'
<i>neu4</i> -HA	5'-TCCAGATTACGCTTAAGAATTCGATATCAA-3'	5'-ACATCGTATGGGTAAGACACAGAGCACATC-3'
<i>csta FLAG</i>	5'-ATGACGACAAGTGAGAATTCGATATCAAGC-3'	5'-CGTCTTTGTAGTCGTAGGGCAGCTTCTTGA-3'



**Table 2-5.** C-terminal HA – tagging PCR reaction mixtures.

<b>KOD plus Neo Polymerase</b>	
<b>Reagent</b>	<b>Volume</b>
10x Buffer for KOD -Plus- Neo	2.5 µl
2mM dNTPs	2.5 µl
25mM MgSO <sub>4</sub>	1.5 µl
10 pmol/µl Primer (sense)	1 µl
10 pmol/µl Primer (antisense)	1 µl
Template DNA	1 µl
PCR grade water	15 µl
KOD-Plus- (1.0 U/µl)	0.5 µl
<b>Total volume</b>	<b>25 µl</b>

Tilapia *neu3* genes were HA-tagged using PCR with Prime Star GXL polymerase (TAKARA) and pBluescript SK(+) ligated *neu3* cDNAs were used as templates at the following conditions; an initial denaturation at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 68°C for 5 minutes. The reaction mixture was prepared as shown in the Table 2-4. After PCR, the samples were electrophoresed using 1% agarose gel, the DNA was extracted from the gels and purified using Genopure Gel/PCR purification kit as outlined above. The PCR products were ligated using the reaction conditions summarized in Table 2-6.

**Table 2-6.** Ligation reaction mixture.

<b>Reagent</b>	<b>Volume</b>
Ligation Mix	7.5 $\mu$ l
DNA template	5 $\mu$ l
Polynucleotide kinase	1 $\mu$ l
ATP	1.5 $\mu$ l
<b>Total</b>	<b>15 <math>\mu</math>l</b>

The ligated samples were transformed into *E. coli* HST08 competent as outlined above and resulting plasmids were sequenced to confirm the presence of HA-tag sequence. After successful sequencing, the insert containing the C-terminal HA-tag was clipped-out of the pBluescript vector and subsequently sub-cloned into pcDNA3.1 (+) expression vector using the same restriction enzymes.

#### **2.4.14. Transfection for Biochemical characterisation**

The HA-tagged pcDNA 3.1-inserted tilapia sialidase genes were transfected into various cell lines. All tilapia HA-tagged genes were transfected into HEK293T cells for cell fractionation and immunoblotting purposes. For indirect immunofluorescence, tilapia *neul* HA-tagged genes were transfected into tilapia hepatic cells lines (Hepa-T1). All transfections were done using polyethylenimine (PEI) transfection protocol (Longo et al., 2014). Through this transfection procedure, DNA is introduced into a host cell by

transfection with polyethylenimine (PEI), a stable cationic polymer (Boussif et al., 1995). The DNA is condensed by PEI into positively charged particles that bind to anionic cell surfaces. As a result, the DNA/PEI complex is endocytosed by the cells and the DNA released into the cytoplasm (Sonawane et al., 2003). For each 60 mm plate to be transfected, 300  $\mu$ L Opti-MEM was mixed with 6  $\mu$ g of your DNA for each tilapia sialidase gene in a 1.5 mL tube and vortexed for 3 seconds. Then 12  $\mu$ L of PEI (1  $\mu$ g/ $\mu$ L) was added and immediately pulse vortexed for 3 seconds. The mixture was Incubated at room temperature for 15 minutes. During the incubation time, the culture medium was replaced with half volume fresh medium. For 60 mm dish, the medium was replaced with 2.5 mL fresh medium and the transfection mixture was added to the plate dropwise, distributing the drops over the entire plate. The plates were incubated for 24 hours at 37°C in a 5% CO<sub>2</sub> incubator. After 24 hours of incubation, the medium was removed and replaced with 5 mL of fresh medium and the cells were grown for another 24 hours under the same conditions.

#### **2.4.15. Cell harvest and fractionation**

After 48 hours on transfection, the cells were scraped from the plate, suspended in PBS, and recovered by centrifugation at 2,580 xg for 5 minutes. The cells were re-suspended in 9-volume of homogenization buffer containing protease inhibitors (Table 2-7).

**Table 2-7.** Sonication buffer for homogenate preparation.

Reagent	Volume
PBS	1 mL
0.2M PMFS	1 $\mu$ l
0.5M EDTA	2 $\mu$ l
2mg/l Leupeptin	4 $\mu$ l

Cell were disrupted using a hand sonicator 10 times with a 1-second pulse and centrifuged at 600 xg for 5 minutes. The supernatant was used for sialidase activity to confirm the activity level after HA-tagging and later used for immunoblotting analysis to determine the molecular sizes of the expressed sialidase proteins.

Other sialidase Neu3 polypeptides in mammals and fish have been reported to be present at the plasma membrane, as tilapia Neu3 sialidases were further assessed using immunoblotting to provide an insight of their localization. For this analysis, biochemical fractionation was carried as described previously (Shiozaki et al, 2014). HEK 293 cells were transfected with *neu3* HA-tagged genes using PEI method as outlines above. After 48 hours, the cell pellet was collected by scraping, suspending in PBS and centrifuging at 600 xg for 5 minutes. The cell pellets were re-suspended in 9-volume of 10 mM Tris–HCl (pH 7.5) 10 mM NaCl and 1.5 mM MgCl<sub>2</sub> and homogenized using a Dounce homogenizer. Homogenate was mixed with equal volume of 525 mM mannitol, 175 mM sucrose, 12.5 mM Tris– HCl (pH 7.5) and 2.5 mM EDTA (pH 7.5) and centrifuged at 1,300 xg for 5 min.

The supernatant was centrifuged again at 17,000  $\times g$  for 15 min to collect the subsequent supernatant and pellet fractions. The pellet was suspended with equal volume of above buffer.

#### **2.4.16. Immunoblotting analysis**

Western blotting was used to determine the relative sizes of sialidase proteins overexpressed in cells. Firstly, protein concentration was determined using the Dye binding assay (Nacalai, Japan). The samples were prepared by adding 2 X loading dye 20  $\mu g$  of protein and incubated at 95°C for 3 minutes. The samples were loaded onto a 12% acrylamide gels prepared in a structured stacking and running gel (Table 2-8).

**Table 2-8.** Ingredients composition of acrylamide gel for SDS-PAGE

<b>Reagent</b>	<b>Running gel</b>	<b>Stacking gel</b>
30% acrylamide 0.8 Bis	3.4 mL	500 $\mu l$
Tris-HCl	2 mL (1.875M, pH 8.8)	500 $\mu l$ (1.25M, pH 6.8)
Water	4.5 mL	3.9 mL
10% SDS	100 $\mu l$	50 $\mu l$
10% APS	100 $\mu l$	50 $\mu l$
TEMED	10 $\mu l$	5 $\mu l$

The proteins were separated according to size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the membrane was washed for 10 minutes with blotting buffer. Then the protein electro-transferred to a

Hybond-P polyvinylidene fluoride membrane (PVDF) (GE Healthcare) and blocked with 1% non-fat dried skimmed milk in PBS containing 0.1% Tween 20 (PBST). For detection of HA-tagged sialidase polypeptides, the membranes were then incubated with monoclonal primary HA antibody for (3F10, Roche) at a dilution of 1:1000 for 1 hour at 37°C. This was followed by washing in PBST for 30 minutes before incubation with horse radish peroxidase conjugated anti-rat IgG secondary antibody (1:1000) for another hour at room temperature. The membranes were washed again with PBST for 30 minutes and detection of immunocomplexes was done using Peroxidase Stain DAB Kit (Nacalai Tesque).

#### **2.4.17. Indirect immunofluorescence**

Indirect immunofluorescence localization were evaluated in HEK 293 and/or Hepa-T1 transiently transfected with each HA-tagged sialidase gene. HEK293 and Hepa-T1 cells ( $1 \times 10^4$ ) were seeded onto glass coverslips inserted in 12-well plates and transfected with expression vector for *neu1a*-HA for Hepa-T1 cells, *neu3*-HAs and *neu4*-HA for HEK 293 cells. At 48 hours post transfection, the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and washed using PBS for 15 minutes. The cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 minutes and blocked with 1% bovine serum albumin (BSA) for 30 minutes at 37°C. The cells were incubated with 1:100 PBS diluted primary antibodies for HA and the following the following subcellular markers; lysotracker (TAKARA, Japan) for lysosome, E-cadherin (N1C3, GeneTex) for plasma membrane, GAPDH (glyceraldehyde 3-phosphate dehydrogenase, N1C3, GeneTex) for the cytosol and DAPI (Nacalai, Japan) for nucleus. Staining was done by incubation with Alexa 488 or

Alexa 555-conjugated secondary antibody (Invitrogen) followed by mounting onto glass slides and examination by sectioning fluorescence microscopy system (Apotome, Carl-zeiss, Germany).

#### 2.4.18. Tissue expression of tilapia sialidase genes

Tissue mRNA expression levels for tilapia sialidase genes were analyzed from adult fish brain, heart, eyes, liver, muscle and spleen using real-time PCR (Lightcycler Nano, Roche, Germany). KOD SYBR Green PCR Master Mix (TAKARA) was used for the real-time PCR using the following primers: 5′-TGTAACGTCAGCCCTCTGCAGAT-3′ (forward) and 5′-AAGACTTCCTGGCTTCTGCAA-3′ (reverse) for *neu1a*, 5′-TTGACCCCCTGGTGTTTGA-3′ (forward) and 5′-AAGACTTCCTGGCTTCTGCAA-3′ (reverse) for *neu1b*, 5′-CACCTGAAAGAGGATGGATC-3′ (forward) and 5′-AAGGATTCATGGTGCGGTGGT-3′ (reverse) for *neu3a*, 5′-GTAGTACTTGCCCTCTTGCAATTG-3′ (forward) and 5′-AAACTGGAGCCGTTGTCTTCA-3′ (reverse) for *neu3d*, 5′-AGAAGTACTGGCCACTTGCGCT-3′ and 5′-AAACTGGAGCCGTTGTCTTCA-3′ for *neu3e*, 5′-CTATGTGGAGTGGGAGGACA-3′ (forward) and 5′-CAGAAAGAGAGTACCCGTG-3′ (reverse) for *neu4*. The primers for  $\beta$ -actin gene as a reference were adopted from Phang et al. (2012) as follows; 5′-CCTGACAGAGCGTGGCTACTC-3′ (forward) and 5′-TCTCTTTGATGTCACGCACGAT-3′ (reverse). Standard curves were generated by serial dilution of the pBluescript vectors containing entire sialidases gene ORFs obtained in

during cloning. The amplification involved a 2-minutes pre-denaturation phase at 98°C followed by 45 cycles of 10 seconds at 98°C denaturation, 10 seconds at 60°C annealing and 30 seconds at 68°C extension phase.

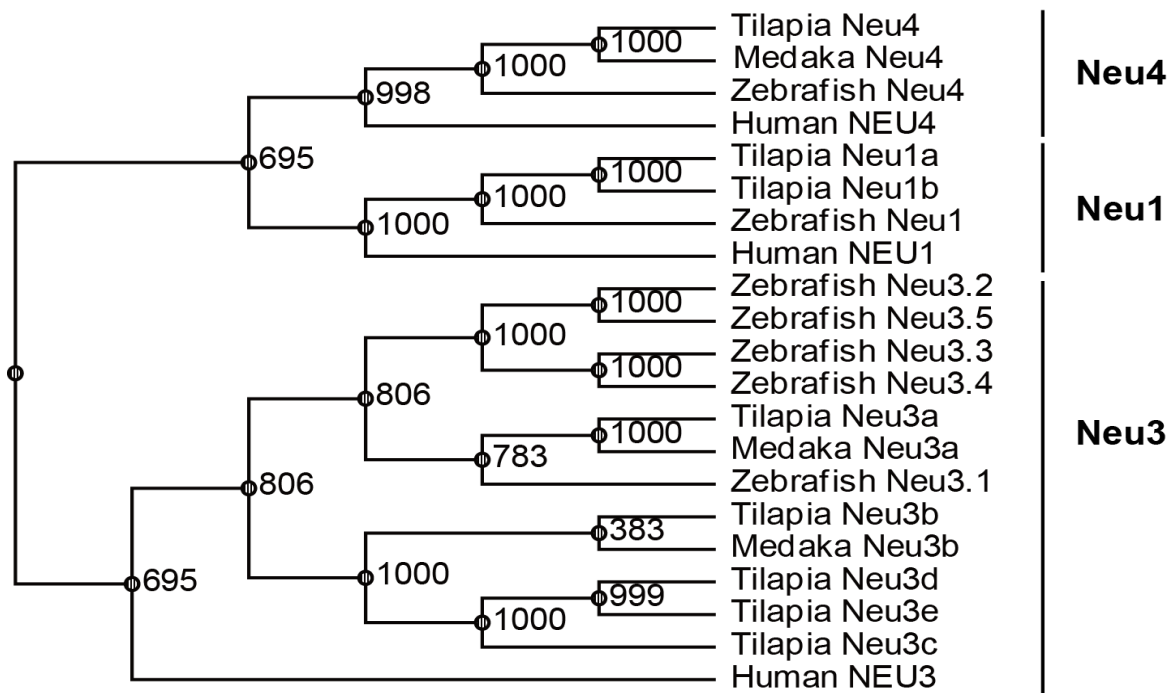
## **2.5. Results**

### **2.5.1. Tilapia *neu1* genes cloning and Neu1 polypeptide characterisation**

#### **2.5.1.1. Identification of tilapia *neu1* genes**

The partial sequence of two *neu1*-like genes in *O. niloticus* genome were identified after BLAST search in Ensembl genome database. Clustered two *neu1*-like genes were on chromosome LG22 (*neu1a*: 2,743,418-2,753,636 and *neu1b*: 2,757,225-2,762,302). The presence of two putative orthologous sequences of *neu1* genes in tilapia genome, indicated the major variation in Neu1 from mammals and other fish, all of which possess a single form of *neu1* gene. Previous reported have shown a single Neu1 in human (Pshezhetsky et al., 1997), mouse (Carrillo et al., 1997), zebrafish (Manzoni et al, 2007) and medaka (Ryuzono et al., 2016). Tilapia *neu1* paralogs were present in the same chromosome (Figure 2-2). The entire coding sequence of putative tilapia *neu1*s were obtained using Ensembl genome browser and University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu/>). A BLASTP homology analysis using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) database revealed that the tilapia Neu1a and Neu1b showed sequence identities of 55.8% and 57.5% in comparison with human NEU1.

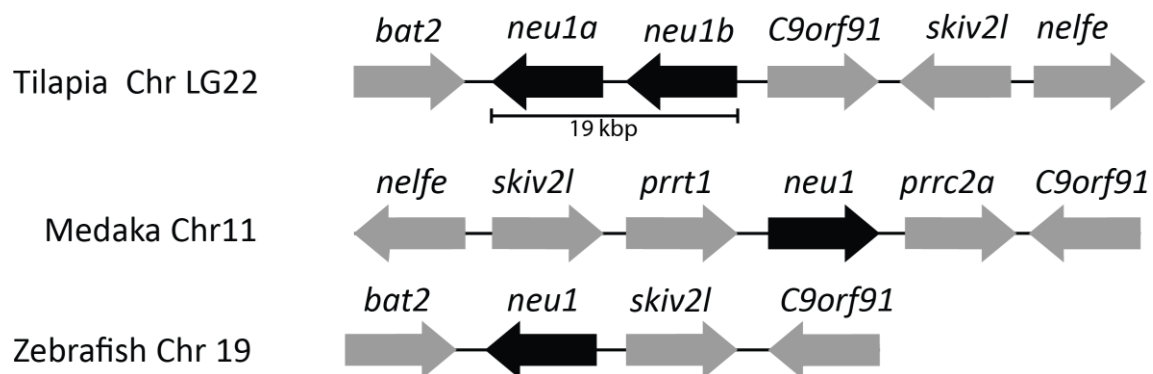




**Figure 2-1.** Tilapia Neu1 sialidase gene organizations and evolutionary relationships.

(A) Unrooted Phylogenetic tree depicting the evolutionary relationship of tilapia sialidases in comparison with other species. The phylogenetic tree was described using the amino acid sequence of NP\_001038374 (zebrafish Neu1), NP\_035023 (mouse Neu1), NP\_000425 (human NEU1), NP\_001161073 (human NEU4), NP\_001018384 (zebrafish Neu4), NP\_006647 (human NEU3), NM\_001079665 (zebrafish Neu3.1), NM\_001003644 (zebrafish Neu3.2), NM\_001077538 (zebrafish Neu3.3), NM\_001109723 (zebrafish Neu3.4) and NM\_001109733 (zebrafish Neu3.5). All medaka amino acid sequences were obtained from previous paper (Shiozaki et al., 2013). Ensembl protein ID for tilapia Neu1a, Neu1b and Neu4 were, ENSONIP00000016967, ENSONIP00000016968, and ENSONIP00000004757, respectively. The tree was constructed by neighbor-joining analysis and numbers at branch points indicate the bootstrap confidence levels with 1000 replications

Amino acid comparison between Neu1a and Neu1b yielded an 87% sequence identity, suggesting a very close association between the two proteins. Furthermore, comparison between tilapia Neu1a and medaka Neu1 yielded 75.5% while tilapia Neu1b and medaka Neu1 yielded 74.3% sequence identities. Tilapia, *neu1* genes are flanked by *skiv2l*, *C9orf91* genes that are conserved in both medaka and zebrafish Neu1. Another flanking gene, *nelfe* was only conserved in medaka but could not be detected in zebrafish.

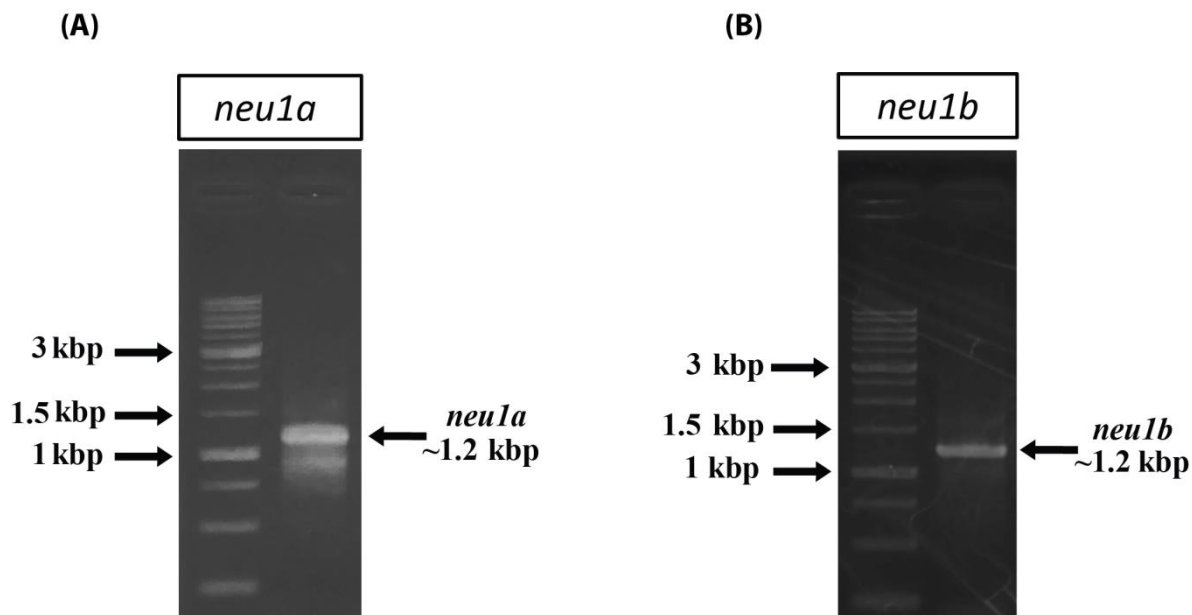


**Figure 2-2.** Syntenic analysis around *neu1s*.

The black arrows indicate sialidase genes. Dark arrows indicate conserved genes in the syntenic regions.

### 2.5.1.2. Actual nucleotide and amino acid properties

The presence of the two different *neu1* genes in tilapia genome was confirmed by PCR and the product were checked using 1% agarose gel electrophoresis. The results showed that only single bands (approximately 1.2 kbp) for each sialidase *neu1* gene (Figure 2-3) similar to each nucleotide predicted sizes.

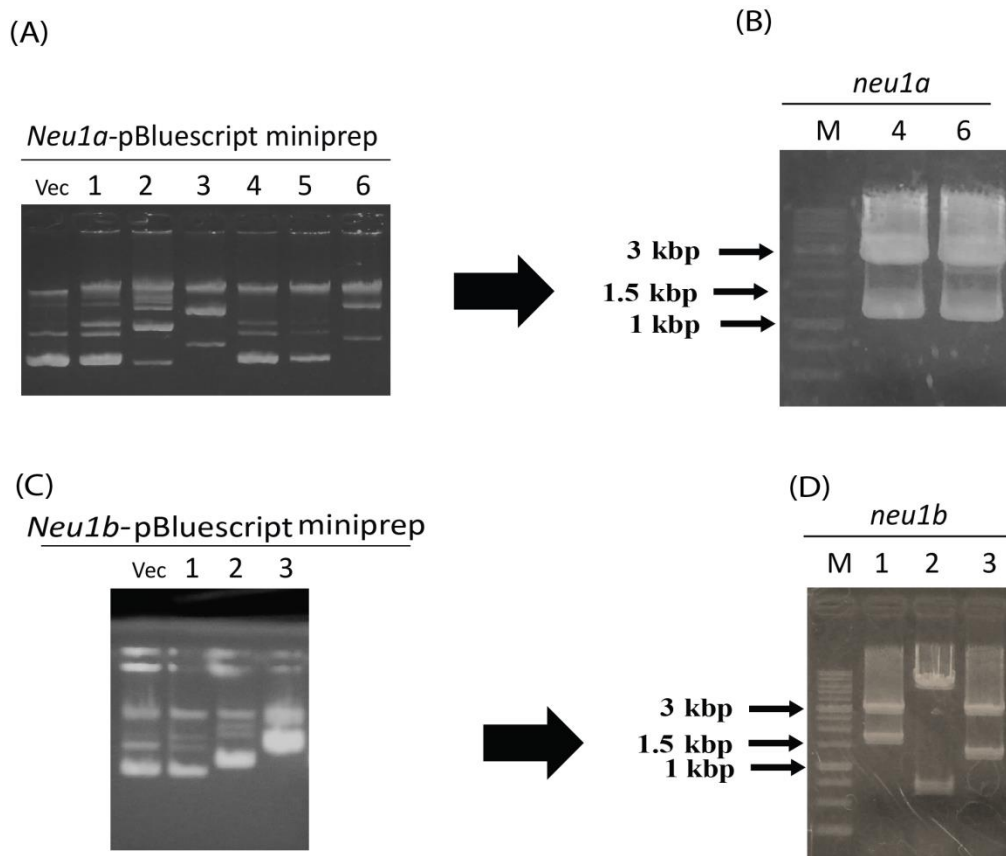


**Figure 2-3.** Tilapia *neu1* genes electrophoresis.

(A) *neu1a* gene amplified using primers designed from the predicted sequence. (B) *neu1b* electrophoresis of the gene product after PCR.

The PCR products were purified and subcloned into pBluescript SK(+) sequencing vector using endonuclease treatment and ligation followed by transformation. Single colonies were picked from the plates and were grown over night. Plasmid DNA was purified using Miniprep Kit. To test for plasmid DNA with correct insert, purified DNA was digested with HindIII and BamHI for *neu1a* and EcoRI for *neu1b*. The resulting DNA fragments were separated on a 1% agarose gel to confirm whether the cloning of *neu1* genes intron pBluescript vector had been successful. After miniprep, the samples were checked for the insert by comparing their size with the empty vector, pBluescript. Samples that showed positive results (Figure 2-4A and B) were further treated with the corresponding

endonuclease to confirm the size of the insert. The restriction enzyme treatment for both *neu1a*-pBluescript and *neu1b*-pBluescript that yielded two bands; one corresponding to each *neuls* and 3 kbp corresponding the linearized vector (Figure 2-4B and D). The samples were later sequenced using ABI3130xl Genetic Analyzer (Applied Biosystems).

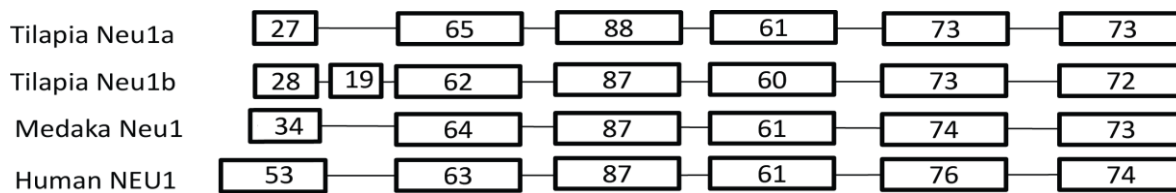


**Figure 2-4.** Insert checking for *neu1a* and *neu1b*.

The transformants were plated on LB agar plates containing 50  $\mu$ g/mL ampicillin. Selected colonies were culture in LB medium also containing 50  $\mu$ g/mL ampicillin. After miniprep, the samples were checked for insert using electrophoresis (A and C). The positive inserts were digested with endonucleases to confirm the insert (B and D).

After sequencing, the actual consensus *neu1a* and *neu1b* nucleotide sequences were

obtained using Genetyx software in comparison with the predicted sequences. Tilapia *neu1a* consisted of 1,164 bp and encoding 388 amino acid Neu1a polypeptide with a predicted molecular weight of 42.4 kDa. On the other hand, *neu1b* nucleotide sequence consisted of 1,218 base pairs, encoding 406 amino acids with a molecular weight of 44.7 kDa. Analysis of the open reading frame for *neu1a* showed the presence of six exons (Figure 2-5) similar to human NEU1 and medaka Neu1 (Ryuzono et al., 2016). Interestingly, Neu1b possessed seven exons, different from other Neu1s from human and medaka.



**Figure 2-5.** Exon analysis.

Exons were determined by sequence alignment alignment of the actual neu1 nucleotide sequences with the genomic nucleotide sequence around the region for the Neu1 sequences.

Several single nucleotide polymorphisms (SNPs) were observed in both *neu1a* and *neu1b*. Comparison between putative and actual *neu1a* nucleotide sequences showed the presence of 18 SNPs synonymous and nonsynonymous, which resulted in nine amino acid change; Q28L, T74S, Y76S, Y77S, A199E, N217Y, A292V and R358H (Figure 2-6A). Apart from having an extra exon, tilapia *neu3b* exhibited lesser SNPs, one of which nonsynonymous resulting into a change E330K (Figure 2-6B). The *neu1a* and *neu1b* sequences were deposited in the DNA Databank of Japan (DDBJ) under the accession numbers LC051024

and LC101496, respectively.

(A)

Putative Neu1a	1	MAAGSRLAALWLQLFVVVSSVCNVSP	QIDPLVFEEQLLWVSGSQGVNTYRIPLLTFT	60
Actual Neu1a	1	MAAGSRLAALWLQLFVVVSSVCNVSP	QIDPLVFEEQLLWVSGSQGVNTYRIPLLTFT	60
Putative Neu1a	61	PKGSLLAFAEARKTSYY	DIGAKFFALRRSTDKGATWSPTTFIIDDGAAPDGINLGSVVVD	120
Actual Neu1a	61	PKGSLLAFAEARKSSSS	DIGAKFFALRRSTDKGATWSPTTFIIDDGAAPDGINLGSVVVD	120
Putative Neu1a	121	EEVGSVILIYSVCFHLYHCSPASIMMVESRDDGLSWTPPRNLSVTLGVKNFAPGPGGLGLO		180
Actual Neu1a	121	EEVGSVILIYSVCFHLYHCSPASIMMVESRDDGLSWTPPRNLSVTLGVKNFAPGPGGLGLO		180
Putative Neu1a	181	KRFNPAKGRLLVCGHGTLE	GDGVFCILSDDHGQTWYNGAALKSIPYNQKKKAQDFNPDEC	240
Actual Neu1a	181	KRFNPAKGRLLVCGHGTLE	GDGVFCILSDDHGQTWYNGAALKSIPYNQKKKAQDFNPDEC	240
Putative Neu1a	241	QPIELTDGTIAINVRNQNNYHCRCRIVVHSYDGGLTLPLEGLIFDEALVDPVVAAGALQK		300
Actual Neu1a	241	QPIELTDGTIAINVRNQNNYHCRCRIVVHSYDGGLTLPLEGLIFDEALVDPVVAAGALQK		300
Putative Neu1a	301	EGVIYFTNPSNEQKRVNLTWKWSLTNGTSWENKAVQIWAGPSGYSSMTSLDSGSVEDEK		360
Actual Neu1a	301	EGVIYFTNPSNEQKRVNLTWKWSLTNGTSWENKAVQIWAGPSGYSSMTSLDSGSVEDEK		360
Putative Neu1a	361	IFVIYEKGQKDYFETVSFTKIHLYGGR		387
Actual Neu1a	361	IFVIYEKGQKDYFETVSFTKIHLYGGR		387

(B)

			Extra exon - 19 aa	
Putative Neu1b	1	MAAWRLSALWLLLSALASSCVLTVPNPQQIDPLVFEE	-----QLLWV	41
Actual Neu1b	1	MAAWRLSALWLLLSALASSCVLTVPNPQQIDPLVFEE	KLLWVSGAQQQIDPLVFEEQLLWV	60
Putative Neu1b	42	SGSQGVNTYRIPLLTFTPKGSLLAFAEARKTSSSDIGAKFIAMRRSTDKGATWSPTSF		101
Actual Neu1b	61	SGSQGVNTYRIPLLTFTPKGSLLAFAEARKTSSSDIGAKFIAMRRSTDKGATWSPTSF		120
Putative Neu1b	102	VDDGMKPDGLNLGSVVVDEEVGSVILIYDVCFYLYHCSPPS	IMMVESLDDGLSWSPPRNL	161
Actual Neu1b	121	VDDGMKPDGLNLGSVVVDEEVGSVILIYDVCFYLYHCSPPS	IMMVESLDDGLSWSPPRNL	180
Putative Neu1b	162	SVTLGVKNFIPGPGLGLQKRFNPAKGRLLVCGHGTLE	GDGVFCILSDDHGQTWYNGAALK	221
Actual Neu1b	181	SVTLGVKNFIPGPGLGLQKRFNPAKGRLLVCGHGTLE	GDGVFCILSDDHGQTWYNGAALK	240
Putative Neu1b	222	SIPYNQKKKAQDFNPDECQPIELTDGTIAINVRNQNRHYHCQCRIVVHSYDGGLTLPLEGL		281
Actual Neu1b	241	SIPYNQKKKAQDFNPDECQPIELTDGTIAINVRNQNRHYHCQCRIVVHSYDGGLTLPLEGL		300
Putative Neu1b	282	IFDEALVDPVVAAGTLQKEGVIYFTNPNCE	EKRNVNLTWKWSLTNGKSWEKKAVQIWSGPS	341
Actual Neu1b	301	IFDEALVDPVVAAGTLQKEGVIYFTNPNCE	EKRNVNLTWKWSLTNGKSWEKKAVQIWSGPS	360
Putative Neu1b	342	GYSCITSLDSGSAEDRKYIYVIYEKGHNQYFETVSFAKIHLYSGH		386
Actual Neu1b	361	GYSCITSLDSGSAEDRKYIYVIYEKGHNQYFETVSFAKIHLYSGH		405

**Figure 2-6.** Putative and actual Neu1 amino acid sequences.

(A) Alignment between predicted and actual Neu1a amino acid sequences. (B) Neu1b amino acid alignment between predicted and actual amino acid sequences. Conserved amino acids are shown in black colour and non-conserved amino acids are shown in white colour.

Several proteins are glycosylated as a post-translational modification. Glycosylation, a protein modification system is present in all domains of life and is characterized by a high structural diversity of N or O-linked glycans found among different species and by a large number of proteins that are glycosylated. Bioinformatic analyses with the deduced amino acid sequence revealed that tilapia Neu1a possessed three putative N-glycosylation (Asn 161, 316, 326) and two O-glycosylation sites (Thr 95, 165) (Figure 2-7). Two of the N-glycosylation sites and one of the O-glycosylation sites were conserved with human NEU1 and medaka Neu1 (Lukong et al., 2001; Ryzono et al., 2015), while one N-glycosylation site was not conserved with medaka Neu1 and one O-glycosylation site was not conserved with both human NEU1 and medaka Neu1.

1	ATGGCAGCAGGAAGCCGGCTCGCTGCTCTTTGGCTACAGCTCTTTGTTGGTGTCTTCC	60
	<u>M</u> A A G S R L A A L W L Q L F V V V S S	
61	TGTGTTTGTAACTGAGCCCTTTCAGATCGACCCCTGGTGTGAGGAGCAGCTGCTG	120
	C V C N V S P L Q I D P L V F E E Q L L	
121	TGGGTGAGCGGATCCCAGGGCCAGGTGAACACTTACAGGATCCCGCTGCTCACCTTCACC	180
	W V S G S Q G Q V N T Y R I P L L T F T	
181	CCCAAAGGAAGCCTGCTGGCGTTTGCAGAAGCCAGGAAGTCTTCTTCTGACATTGGA	240
	P K G S L L A F A E A R K S S S S D I G	
241	GCAAAATTCTTTGCTTTGCGGCGCTCCACCGACAAAGGAGCCACCTGGTCCCCGACCACA	300
	A K F F A L R R S T D K G A <u>T</u> W S P T T	
301	TTTATTATCGATGACGGGGCGGCGCCGGATGGCATAAACCTGGGCTCTGTTGTGGTGGAC	360
	F I I D D G A A P D G I N L G S V V V D	
361	GAGGAAGTGGGCTCAGTGATTCTGATCTACTCAGTGTGCTTCCACCTCTACCACTGTAGC	420
	E E V G S V I L I Y S V C F H L Y H C S	
421	CCGGCTAGCATCATGATGGTGGAGAGCCGGGATGACGGCCTCAGCTGGACCCCGCCAGA	480
	P A S I M M V E S R D D G L S W T P P R	
481	AACCTCTCGGTTACGCTCGGGGTGAAGAACTTTGCTCCTGGGCGGGCCTCGGCCTCCAG	540
	<u>N</u> L S V <u>T</u> L G V K N F A P G P G L G L Q	
541	AAGCGCTTCAATCCTGCAAAGGGAGGTTGGTGGTTTTCGGGTACGCGGACGCTGGAGGGC	600
	K R F N P A K G R L V V C G H G T L E G	
601	GATGGTGTCTTCTGCATCCTGAGCGACGACCACGGACAAACCTGGTACTATGGTGC	660
	D G V F C I L S D D H G Q T W Y Y G A A	
661	CTGAAAAGCATCCCCTACAACCAGAAGAAGAAAGCGCAGGACTTTAACCTGATGAGTGC	720
	L K S I P Y N Q K K K A Q D F N P D E C	
721	CAGCCAATTGAGTTGACTGACGGCACCATCGCCATCAATGTCCGGAATCAGAACAACTAC	780
	Q P I E L T D G T I A I N V R N Q N N Y	
781	CACTGCCGGTGCCGATTGTGGTGCACAGCTATGATGGAGGGTTGACCCTGCCCTTAGAA	840
	H C R C R I V V H S Y D G G L T L P L E	
841	GGCCTAATCTTTGACGAGGCACTGGTGGATCCTGTAGTGGCAGCCGGAGCTCTGCAAAAA	900
	G L I F D E A L V D P V V A A G A L Q K	
901	GAGGGCGTGATCTACTTCACCAACCCCTCCAATGAGCAAAAGAGAGTGAATCTAACCTTG	960
	E G V I Y F T N P S N E Q K R V <u>N</u> L T L	
961	AAATGGTCGCTGACAAACGGCACGTCTTGGGAGAAACAAAGCTGTCCAGATATGGGCGGGA	1020
	K W S L T <u>N</u> G T S W E N K A V Q I W A G	
1021	CCGAGCGGCTACTCCAGTATGACATCACTGGACAGCGGCTCTGTAGAAGACCACAAGTAC	1080
	P S G Y S S M T S L D S G S V E D H K Y	
1081	ATCTTTGTCATCTACGAGAAGGGCCAGAAAGACTATTTTCGAGACCGTCTCGTTACCAAG	1140
	I F V I Y E K G Q K D Y F E T V S F T K	
1141	ATCCACCTGTATGGTGGTCGTTAA	1164
	I H L Y G G R *	
	.....	

**Figure 2-7.** Nucleotide and deduced amino acid sequence for Neu1a.

Single letters for the top sequence represent nucleotides of *neu1a* while single letters for the lower sequence represent amino acids of Neu1a. Start and stops codons are represented by solid horizontal line and asterisk, respectively. Predicted N-glycosylation site are boxed while O-glycosylation sites are circled. Vertical lines represent exon-intron boundaries while the lysosomal targeting sequence is underlined by a dotted vertical line



For Neu1b, two N-glycosylation and one O-glycosylation sites were predicted using NetNGlyc (Figure 2-8) all of which were conserved in both human and medaka. Search for sialidase specific motifs in tilapia Neu1 amino acid sequence was done by comparison with human NEU1 and medaka Neu1 (Figure 2-9). A total of five Asp-boxes (Ser-X-Asp-X-Gly-X-Thr-Trp), RIP and PGPG motifs were conserved strictly conserved both tilapia Neu1a and Neu1b similar to human and medaka. In addition, six key residues involved in the catalytic site architecture; such as Arg triad, the Tyr/Glu nucleophile pair and as Asp acting as the acid/base catalyst (Giacopuzzi et al., 2012) were determined to be conserved only in Neu1a (Figure 2-9).

Previously, it has been demonstrated that mammalian Neu1 possesses lysosomal targeting motif (Tyr-X-X-hydrophobic residue) at C-terminus (Guarnieri et al., 1993; Ryuzono et al., 2016). Neu1 studies revealed that the both tilapia Neu1a possessed a partial lysosomal targeting motif as Tyr-Gly-Gly-Arg (Figure 2-7) similar to medaka Neu1 but not conserved with Human NEU1. A partial lysosomal targeting motif (Tyr-Ser- Gly –His) was also present in Tilapia Neu1b (Figure 2-8). SignalP analysis predicted the presence and location of signal peptide cleavage sites in Neu1a between Cys23 and Asn24, while Neu1b showed the predicted signal peptide cleavage sites between positions Thr23 and Val24.

1	ATGGCAGCATGGCGGCTCTCTGCACTCTGGTTACTGCTCTCTGCTTTGGCGTCTTCGTGT	60
	<u>M</u> A A W R L S A L W L L L S A L A S S C	
61	GTCCTCACCGTCAACCCTCAGCAGATTGACCCCTGGTGTGTTGAGGAGAAGCTGCTGTGG	120
	V L T V N P Q Q I D P L V F E E K L L W	
121	GTGAGCGGAGCCCAGGGCCAGATCGACCCCCTGGTGTGTTGAGGAGCAGCTGCTGTGGGTG	180
	V S G A Q G Q I D P L V F E E Q L L W V	
181	AGTGGATCCCAGGGCCAGGTGAACACTTACAGGATCCCCTGCTCACCTTCACCCCCAAA	240
	S G S Q G Q V N T Y R I P L L T F T P K	
241	GGAAGCCTGTTGGCATTTCAGAGAAGCCAGGAAGACTTCTTCTTCTGACATTGGAGCAAAG	300
	G S L L A F A E A R K T S S S D I G A K	
301	TTCATTGCAATGCGGCGCTCCACTGACAAAGGAGCCACCTGGTCCCCGACCAGCTTCATC	360
	F I A M R R S T D K G A <span style="border: 1px solid black; border-radius: 50%; padding: 0 2px;">T</span> W S P T S F I	
361	GTCGATGACGGGATGAAGCCGGATGGCCTGAACCTGGGCTCCGTTGTGGTGGACGAGGAA	420
	V D D G M K P D G L N L G S V V V D E E	
421	GTGGGCTCAGTGATTCTGATCTACGACGTGTGCTTCTACCTCTACCACTGCAGCCCCACCC	480
	V G S V I L I Y D V C F Y L Y H C S P P	
481	AGCATCATGATGGTGGAGAGCCTGGACGACGGCCTCAGCTGGAGCCCCGCCAGAAACCTC	540
	S I M M V E S L D D G G L S W S P P R <span style="border: 1px solid black; border-radius: 50%; padding: 0 2px;">N</span> L	
541	TCGGTTACGTCGGGGTGAAGAATTCATTCCCGGGCCGGCCTCGGCCTCCAGAACGCGC	600
	S V T L G V K N F I P G P G L G L Q K R	
601	TTCAATCCCGCAAAGGGGAGGTTGGTGGTTTTCGGTACGGGACGCTGGAGGGCGACGGT	660
	F N P A K G R L V V C G H G T L E G D G	
661	GTTTTCTGCATCCTGAGCGATGACCACGGGGAAACCTGGTACAACGGTGCGGCGCTGAAA	720
	V F C I L S D D H G E T W Y N G A A L K	
721	AGCATCCCCTACAACCAGAAGAAGAAAGCACAAGACTTTAACCTGATGAGTGCCAGCCCG	780
	S I P Y N Q K K K A Q D F N P D E C Q P	
781	ATTGAGTTGACTGATGGCACCATCGCCATCAACGTGCGGAACCAGAACAGATACCACTGT	840
	I E L T D G T I A I N V R N Q N R Y H C	
841	CAGTGTGCGATTGTGGTGCACAGCTATGATGGAGGGTTGACCCTGCCCTTGAGGGGCTG	900
	Q C R I V V H S Y D G G L T L P L E G L	
901	ATCTTTGACGAGGCACTGGTGGATCCTGTAGTGGCAGCTGGAACCTCTGCAAAAAGAGGGC	960
	I F D E A L V D P V V A A G T L Q K E G	
961	GTGATCTACTTCACCAACCCCTGCAACAAGGAAAAGAGAGTGAACCTAACCTTGAAATGG	1020
	V I Y F T N P C N K E K R V <span style="border: 1px solid black; border-radius: 50%; padding: 0 2px;">N</span> L T L K W	
1021	TCGCTGACAAACGGCAAATCTTGGGAGAAGAAAGCCGTGCAGATATGGTCGGGACCGAGC	1080
	S L T N G K S W E K K A V Q I W S G P S	
1081	GGCTACTCCTGCATCACTTCACTGGACAGCGGTTCTGCAGAGGACCGCAAGTACATCTAT	1140
	G Y S C I T S L D S G S A E D R K Y I Y	
1141	GTCATCTACGAGAAAGGCCACAATCAGTATTTGAGACCGTCTCGTTCGCCAAGATCCAC	1200
	V I Y E K G H N Q Y F E T V S F A K I H	
1201	CTGTATAGTGGCCATTAA	1218
	L Y <u>S G H</u> *	

**Figure 2-8.** Nucleotide and deduced amino acid sequence for Neu1b.

Single letters for the top sequence represent nucleotides of *neu1b* while single letters for the lower sequence represent amino acids of Neu1b. Start and stop codons are represented by solid horizontal line and asterisk, respectively. Predicted N-glycosylation site are boxed while O-glycosylation sites are circled. Vertical lines represent exon-intron boundaries while the lysosomal targeting sequence is underlined by a dotted vertical line

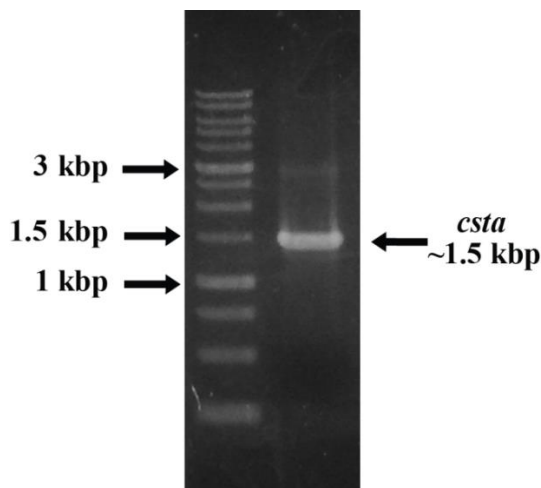
Tilapia Neu1a	1	-----MAAGSRLAALWLQLFVVVSSVCVNSPLQIDPLVFEEQLLWVSGSQ-GQVNTYRIP	55
Tilapia Neu1b	1	-----MAAWRLSALWLLLSALASSCVLTVPNPQQIDPLVFEEKLLWVSGAQGQIDPLVFEEQLLWVSGSQ-GQVNTYRIP	73
Medaka Neu1	1	-----MKVSGRSCDALTGPNLVWLLCALLPRVCYNTSAQIDPLVYEEQLLWVSGGQ-GHVSTYRIP	61
Human NEU1	1	MTGERPSTALPDRRWGPRILGFWGGCRVWVFAAIFLLLSLAASWSKAENDFGLVQPLVTMEQLLWVSGRQIGSVDTFRIP	80
Tilapia Neu1a	56	LLTFTPKGSLLAFAEARKSSSSDIGAKFFALRRSTDKGATWSPTTFIIDDGAAPDGINLGSVVVDEEVGSVILIYVCFH	135
Tilapia Neu1b	74	LLTFTPKGSLLAFAEARKTSSSSDIGAKFIAMRSTDKGATWSPTSFIIVDDGMKPDGINLGSVVVDEEVGSVILIYDVCFY	153
Medaka Neu1	62	VLTFTPKGSLLAFAEGRKSSSSDIGAKFIAMRSTDKGNTWSPTVFIIVDDVEHPDGINLGSVVVDEEVGSVFLIYSICFH	141
Human NEU1	81	LITATPRGTLTLLAFAEARKMSSSDEGAKFIATRRSMDQGSTWSPTAFIIVNDGVDPDGINLGAVVSDVETGVVFLFYSLCAH	160
Tilapia Neu1a	136	LYHCSPASIMMVESRDDGLSWTPPRNLSVTLCVKNFEPGPGGLGLOKRFNPAKGRIVVCGHGTLEGDGVFCILSDDHGQTW	215
Tilapia Neu1b	154	LYHCSPSPIMMVESLDDGLSWSPPRNLSVTLCVKNFEPGPGGLGLOKRFNPAKGRIVVCGHGTLEGDGVFCILSDDHGQETW	233
Medaka Neu1	142	LYRCSPSSIMMVESRDDGLSWSPPRNLSIETGVRAFAPGPGFGIQKRHSPAKGRIVVCGHGTIEGDGVFCILSDDHGKGW	221
Human NEU1	161	KAGCQVASTMLVMSKDDGVSWSTPRNLSLDIGTEVFAPGPGSGIQKQREPRKGRLIVCGHGTIERDGVFCILSDDHGASW	240
Tilapia Neu1a	216	YYGAALKSIPYNQKKKAQDFNPDECQPIELTDGTIAINVRNQNNYHCRICRIIVVHSYDGGTLPLEGLIFDEALVDPVVA	295
Tilapia Neu1b	234	YNGAALKSIPYNQKKKAQDFNPDECQPIELTDGTIAINVRNQNNRYHCQCRIVVHSYDGGTLPLEGLIFDEALVDPVVA	313
Medaka Neu1	222	RNGAALKSIPYNQKKKAQDFNPDECQPEMPDGSILINVRNQNNYHCRICRMARSIDGGESLPVEELYFDSELVDPVVA	301
Human NEU1	241	RYGSGVSGIPYGQPKQENDFNPDECQPIELPDGSVVINARNQNNYHCHCRIVLRSYDACDTLRPRDVTFDPELVDPVVA	320
Tilapia Neu1a	296	GAL-QKEGVIIYFTNPSNEQKRVNLTSLTNGTSWENKAVQIIVAGPSGYSSMTSLDSG--SVEDHKYIFVIYEKGQKDY	372
Tilapia Neu1b	314	GTL-QKEGVIIYFTNPNCKEKRNVNLTSLTNGKSWEKKAVQIIVAGPSGYSCITSLDSG--SAEDRKYIYVIYEKGHNQY	390
Medaka Neu1	302	GAL-QKNGVLYFTNPSNDRNRVNLTSLTNGLSWEKKAVQIIVAGPSGYSCMSSLDTG--SVEDQKFVFLYEKGHKDY	378
Human NEU1	321	GAVVTSSGIVFESNPAHPEFRVNLTSLTNGTSWRKETVQLWPGPSGYSSLATLEGSMDGEEQAPQLYVLYEKGGRNHY	400
Tilapia Neu1a	373	FETVSFTKIHLIYGGR	387
Tilapia Neu1b	391	FETVSFAKIHLIYSGH	405
Medaka Neu1	379	FETISFVKVHLYGGR	393
Human NEU1	401	TESISVAKISVYGTL	415

**Figure 2-9.** Multiple alignment of amino acid sequences from tilapia Neu1s, human NEU1 and medaka Neu1.

Actual amino acid sequences were used for Neu1a and Neu1b. Identical residues are shown by black background (100% identity) and gray background (partial identity). Conserved motifs YRIP, VPGP and Asp-box are boxed with a dotted line, a bolded line and a single line, respectively. Highly conserved residues involved in the formation of active sites are shown by closed diamonds.

### 2.5.1.3. Identification and cloning of Cathepsin A gene (*ctsa*)

It has previously been observed in human and medaka that Neu1 forms a complex with CathepsinA and  $\beta$ -galactosidase. Activation of medaka Neu1 has recently been investigated (Ryuzono et al., 2016). To gain insights into the role of Cathepsin A in tilapia Neu1s activation, search for putative Cathepsin A orthologues was done in tilapia genome using human Cathepsin A *in silico*. BLAST search revealed the presence of a single *ctsa*-like gene in the tilapia genome. PCR amplification using primers (Table 2-1) designed based on the putative *ctsa* sequences yielded a PCR product with molecular size corresponding to the predicted tilapia *ctsa* gene. Information of putative tilapia *ctsa* sequence (XM\_003454521) was identified from the BLAST search in NCBI database.



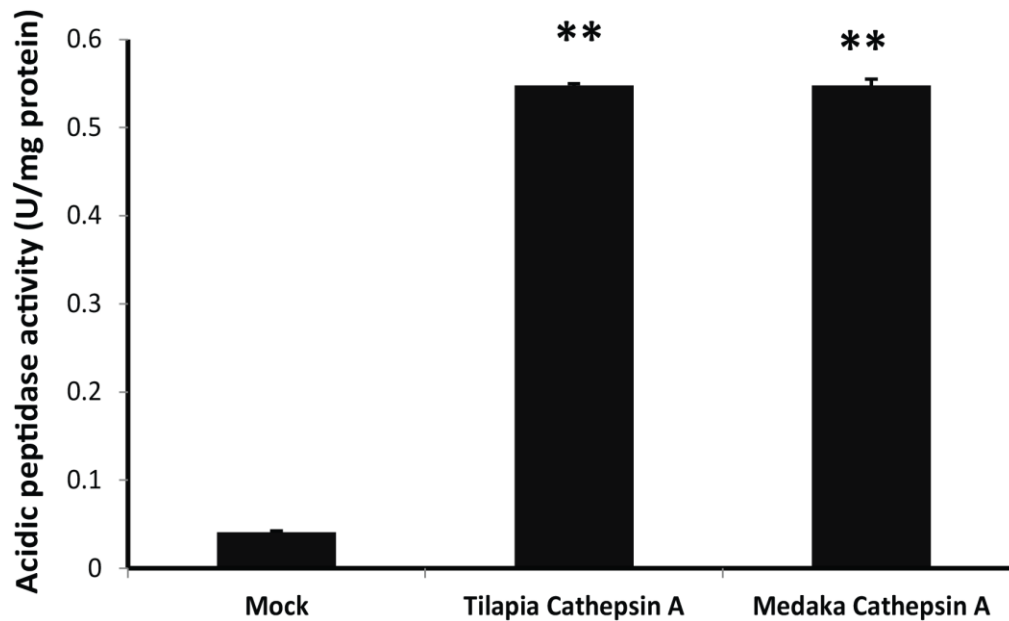
**Figure 2-10.** Electrophoresis of the *ctsa* PCR product.

Tilapia *ctsa* putative was predicted using BLAST and primers were designed accordingly. Amplification was done by PCR using cDNA generated from brain tissues

No other candidates of first ATG in NCBI database were found by tilapia genome analysis. The PCR amplified *csta* gene showed similar size as predicted (Figure 2-10). The *csta* cDNA was cloned into pBluescript similar to other genes using endonuclease EcoRI. The samples were sequenced using ABI3130xl Genetic Analyzer. The sequenced *csta* cDNA consisted 1,422 bp nucleotide sequence encoding a 373 amino-acid-polypeptide similar to the predicted sequence. Comparison with medaka Cathepsin A, showed a sequence identity of 83.7%, suggesting that the transcript the identified transcript in tilapia was indeed orthologue of medaka Cathepsin A. Tilapia *csta* was deposited at the DDJB under accession number LC051025.1.

#### **2.5.1.4. Cathepsin A activity**

To confirm the correct cloning and that the right *csta* gene was inserted in the expression vector, pcDNA3.1-*csta*- were transfected into HEK293T cells and the resulting homogenate was used for the estimation of Cathepsin A activity using acidic carboxypeptidase activity measuring kit (Kikkoman, Japan). Comparison of Cathepsin A activity in mock and *csta* transfected cell showed significantly higher acidic carboxypeptidase activity in *csta* overexpressing cells than mock cells. The acidic carboxypeptidase activity was more than ten times higher than the mock cells (Figure 2-11). Tilapia Cathepsin A activity was compared to medaka Cathepsin A and the results showed similar acidic carboxypeptidase activity in both cases but significantly different from the mock cells. This confirmed that amplified nucleotide is tilapia *csta* and that it encoded an active polypeptide.



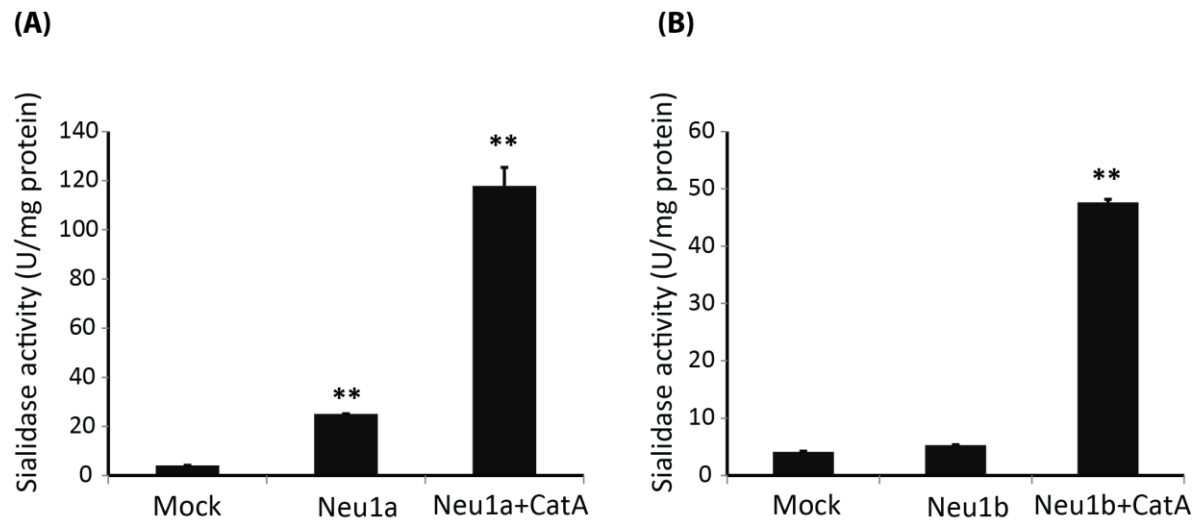
**Figure 2-11.** Cathepsin A activity.

Tilapia *ctsa* sb-cloned into pcDNA 3.1 expression vector was transfected into HEK293T cells and cultured for 48 hours. Cathepsin A activity was estimated as the amount of acidic peptidase activity after exposure to homogenate from transfected cells. The empty vector transfected cells were used as control. \*\*,  $p < 0.01$  compared with mock cells.

#### **2.5.1.5. Cathepsin A activation of tilapia Neu1a and Neu1b**

To determine the enzymatic properties of the tilapia Neu1s, cloned genes (*neu1a* and *neu1b*) were subcloned into pcDNA3.1 vector. The expression vectors were transfected into HEK293T cells to express each of the sialidase Neu1 protein. Sialidase activity was estimated as amount of release sialic acid from 4MU-NANA after incubation with the prepared cell homogenates and incubation at pH 4.6 (based on medaka Neu1) (Ryuzono et al., 2016). Based on the previous reports that sialidase Neu1 required activation by

Cathepsin A (Ryuzono et al., 2016; Bonten et al., 2009), co-transfection of both *neu1a* and *neu1b* was with Cathepsin A in HEK293T. This yielded three types of transfection conditions for each *neu1* gene; mock, overexpressed Neu1a/Neu1b and overexpressed Neu1a/Neu1b + Cathepsin A. Sialidase activity towards 4MU-NANA was compared for each transfection condition in each Neu1 group to confirm the Cathepsin A activation. Toward 4MU-NANA, mock transfected cells showed negligible sialidase activity (< 5 nmol/hr/mg protein, (Figure 2-12).



**Figure 2-12.** Tilapia Neu1 activation by Cathepsin A.

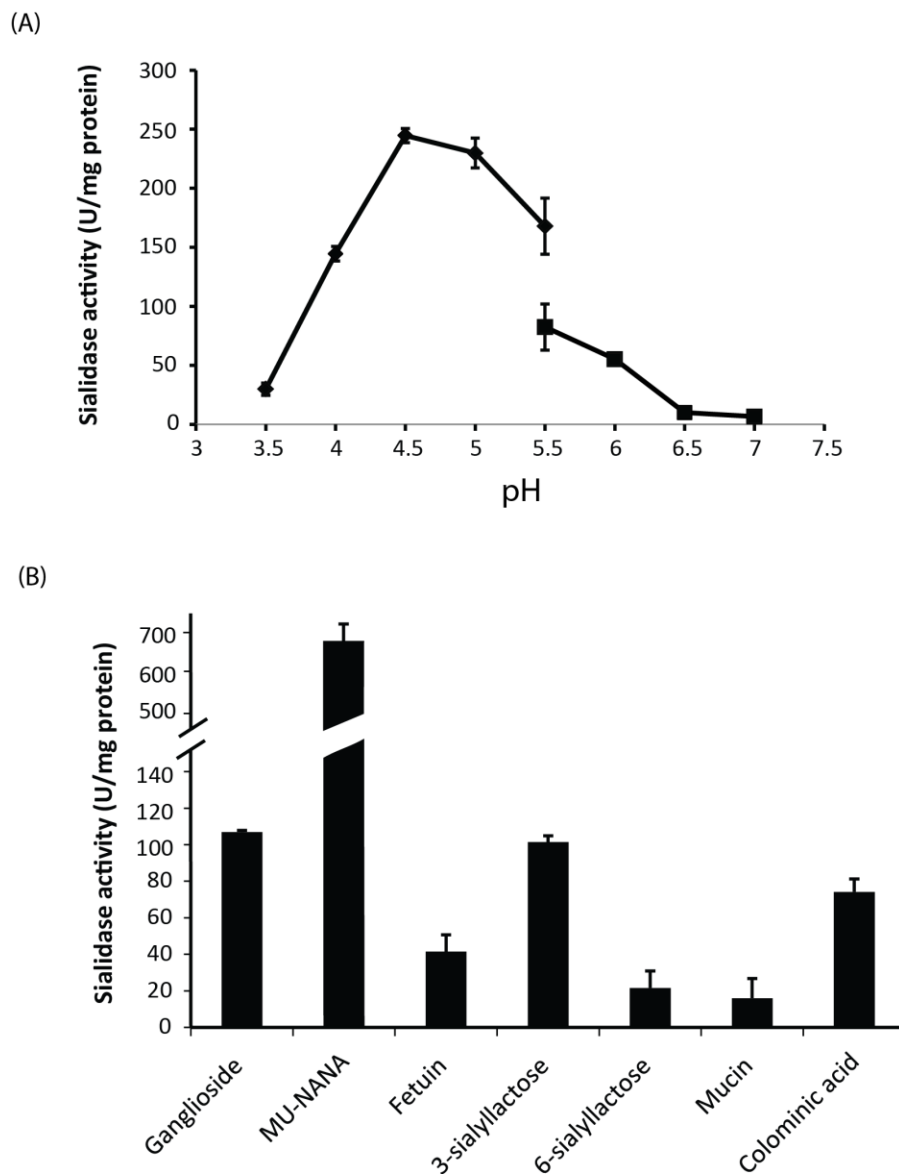
HEK293T cells were co-transfected with each *neu1* gene and *ctsa*. In addition, each *neu1* was also transfected into HEK293 without *ctsa*. As a control, mock transfected cells were also prepared. Homogenate was prepared from each transfectant and used for estimation of sialidase activity. \*\*,  $p < 0.01$  compared with mock cells. The results were prepared from three independent experiments.

Sialidase activity was significantly higher in co-transfection of both *neu1a* with *ctsa* gene and *neu1b* with *ctsa* genes. Interestingly, transfection with *neu1a* only also exhibited significant higher activity than mock (Figure 2-12A) suggesting that, Cathepsin A activation is crucial for Neu1a. On the other hand, Neu1b sialidase activity was completely diminished in the absence of Cathepsin A. The activity of co-transfected *neu1b* and *ctsa* showed significantly increased activity when compared with mock cells (Figure 12B). Tilapia *neu1b* lone transfection in HEK293T yielded sialidase activity similar to mock cells. These results strongly suggest the activation of tilapia Neu1b by Cathepsin A. Therefore, all proceeding overexpression of tilapia sialidase Neu1s experiments were done in the presence of Cathepsin A.

#### **2.5.1.6. Tilapia Neu1s sialidase activity**

Sialidase activity was estimated using homogenates from HEK293T cells co-transfected with *neu1a/ctsa* and *neu1b/ctsa* at various pH values to determine the effect of pH and towards various substrates. Neu1a optimal pH was observed at pH 4.5 (Figure 2-13A), similar to human NEU1 (pH 4.6) (Miyagi et al., 2008), and medaka Neu1 (pH 4.0) (Ryuzono et al., 2016).

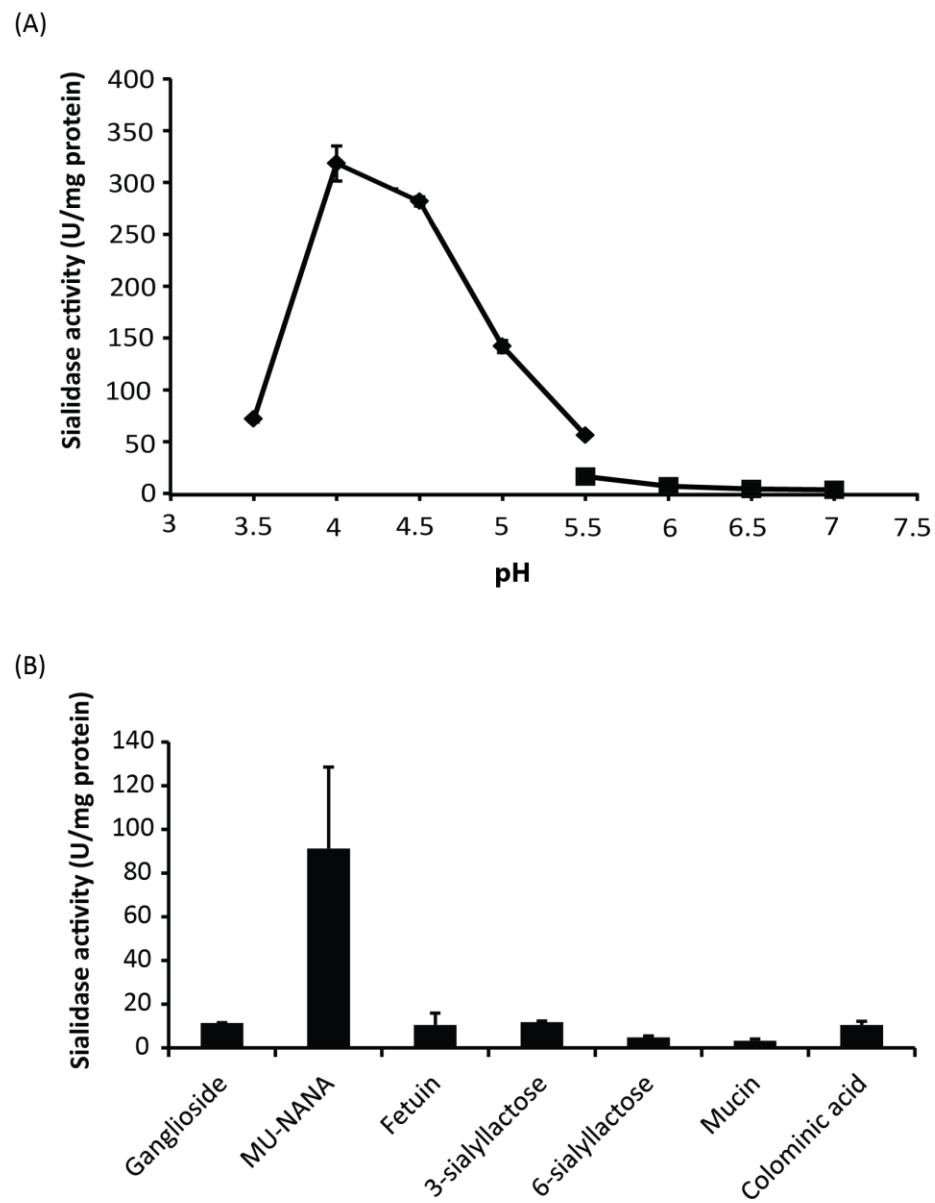




**Figure 2-13 .** Optimal pH and substrate specificity of tilapia Neu1a sialidase.

(A) Enzymatic reaction was done with neu1a-transfected cell lysate using two types of buffers: sodium acetate buffer for pH 3.5-5.5 (diamond) and sodium phosphate buffer for pH 5.5–7.0 (square). (B) Sialidase activity of neu1a-transfected cell lysate was assayed towards various glycoconjugates at pH 4.0 for Neu1a transfected cells. The results are shown as means  $\pm$  standard deviation from three independent experiments.

The recorded optimal pH was very different from that observed in zebrafish Neu1 (Manzoni et al., 2007). Tilapia Neu1b showed highest sialidase activity at pH 4.0 (Figure 2-14A), very similar to medaka Neu1. An extra exon was observed in Neu1b, interestingly, Neu1b still showed significantly higher sialidase, suggesting that the presence of the extra exon did not affect the catalytic site architecture. Further, sialidase activity for tilapia Neu1a and Neu1b were evaluated towards various substrates at the optimal pH levels (pH 4.5 for Neu1a and pH 4.0 for Neu1b). Neu1a/Cathepsin A co-transfected homogenate showed highest activity towards 4MU-NANA, ganglioside and 3-sialyllactose (Figure 2-13B) Lower Neu1a activities were observed in mucin and 6-sialyllactose, similar to medaka Neu1. Neu1b/cathepsin A showed significantly higher sialidase activity towards 4MU-NANA only (Figure 2-14B). Generally, lower sialidase activity (<20 U/mg protein) were observed in all substrates apart from 4MU-NANA (Figure 2-14B), suggesting that Neu1b has a very narrow substrate specificity.

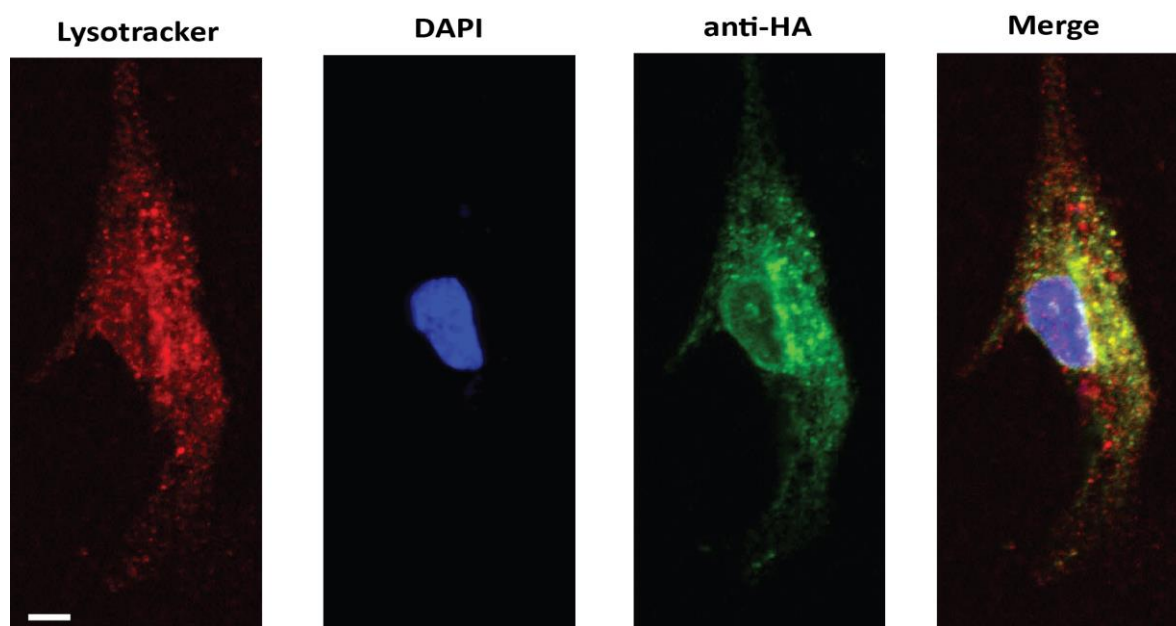


**Figure 2-14.** Optimal pH and substrate specificity of tilapia Neu1b sialidase.

(A) Enzymatic reaction was done with neu1b-transfected cell lysate using two types of buffers: sodium acetate buffer for pH 3.5–5.5 (diamond) and sodium phosphate buffer for pH 5.5–7.0 (square). (B) Sialidase activity for neu1b transfected cells was estimated towards pure gangliosides various sialo-glycoconjugates. The results are shown as means  $\pm$  standard deviation from three independent experiments.

### 2.5.1.7. Immunoblotting analysis

Based on the properties of Neu1a polypeptide, it was concluded that Neu1a is the main Neu1 in tilapia, and all other subsequent analyses were done using Neu1a. Neu1a-HA was transiently transfected in Hepa-T1 cells to determine its subcellular localization through confocal analysis. As a result, Neu1a showed a significant colocalization with lysotracker, a lysosomal marker (Figure 2-15), suggesting that a significant part of tilapia Neu1a is localised at the lysosomal similar to medaka Neu1 (Ryuzono et al., 2016).

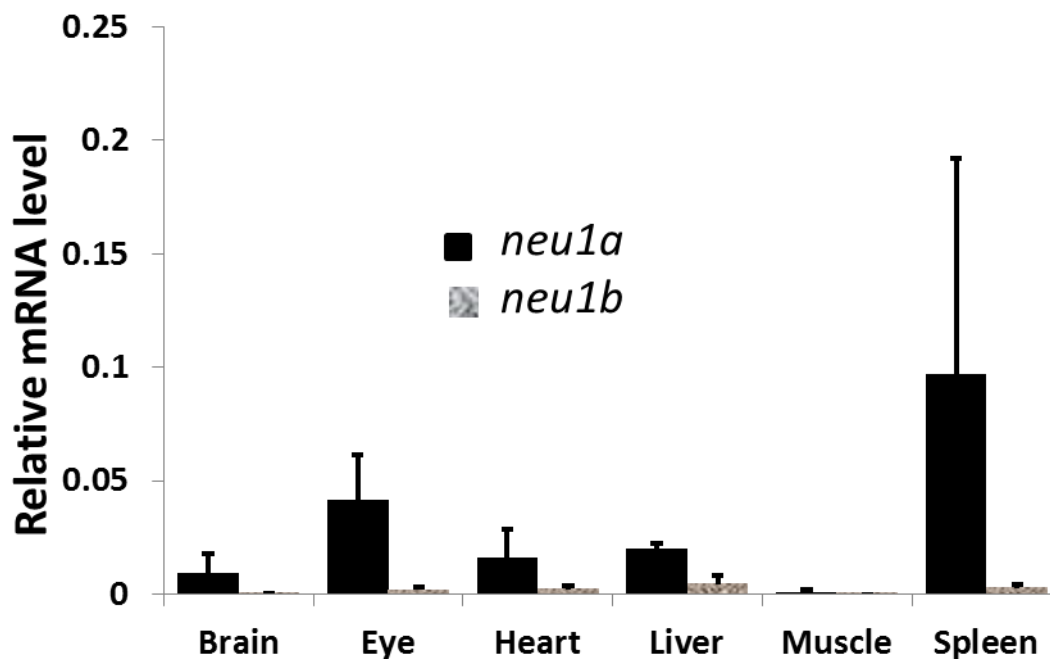


**Figure 2-15.** Neu1a localization.

For subcellular localization of Neu1a, Hepa-T1 cells grown on coverslips were transfected with HA-tagged *neu1a* gene. Expressed sialidase proteins were detected by anti-HA antibody and Alexa 555-anti-rat IgG while lysosomes were detected by lysotracker. The observation was done using sectioning fluorescence microscopy.

#### 2.5.1.8. Tilapia *neu1* tissue expression

To clarify tilapia *neu1a* and *neu1b* expression patterns in various tissues, real-time PCR was carried using cDNA prepared from total RNAs of various tilapia tissues. Tilapia *neu1a* mRNA showed highest expression in the spleen, eye and liver (Figure 2-16). On the other hand, *neu1b* expression was generally low in all tissues used in this study (Figure 2-16). This confirmed that *neu1a* is the main *neu1* gene in tilapia.



**Figure 2-16:** Tissue expressions of tilapia *neu1* sialidases.

Tilapia *neu1a* and *neu1b* expression levels in tilapia brain, heart, eye, muscle and spleen estimated by real-time PCR.  $\beta$ -actin isoform expression in tilapia was also examined as an internal reference. For the comparison, standard curves were generated by serial dilution of the pBluescript vectors containing the entire *neu1a* and *neu1b* ORFs. The results are shown as means  $\pm$  S.D. of three independent experiments

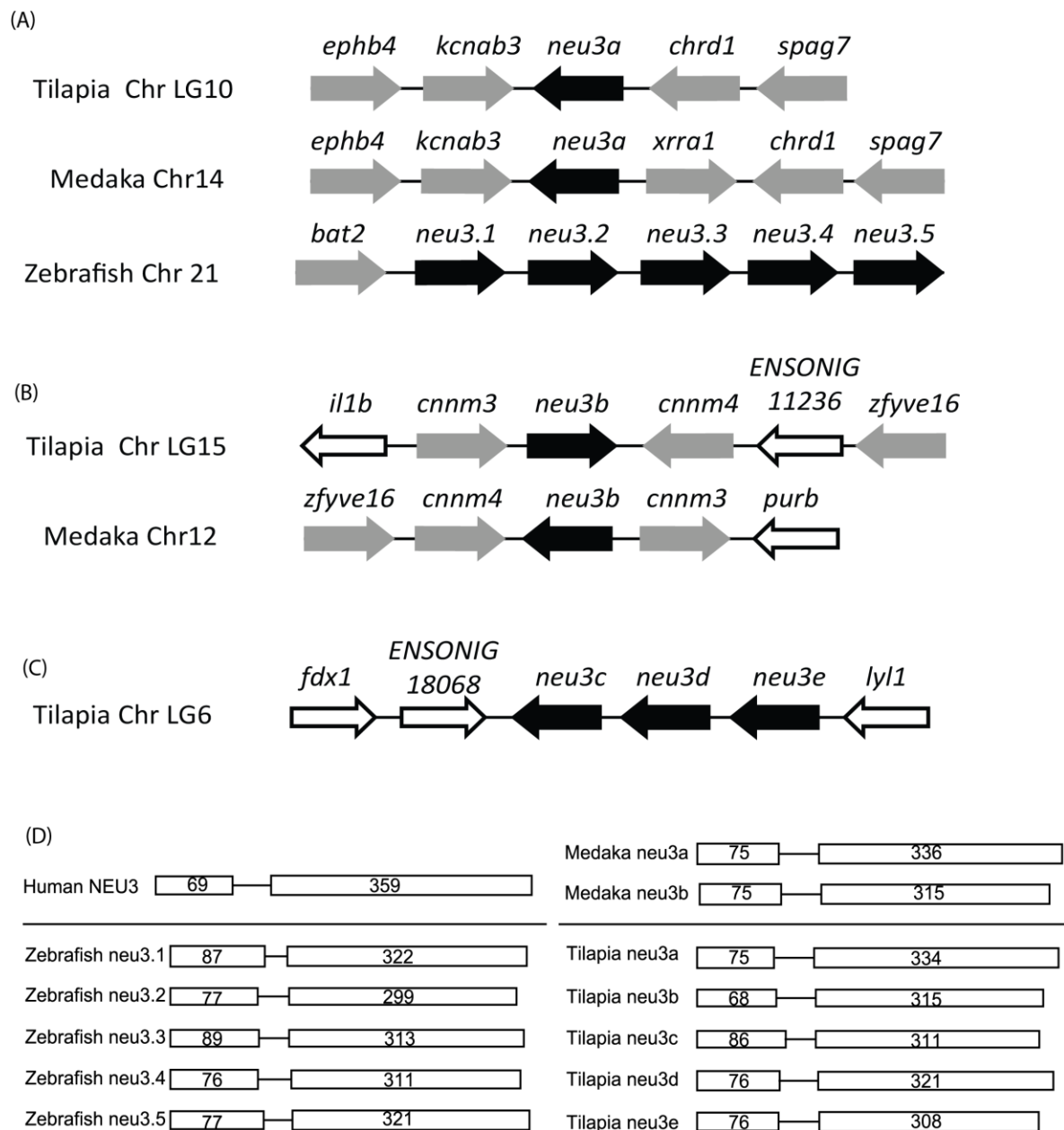
## 2.5.2. Neu3 gene cloning

### 2.5.2.1. Identification of sialidase genes in *O. niloticus*

*O. niloticus* genomic sequence databases were queried by BLAST search for sequences showing similarities to human *NEU3* genes revealing five putative *neu3*-like sialidase genes were found in tilapia genome. One *neu3*-like sequence, not predicted in Ensembl database was further analysed *in silico* using gene predictions available within the UCSC Genome Browser. On the basis of further bioinformatic analyses the genes were named as follows; *neu3a*, *neu3b*, *neu3c*, *neu3d* and *neu3e*. The putative *neu3* genes were present on chromosome LG10 (*neu3a*: 9,658,629-9,659,969) and LG15 (*neu3b*: 10,406,089-10,407,391). Three *neu3*-like genes (*neu3c*, *neu3d* and *neu3e*) were clustered on chromosome LG6 (*neu3c*: 19,806,859-19,808,151, *neu3d*: 19,813,048-19,814,343 and *neu3e*: 19,864,775-19,867,445) and LG14 (*neu4*: 19,201,481-19,209,685).

The analysis of the genomic regions surrounding putative tilapia *neu3* genes showed that tilapia *neu3a* gene is flanked by *chrdl2*, *spag7*, *kcnab3*, *ephb4a* genes, which are also conserved in the region flanking medaka *neu3a* gene (Figure 2-16A). On the other hand, tilapia *neu3b* gene is flanked by genes homologous to medaka *cnnm3*, *cnnm4* and *zfyve16* genes (Figure 2-16B). As shown in Figure 16C, there was no evidence of conserved synteny among tilapia *neu3c/neu3d/neu3e* cluster, zebrafish and medaka *neu3* flanking regions. Different from zebrafish which also possesses five *neu3* genes clustered on a single chromosome (Manzoni et al., 2007), tilapia's five sialidase genes were present on different chromosomes. Bioinformatics approach allowed us to recover the entire coding sequence of

*neu1a*, *neu1b*, *neu3a*, *neu3b* and *neu4*. Only partial sequences were available for *neu3c*, *neu3d* and *neu3e* in public databases, without upstream and downstream sequences precisely known. Using *in silico* manual inspection of the upstream and downstream of partial *neu3c*, *neu3d* and *neu3e* sequences, putative first ATG and stop codons were subsequently identified. To determine tilapia sialidase protein evolution, we constructed a phylogenetic tree using deduced sialidase amino acid sequences from tilapia, human, zebrafish and medaka. A phylogenetic tree showed three distinct clusters, Neu1, Neu3 and Neu4 (Figure 2-1).



**Figure 2-17.** Syntenic analysis and exon intron organisation for *neu3* genes.

Syntenic analysis around *neu3a* (A), *neu3b* (B) *neu3c*, *neu3d* and *neu3e* (C). The black arrows indicate sialidase genes. Dark arrows indicate conserved genes in the syntenic regions and white arrows indicate non-conserved genes. (D) Exon-intron organization of sialidase genes. Exons are represented by boxes and their sizes are indicated by the number of encoded amino acids.



Tilapia Neu3c, Neu3d and Neu3e proteins were present in their own sub-cluster within Neu3 cluster, suggesting that they could be unique and might have evolved together in the species. Among the five putative tilapia Neu3 sialidases, it is worth noting that Neu3d and Neu3e are very similar (81.5%) in their nucleotide and amino acid sequences. All tilapia *neu3s* are organized into two exons and possess similar intron positions to that of human, medaka and zebrafish (Figure 2-16D). From these results, it could be suggested that all putative tilapia Neu3 sialidases are closely related to human NEU3 and medaka Neu3s, although no synteny was observed in the region flanking tilapia *neu3c*, *neu3d* and *neu3e*. Intron-exon organization, phylogenetic analysis and amino acid sequence analysis provided strong evidence to suggest that these genes are closely related to NEU3.

#### **2.5.2.2. Molecular cloning of tilapia *neu3* genes and sequence analysis**

Putative tilapia *neu3a*, *neu3d* and *neu3e* coding regions were amplified by PCR using the first strand cDNA as template and were sequenced. Tilapia *neu3a*, *neu3d* and *neu3e* ORF were 1227 bp, 1194 bp and 1155 bp, respectively as predicted in the bioinformatics analysis (Figure 2-17; 2-18 and 2-19). The PCR products visualised on 1% agarose gel, showed bands with molecular sizes corresponding to predicted sizes for *neu3a*, *neu3d* and *neu3e* (Figure 2-20A, B)

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1  ATGGGAAACACGTCGTCGAAGGGAGACAGCGGAGAAGAACCTCCTAAAACAACCTTTGTTT      60
   M G N T S S K G D S G E E P P K T T L F
61  GAAAGGGAGCCAAGTGGGATAACATACAGAATCCCTGCTCTCGTCTATCTGAGGCACTGT      120
   E R E P S G I T Y R I P A L V Y L R H C
121 CACACCTTCCTCGCCTTTGCAGAGAAAAGATCCTCACCCCTGTGACAATGACGCCAAAATT      180
   H T F L A F A E K R S S P C D N D A K I
181 TTCGTTATGAGGAGAGGCACCCTGAAAGAGGATGGATCCATGCAGTGGTCATCCAGTCAG      240
   F V M R R G T L K E D G S M Q W S S S Q
241 GAGCTGTCCTCGGTGTGCCTGCCAAACCACCGCACTATGAATCCTTGCCCGGTGTATGAA      300
   E L S S V C L P N H R T M N P C P V Y E
301 AAAAACACCAAACGCTCTTTTTGTTTTTCATCTGTGTGTGGGGCACAACCACGGAAGCG      360
   K N T K T L F L F F I C V W G T T T E A
361 AAGCAGATCCTAACAGGTAAGAACAAGACTCGCCTTTGCTACGTCAGCAGCCGAGACGAC      420
   K Q I L T G K N K T R L C Y V S S R D D
421 GGCCAGACTTGGAGCCAAGTCACAGACTTAAGTGAAGTGTGATCGGCGAGGCCATCCAC      480
   G Q T W S Q V T D L T E S V I G E A I H
481 AAGTGGGCCACGTTCGCCGTGGGCCCCGGCCACGGTGTTCAGCTTGAGAACGGCCGACTG      540
   K W A T F A V G P G H G V Q L E N G R L
541 ATCATCCCTGCGTACGCCTACTACATTCCTTACAGATGCTGTTCCCTTCCCCATCCCTTG      600
   I I P A Y A Y I P Y R C C S F P I P C
601 ACAGTCTACCCACGTGCACTGTCACTATATAGCGAGGACTTTGGACAGACGTGGCACATA      660
   T V Y P R A L S V Y S E D F G Q T W H I
661 GGTAAGATGCTGCGGAAGAAGTCGTGCGAATGTGAAATGGCCGAGATCATAGACCACGAG      720
   G K M L R K K S C E C E M A E I I D H E
721 GGCAGGAGCCACCTCTACTGCAATGCTCGTAATGCCGGGGGCCACAGGTGTGAGGCTCTC      780
   G R S H L Y C N A R N A G G H R C E A L
781 AGTGA AACAGCGGCTCTACTTCGACAAGCCCCACATGGCTCCGGAGCTCGTCGAAACG      840
   S E N S G V Y F D K P H M A P E L V E T
841 CGCTACGGATGTCAGGGAAGCATCATCGGCTTTCCCGCTCCGGAATTTGTCCCAAACGAC      900
   R Y G C Q G S I I G F P A P E F V P N D
901 GACGCGGAAAGCAAAGCCTGCGGCACGTGCTCCTGTCAACAGACACGCAAACCTGGCTC      960
   D A E S K A C G T S L L S P D T Q T W L
961 CTCTTCATGCACCCGACGAGCAAGTCCAGAAGAAGGGACATGGGTGTGTATTTGAACCGA      1020
   L F M H P T S K S R R R D M G V Y L N R
1021 TCCCCCTGCACTCATCTGGATGGGACAGACCCAGGATCATCCACAGGGGGCCAGCGGC      1080
   S P L H S S G W D R P R I I H R G P S G
1081 TACTCGGACCTGGCTTACAACGGGGACAAGGATCAGTTTTTCGTGCCTGATGGAGTGCGGG      1140
   Y S D L A Y N G D K D Q F S C L M E C G
1141 AAAGAGAGTGAAGTTCGAGCAGATTGCCTTCGTGTCGTTCTCCCTTAATGATGTCATGCAG      1200
   K E S E L E Q I A F V S F S L N D V M Q
1201 ACAGGCGGCAGGAAAGAAAAGCGTTGA      1227
   T G G R K E K R *

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**Figure 2-18.** Nucleotide and deduced amino acid sequence for *neu3a*.

Single letters for the top sequence represent nucleotides of *neu3a* while single letters for the lower sequence represent amino acids of Neu3a. Start and stops codons are represented by solid horizontal line and asterisk, respectively. Predicted N-glycosylation site are boxed while O-glycosylation sites are circled. Vertical lines represent exon-intron boundaries

1	<b>ATG</b> GGCTGTGGACATTTCAGCAACAGCTCACCTACAGAAACAACCTGTCTTTGAATCTAAT	60
	M G C G H S A T A H L Q K Q P V F E S N	
61	GAAGCTGCTCGACGCGTTTACAGGATTCTGTCTTTCTGTATGAGAGAGAAAGTAAACT	120
	E A A R R V Y R I P A L L Y E R E <b>S</b> K T	
121	TTATTTGCCTTTGCAGAGCAAAGGAAGACTTCAGATGATGGCAGTGGAGAAAAGCTGGTT	180
	L F A F A E Q R K <b>T S</b> D D G <b>S</b> G E K L V	
181	ATGAGAACAGGAAAGATCAAGGAGGAATCTCTGAGAAGACGATTGAGTGGTCAGAGCTC	240
	M R T G K I K E E <b>S S</b> E K <b>T</b> I E W S E L	
241	AAACAGGTGGAAGGCACTTATTAAAGGACACCGTCCAATGAACCCCTGCCCGGTGTAT	300
	K Q V E K A L I K G H R P M N P C P V <b>Y</b>	
301	GACAAGGTCAACAAAAAATTTTCTGTCTTTTCATCTGTGTTGAAGACGGCATCACTGCG	360
	D K V N K K L F L F F I C V E D G I T A	
361	AGGGACCAATAGAAAACTATGACAACAGGACCCGTCTCTGCTACATCACAAGTAACAAT	420
	R D Q I E N Y D <span style="border: 1px solid black;">N</span> R T R L C Y I T S N N	
421	CTTGAAAAACCTGGAGCGATGTGACCGATTTGACGGACAAAGTGAGTGACATAACAAAC	480
	L G K T W S D V T D L <b>T</b> D K V S D I T N	
481	TGGTCCACATTTGCTGTTGGGCCAGGCCATGGGATTCAAACAGAGGGTGGTAGACTGATT	540
	W S T F A V G P G H G I Q <b>T</b> E G G R L I	
541	ATCCAGTTTATGCTTATTGCAGTAGTACTTGCTCTTGCATTGTTGTTGTAAACCAGTT	600
	I P V Y A Y C S S T C L L H C C C K P V	
601	GCCTGTTTCGTTTCAGTTGCTGTTGTAAATCATATGCACTCTCACTGTATAGTGATGATCCT	660
	A C S F S C C C K S Y A L <b>S</b> L Y S D D P	
661	GATAAAAGTTGGAATTTGGTCAAACGTTGCAATCAGAATCAGGCGAATGTCAAATGGCA	720
	D K <b>S</b> W K F G Q T L Q <b>S</b> E <b>S</b> G E C Q M A	
721	GAGGTTTTTGAAGACAACGGCTCCAGTTTCATTTATTGCAATGCTCGCAGTTTTTGTGTC	780
	E V F E D <span style="border: 1px solid black;">N</span> G S S F I <b>Y</b> C N A R S F C C	
781	TCTCGAGTGGAAGCTGTGAGTGAATAAAGGTTTTAACTTTAGCAAATTTAGATGCACT	840
	<b>S</b> R V E A V <b>S</b> E N K G F <span style="border: 1px solid black;">N</span> F S K F R C T	
841	AAACTTACAGAAACAAAGTGCTGCTCAGGCTGTCAGGGAAGTGTGGTCTCCTATCCAGCT	900
	K L T E T K C C S G C Q G S V V S Y P A	
901	CAAGAACAAAGTGGAAGTGCACAAGGTGATCCAAACCAAAACAAGTGGCTGCTGTTTACA	960
	Q E Q S G T A Q G D P N Q N K W L L F T	
961	CACCCAACCAGTAAATCACGCACGATTGATCTCGGTGTGTATGTGAACAAATCCCCACGT	1020
	H P <b>T S</b> K S R T I D L G V <b>Y</b> V N K <b>S</b> P R	
1021	GATCCAAAGGCATGGAGCAACCCCTGCATCATTAAATAATGGACTCAGTGGTTACTCAGAC	1080
	D P K A W S N P C I I N N G L <b>S</b> G Y S D	
1081	CTGGCTTACATTGATGATGGCTGCTTTGCATGTCTGTTTGAGTGTGGGAAGCACGAAATA	1140
	L A <b>Y</b> I D D G C F A C L F E C G K H E I	
1141	AATGAGCAGATAGCCTCTGTTGTCTTTAGCTATGAGGACATCACCAAGAAATAA	1194
	N E Q I A S V V F <b>S Y</b> E D I <b>T</b> K K *	

**Figure 2-19.** Nucleotide and deduced amino acid sequence for *neu3d*.

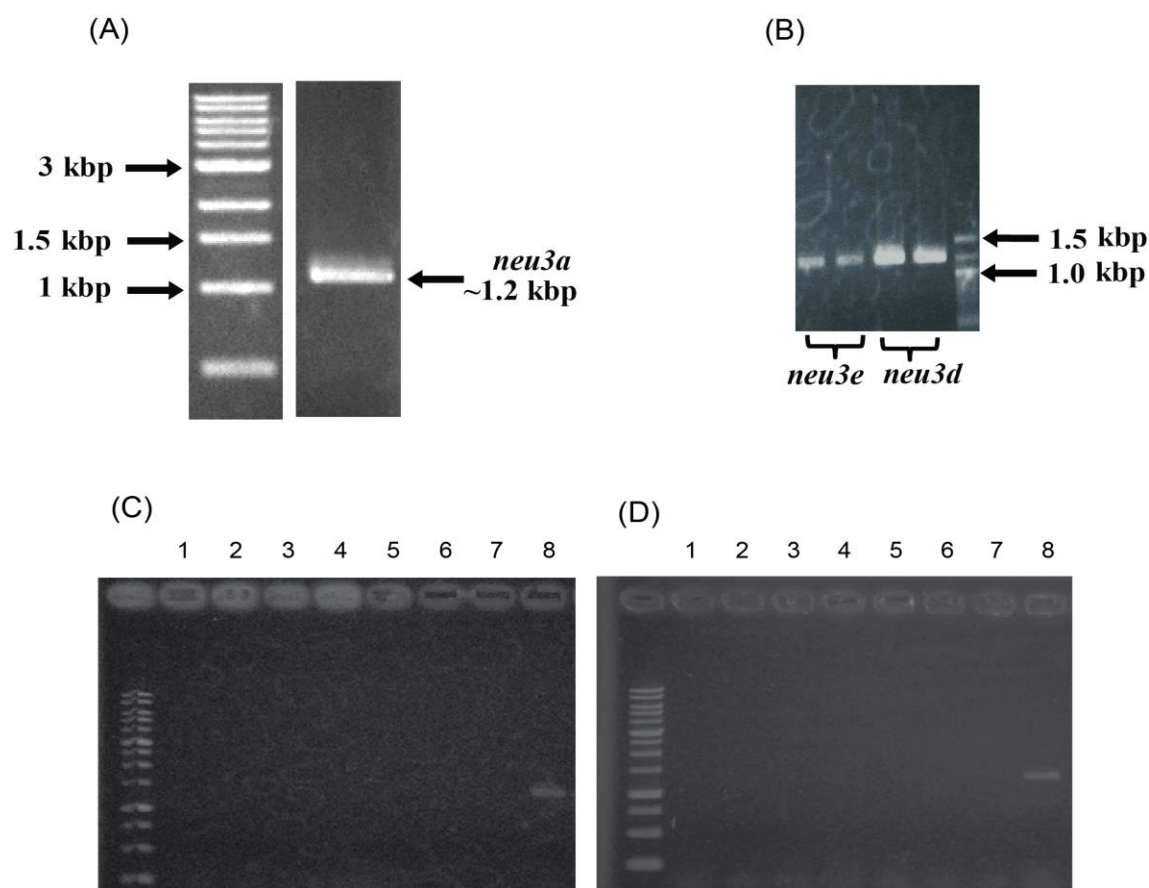
Single letters for the top sequence represent nucleotides of *neu3d* while single letters for the lower sequence represent amino acids of *Neu3d*. Start and stops codons are represented by solid horizontal line and asterisk, respectively. Predicted N-glycosylation site are boxed while O-glycosylation sites are circled. Vertical lines represent exon-intron boundaries

1	<b>ATG</b> GACTGTGGATATTCAACAAAAGCTCAGCTACAGAAACAACCTGTCTTTGAATCTAAA	60
	M D C G <b>Y S</b> T K A Q L Q K Q P V F E <b>S K</b>	
61	GAAGCTGCTCGATGTGTTTACAGGATCCCTGCTCTTCTGTATGAGAGAGAAAGTAAACT	120
	E A A R C V Y R I P A L L Y E R E <b>S K T</b>	
121	TTACTTGCCTTTGCAGAGCAGAGGAAGACTCCAGATGATGCCAGTGGAGAAAAGCTGGTT	180
	L L A F A E Q R K <b>T</b> P D D A <b>S G E K L V</b>	
181	ATGAAAACAGGACAGATCAAGGAGGGATCCTCTGAGAAGACGGTTGAGTGGTCAGAGCTC	240
	M K T G Q I K E G <b>S S E K T</b> V E W S E L	
241	AAAGAGGTGGAAAAGGCACTTATTAAAGGACACCGTCCAATGAACCCCTGCCCGGTGTAT	300
	K E V E K A L I K G H R P M N P C P V <b>Y</b>	
301	GACAAGATCAACAGGAACTTTTCCTGTTTTTCATCTGTGTTGAAGACGGTATCACTGAG	360
	D K I N R K L F L F F I C V E D G I T E	
361	AGGTACCAGATAAACAGCAATGACAACAGGACCCGCTCTCTGCTACATCACAAGTGATGAT	420
	R <b>Y</b> Q I N S N D <b>N</b> R T R L C Y I T S D D	
421	CTTGAGAAACCTGGAACAATGTGATCGATTTGATGGACGAACTGAGTGAAATAAAAAAC	480
	L G E T W N N V I D L M D E L S E I K N	
481	TGGGCCACATTTGCTGTTGGGCCAGGCCATGGGATTCAAACAGAGAGTGGTAAACTGATT	540
	W A T F A V G P G H G I Q <b>T E S G K L I</b>	
541	ATCCAGTTTATGCTTATTGCAGAAGTACTGGCCACTTGCCTGTTGTAAATCATATGCA	600
	I P V Y A Y C R S T G H L R C C K S Y A	
601	CTCTCACTGTATTATGATCCTGATAAAAGTTGGAATTTGGTCAAACGTTGCAATCAGAA	660
	L S L Y Y D P D K <b>S W K F G Q T L Q S E</b>	
661	TCAGGCGAATGTCAAATGGCAGAGGTTTTTTGAAGACAACGGCTCCAGTTTCATTTATTGC	720
	<b>S G E C Q M A E V F E D</b> <b>N</b> G S S F I <b>Y C</b>	
721	AATGCTCGCAGCTTCCACTCCTCTCGACTGGAAGCTGTCAGTGAGAATACAGGTTTTACC	780
	N A R S F H <b>S S R L E A V S E N T G F T</b>	
781	TTCACCAAATCCAGTTGCTACTAAGCTTACAGAAACAAAGCGCTATTCAGGCTGTCACGGA	840
	F T K S S C T K L T E T K R Y <b>S G C H G</b>	
841	AGTGTGGTCTCCTTTCCAGCTCAAGAACAAAGTGGAAGTGCACAAAGTGATCCAAACCGA	900
	S V V S F P A Q E Q S G T A Q S D P N R	
901	AACAAGTGGCTGCTGTTTACACACCCCAACCAGTAAGTCAGACAGGATTGATCTCGGTGTG	960
	N K W L L F T H P <b>T S K S D R I D L G V</b>	
961	TATGTGAACAAATACCCACGTGATCCAAAGGCATGGAGCCACCCCTGCATCATTAATGAT	1020
	<b>Y V N K Y P R D P K A W S H P C I I N D</b>	
1021	GGATGCAGTGGTTACTCAGACCTGGCTTACATTGACGATGGCTGGTTTGCATGTCTGTTT	1080
	G C <b>S G Y S D L A Y I D D G W F A C L F</b>	
1081	GAGTGTGGGCAGCACAAAATACATGAGCAGATAGCCTCTGTTGTATTTAGCTATGAGGAC	1140
	E C G Q H K I H E Q I A S V V F <b>S Y E D</b>	
1141	ATCACCAAGAAATAA	1155
	I <b>T K K *</b>	

Figure 2-20. Nucleotide and deduced amino acid sequence for *neu3e*.

Single letters for the top sequence represent nucleotides of *neu3e* while single letters for the lower sequence represent amino acids of Neu3e. Start and stops codons are represented by solid horizontal line and asterisk, respectively. Predicted N-glycosylation site are boxed while O-glycosylation sites are circled. Vertical lines represent exon-intron boundaries

On the other hand, tilapia *neu3b* and *neu3c* could not be amplified using cDNA from all tissues suggesting that these genes are not expressed in the assayed tissues (Figure 2-20). To confirm PCR conditions and as a positive control, PCR amplification using genomic DNA yielded bands corresponding to *neu3b* and *neu3c* (Figure 2-20A and B). Hence, these two genes were not considered in the subsequent analysis.



**Figure 2-21.** PCR amplification of tilapia *neu3* genes.

(A, B) *Tilapia neu3a* (A), *neu3d* and *neu3e* (B) PCR products from brain cDNA. *Tilapia neu3b* (C) and *neu3c* (D) gene amplification were carried out using cDNAs from tilapia tissues (lane 1: brain, lane 2: heart, lane 3: eye, lane 4: liver, lane 5: muscle, lane 6: skin and lane 7: spleen) as templates. The PCR products were then analyzed using 1% agarose electrophoresis. As a positive control, tilapia genomic DNA was used as template for PCR (lane 8).

Assembled consensus sequences for tilapia *neu3a*, revealed the presence of two synonymous single nucleotide polymorphisms (SNPs) compared with tilapia genomic database in *neu3a* (Table 2-9).

**Table 2-9.** Observed single nucleotide polymorphisms (SNPs).

SNPs were determined by comparing the actual nucleotide sequences and predicted sequences from tilapia genome databases. Nucleotide sequence translation to amino acids revealed the nature of the SNPs (synonymous or Non-synonymous). For Non-synonymous SNPs, the resulting amino acid substitution is presented alongside the effector non-synonymous SNP.

Synonymous SNPs		Non-synonymous SNPs and corresponding amino acid shift					
Neu3a	Neu3d	Neu3d					
		NS-SNPs	Amino acid Substitution	NS-SNPs	Amino acid substitution	NS-SNPs	Amino acid substitution
57G→A 276T→C	141G→A	30G→C	Q10H	305C→A	T103K	827C→A	T276K
	201A→G	43A→C	T15P	359A→C	E120A	893T→A	F298Y
	351T→C	60A→T	K20N	363T→G	S121R	898T→G	S300A
	585C→T	71A→G	Q24R	380G→A	C127Y	911T→G	I304S
	603A→C	73T→C	C25R	430G→A	A144T	925A→G	S309G
	642G→A	107A→G	K36R	460G→A	E154K	945T→G	N315K
	726C→T	124C→T	L42F	463C→G	L155V	980A→G	H327R
	768A→C	161C→G	A54G	471A→C	E157D	983G→C	R328T
	789A→G	198G→C	M66I	476A→C	K159T	1057G→A	E353N
	822C→T	221G→C	R74T	526A→G	S176G	1059G→T	E353N
	843G→A	222A→G	R74T	686T→C	M229T	1064C→T	P355L
	870A→C	223G→A	V75I	703T→G	C235G	1104G→C	W368C

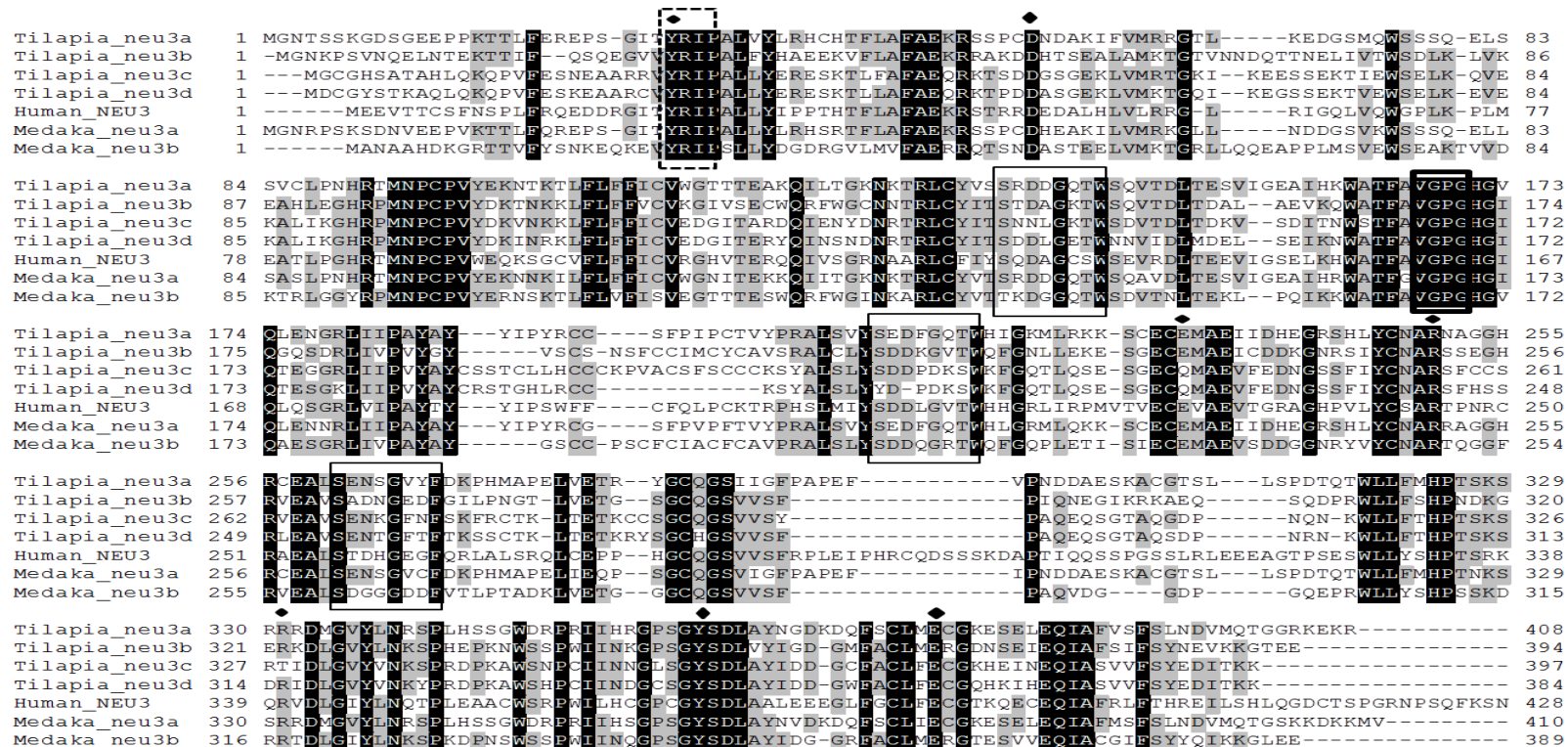
	960G→A	233T→C	L78S	728A→T	Y243F	1129C→A	Q377K
	978G→A	244G→C	E82Q	796A→G	I266V	1134G→C	Q378H
		271→TC	Y91H	803T→A	V268E	1141C→A	H381N
		277T→C	S93P	818C→A	T273N		

The amount of SNPs in *neu3d* were more pronounced most of which were non-synonymous, while no SNPs were present in *neu3e* (Table 2-9) The deduced Neu3 sequences were 100% conserved in all three individual fish from the Ibusuki *O. niloticus* population that were used in this study. The sequence identity analysis between tilapia and medaka sialidases showed highest degree of amino acid identity between tilapia Neu3a and medaka Neu3a (85.5%), while tilapia and human NEU3 amino acid identities were 45.9%, 40.2% and 41.6% with tilapia Neu3a, Neu3d and Neu3e, respectively. DNA sequences were deposited in the DDBJ under the accession numbers AB933557.1 (tilapia *neu3a*), AB933558.1 (tilapia *neu3b*), AB933559.1 (tilapia *neu3c*), AB933560.1 (tilapia *neu3d*) and AB933561.1 (tilapia *neu3e*).

As shown in Figure 2-21, typical exosialidase YRIP motif was present and located near the N-terminal in all tilapia Neu3 proteins, similar to human NEU3 and medaka Neu3 proteins, along with three Asp-boxes (SXDXGXTW) and VGPG motifs. The six key residues in the formation of catalytic sites were well conserved in all sialidases, except one residue at position 328 in Neu3d in the multiple alignment. Tilapia Neu3a showed a single predicted N-glycosylation site different from Neu3d and Neu3e (Figure 2-18 and 2-19) and also different from medaka Neu3a and zebrafish Neu3.1 (Manzoni et al., 2007, Shiozaki et al.,

2013). Similar to medaka Neu3 sialidases (Shiozaki et al., 2013), tilapia Neu3a, Neu3d and Neu3e sialidases showed no predicted O-glycosylation sites. Neu3a, Neu3d and Neu3e sialidases showed the presence of several putative phosphorylation sites same as medaka Neu3s and zebrafish.





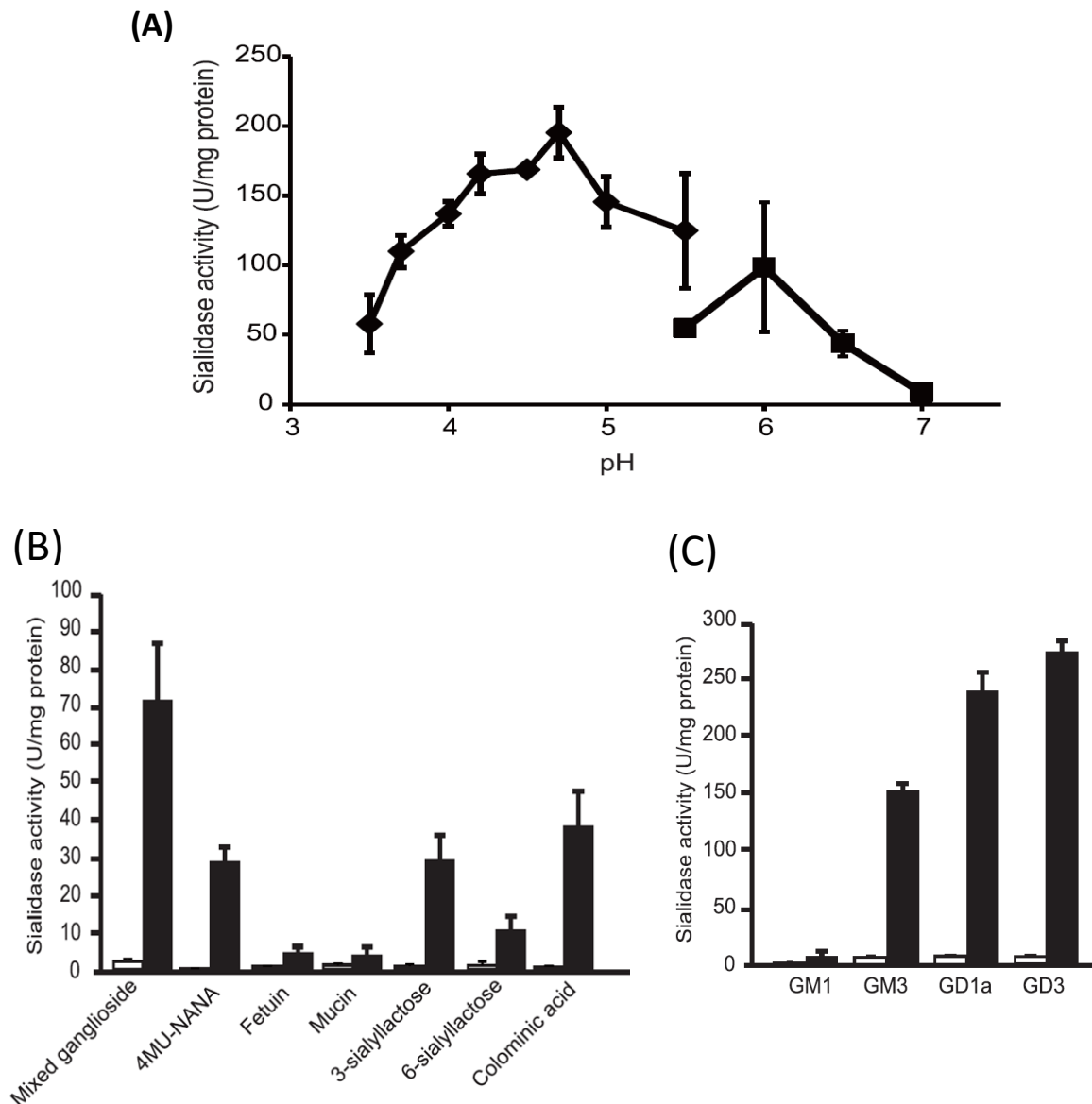
**Figure 2-22.** Multiple alignment of amino acid sequences from tilapia Neu3a, Neu3b, Neu3c, Neu3d and Neu3e, human NEU3, medaka Neu3a and Neu3b.

The alignment was performed as described in materials and methods. Actual amino acid sequences were used for Neu3a, Neu3d and Neu3e, while putative sequences were used in case of Neu3b and Neu3c due to lack of gene amplification. Identical residues are shown by black background (100% identity) and gray background (partial identity). Conserved motifs YRIP and Asp-box are boxed with a dotted line, a bolded line and a single line, respectively. Highly conserved residues involved in the formation of active sites are shown by closed diamonds.

The consensus nucleotide sequences were translated into amino acid sequences with *neu3a* transcribing 409 amino acids, *neu3d* transcribing 398 amino acids and *neu3e* transcribing 385 amino acids. The differences in molecular weights were modest with 45.9 kDa, 44.4 kDa and 43.6 kDa for Neu3a, Neu3d and Neu3e, respectively. These values were later confirmed by immunoblotting analyses using lysate from each *neu3* transfected cells (Figure 2-24A).

### **2.5.2.3. Enzymatic characterisation of tilapia neu3 sialidases**

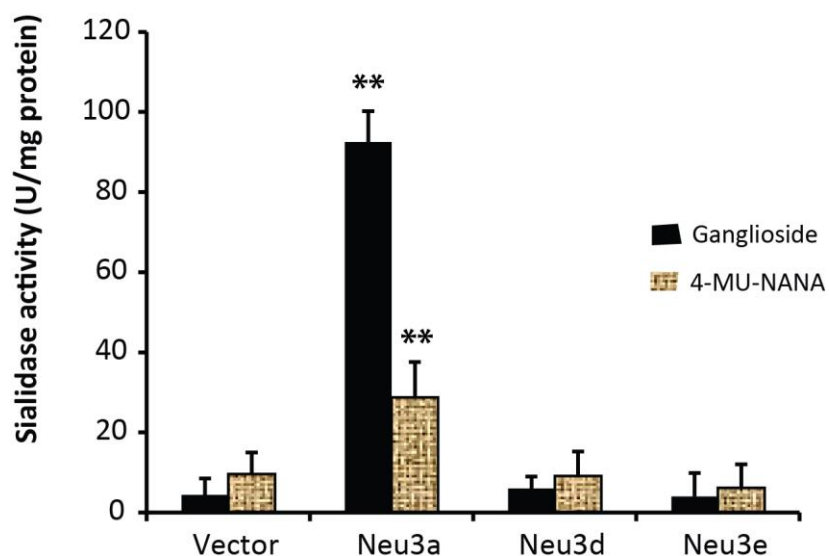
Enzymatic characterisation of Neu3 sialidases was done using recombinant polypeptides. Tilapia *neu3a*, *neu3d* and *neu3e* subcloned into pcDNA3.1 vector were expressed in HEK293 cells followed by lysate preparation. The prepared lysate was used in the determination of sialidase activity at various pH values towards various substrates. Tilapia Neu3a activity showed highest sialidase activity at pH 4.6 (Figure 2-22A), a similar behaviour to Neu3 sialidase from other species, pH 4.6 for human NEU3 (Wada et al., 1999) and pH 4.2 for medaka Neu3a (Shiozaki et al, 2013). For determination of substrate specificity, seven different substrates were used as described in section 2.12.2. Estimation of endogenous sialidase activities in HEK293 cells yielded significantly low activity (less than 3 U/mg protein) towards all substrates (Figure 2-22B). Highest sialidase activity was detected towards ganglioside mix for tilapia Neu3a. Tilapia Neu3a also showed significant activity towards 3-sialyllactose, colominic acid and 4-MU-NANA.



**Figure 2-23.** Optimum pH and substrate specificity of tilapia Neu3a sialidase.

(A) Enzymatic reaction was done with *neu3a*-transfected cell lysate using two types of buffers: sodium acetate buffer for pH 3.5-5.5 (diamond) and sodium phosphate buffer for pH 5.5-7.0 (square). (B) Sialidase activity of *neu3a*-transfected cell lysate was assayed towards various glycoconjugates at pH 4.6 for wild and Neu3a transfected cells. (C) Sialidase activity was then assayed towards pure gangliosides, GM1, GM3, GD1a and GD3. The results are shown as means  $\pm$  standard deviation from three independent experiments.

The sialidase activities for Neu3a towards 6- sialyllactose and glycoproteins (fetuin and submaximally mucin) were significantly low, indicating that tilapia Neu3a hardly cleaves sialic acids from these sialoglycoconjugates. Further examination of tilapia Neu3a sialidase activities towards pure gangliosides showed high activity towards GD3, GD1a and GM3 (Figure 2-22C). Only trace Neu3a activity was detected towards GM1 (4.2 U/mg protein), which was similar to the endogenous sialidase activity towards all pure gangliosides. Neu3d and Neu3e polypeptides did not show any significant sialidase activity at various pH and toward all seven substrates (Figure 2-23)

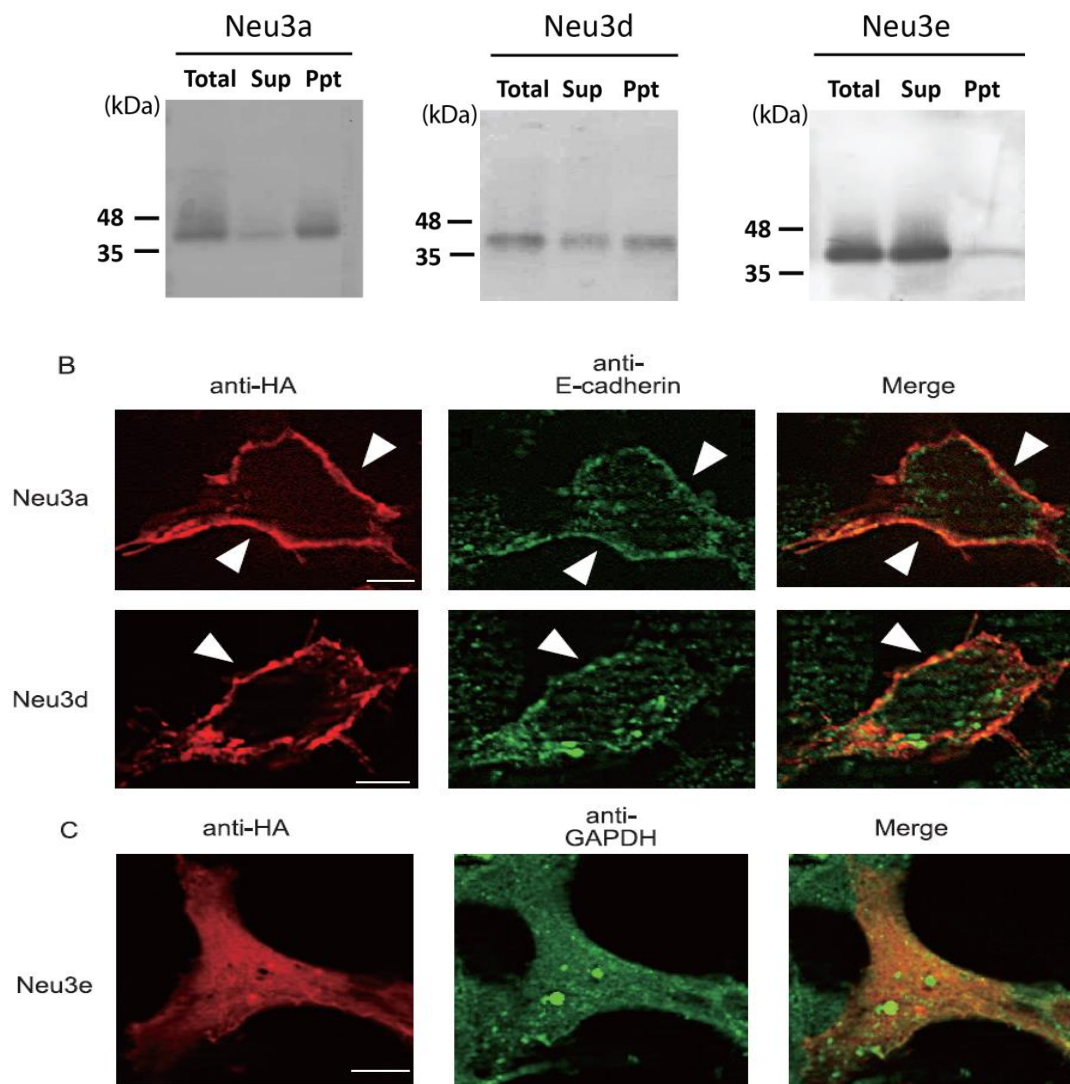


**Figure 2-24.** Neu3s sialidase activity comparison with mock.

Cell homogenates from vector, *neu3a*, *neu3d* and *neu3e* transfected cells were prepared and used for estimation of silaidase activity towards ganglioside mix and 4-MU-NANA. \*\*,  $p < 0.01$  compared with mock cells. The results are shown as means  $\pm$  standard deviation from three independent experiments.

#### **2.5.2.4. Tilapia *neu3* sialidase subcellular localization and fractionation**

Biochemical fractionation was carried out as described in materials and methods to approximate and confirm subcellular distribution of tilapia Neu3a, Neu3d and Neu3e using HA-tagged *neu3* transfected HEK293 cells. The cells were fractionated into supernatant and pellet by centrifugation at 17, 000 xg and each fraction was analyzed for the presence of respective Neu3-HA tagged protein using western blotting. Most Neu3a was recovered in the pellet fraction, while Neu3d was mainly recovered in pellet with a significant amount also recovered in the supernatant (Figure 2-24A). On the other hand most of the Neu3e proteins were recovered in the supernatant (Figure 2-24A). To obtain further information on subcellular localization of tilapia Neu3a, Neu3d and Neu3e, indirect immunofluorescence examination of sialidases was done in HEK293 cells transiently transfected with HA-tagged sialidase genes. The analysis showed that tilapia Neu3a strictly localized at the plasma membrane (Figure 2-24B), same as its orthologs; human NEU3 (Miyagi et al., 1999) and medaka Neu3a (Shiozaki et al., 2013). Tilapia Neu3d showed both plasma membrane and intracellular localization, although its intracellular labelling pattern could not be superimposable with any organellar markers used in this study. Tilapia Neu3e showed localization both at plasma membrane and in the cytosol as shown by its restricted distribution with E-cadherin (plasma membrane marker) and GAPDH (cytosolic marker) (Figure 2-24B).

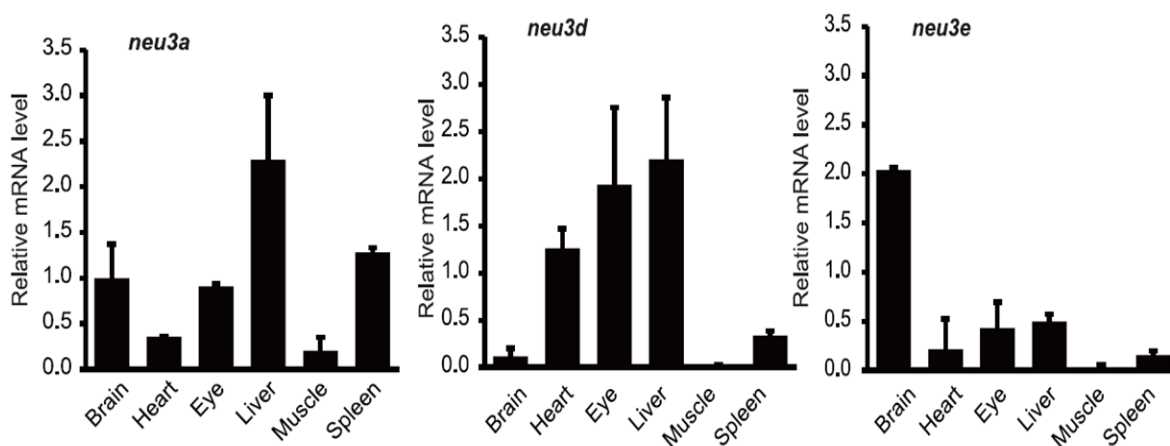


**Figure 2-25.** Subcellular localization of tilapia Neu3s.

(A) The fractionation was carried out as described in Section 2.15. Supernatant (Sup) and precipitate (Ppt) were obtained after centrifuging the original homogenate at 17,000 xg for 15 min and Neu3-HA proteins detected by immuno-blotting analysis. Protein separated using 12% SDS -PAGE and expressed Neu3a, Neu3d and Neu3e proteins were detected by using monoclonal antibody for HA and HRP-anti-rat IgG. (B, C) For subcellular localization of Neu3a, Neu3d and Neu3e, HEK293 cells grown on coverslips were transfected with each HA-tagged neu3 gene were used. Expressed sialidase proteins were detected by anti-HA antibody and Alexa 555-anti-rat IgG while plasma membrane, and cytosol were detected by E-cadherin (B) and GAPDH (C), respectively. The observation was done using sectioning fluorescence microscopy

### 2.5.2.5. Tilapia *neu3* tissue expression

To determine tilapia transcription levels of Neu3 sialidases in various tissues, we analyzed the expression levels of *neu3* mRNA using real-time PCR. As shown in Figure 2-25, *neu3a* exhibited significant expression in liver, brain, spleen and eye while expression was generally low in muscle and heart tissues. Interestingly, despite showing little activity in the sialidase activity assay *neu3d* and *neu3e* showed significant expression in various tissues. Tilapia *neu3d* showed significant expression in heart, liver and eye while Neu3e had significant transcription in brain, liver and spleen tissues (Figure 2-25).



**Figure 2-26.** Tissue expressions of tilapia *neu3* sialidases.

Expression level of *neu3a* and *neu3d* and *neu3e* genes in tilapia brain, heart, eye, muscle and spleen were estimated by real-time PCR.  $\beta$ -actin isoform expression in tilapia was also examined as an internal reference. For the comparison of these *neu3* gene expressions, standard curve for *neu3a*, *neu3d* and *neu3e* cDNAs were generated by serial dilution of the pBluescript vectors containing the entire *neu3a*, *neu3d* and *neu3e* ORFs. *neu3a*  $\beta$ -actin expression level in brain was considered as one. The results are shown as means  $\pm$  S.D. of three independent experiments.

### **2.5.3. Tilapia *neu4* cloning and characterisation**

#### **2.5.3.1. Tilapia *neu4* gene bioinformatic analysis**

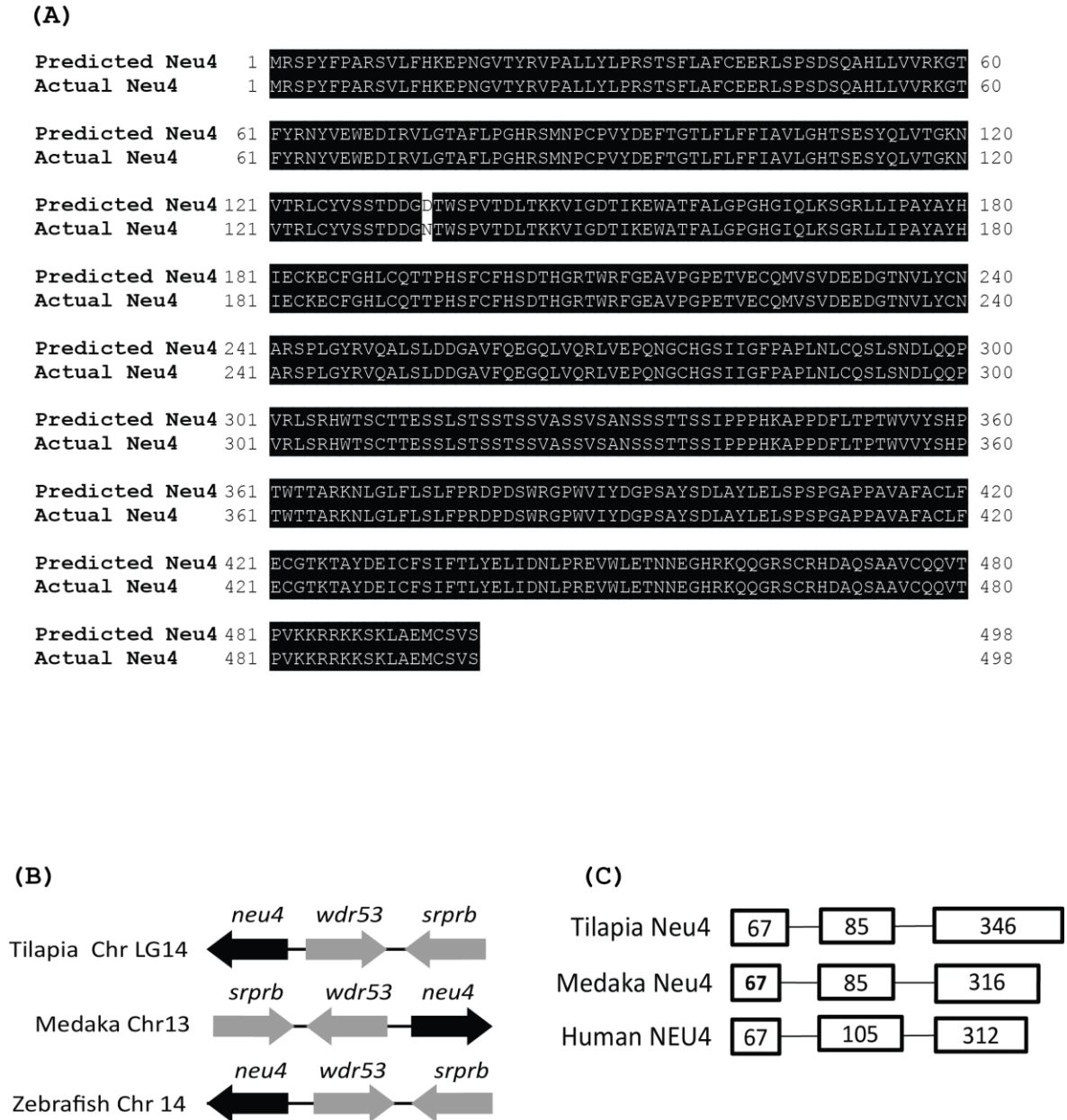
Orthologue of human NEU4 sequence in tilapia genome was determined using Ensembl genome browser (<http://www.ensembl.org/index.html>) and University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu/>). BLAST search for sequences showing similarities to human NEU4 genes in tilapia genomes yielded a single *neu4*-like putative sialidase gene. One first ATG was detected which yielded a nucleotide sequence without any premature stop coding. Further search for ATG in the upstream of the 5' end, yielded nucleotide sequences with several stop codons after translation. The syntenic analysis showed that the predicted tilapia *neu4* gene was flanked by *srprb* and *wd53* (Figure 24B) on chromosome LG 14 which were also conserved in medaka and zebrafish. These flanking genes were not present in other putative tilapia sialidase genes.

#### **2.5.3.2. Tilapia *neu4* gene cloning**

For cloning of tilapia, PCR primers were designed based on the 5' and 3' ends of the putative *neu4* nucleotide sequence, while incorporating endonuclease cleaving and Kozak sequences. The PCR product was electrophoresed on 1% agarose gel and the results showed a single band with molecular weight corresponding to the predicted weight (~1,500 bp). The PCR product was purified and sub-cloned into pBluescript SK (+) cloning vector and sequenced. The sequenced results showed that the actual ORF was 1,497 bp. Previous reports in mammalian *Neu4* sialidase genes have reported the presence of splicing variants



(Yamaguchi et al., 2005). Only a single form of *neu4* gene was detected in tilapia using cDNA from brain and liver tissues after PCR amplification. Four SNPs were present in the actual sequence when compared with predicted sequence, one of which was nonsynonymous and resulted in the amino acid changed (D134N) (Figure 2-27A). Analysis of the region surrounding *neu4* genes in tilapia and other fish species showed that tilapia *neu4* is flanked by similar genes to medaka and zebrafish (Figure 2-27B). In addition, the actual tilapia *neu4* gene was organized into three exon (Figure 2-27C) similar to medaka *neu4* (Shiozaki et al, 2014) and zebrafish *neu4* (Manzoni et al., 2007) but different to *neu1* and *neu3* groups. A comparison of the putative encoded amino acid sequence, showed sequence identities of 81.6% and 68.3% with medaka and zebrafish Neu4, respectively. The BLAST search revealed that the encoded protein of tilapia Neu4 showed sequence identities of 30.7%, 46.2%, 42.9% and 49.8% when compared with human NEU1, NEU2, NEU3 and NEU4, providing further evidence that the predicted sequence in tilapia genome is indeed Neu4. The *neu4* DNA sequence was deposited in the DDJB under the accession number LC055682.1.



**Figure 2-27.** Sequence alignment, syntenic analysis and exon organisation.

(A) Comparison between putative and actual tilapia Neu4 sequences. (B) Analysis of the flanking genes for *neu4* in tilapia, medaka and zebrafish genomes. The syntenic analysis was determined using Ensembl genome browser. (C) Exon organisation of tilapia, medaka and human Neu4.

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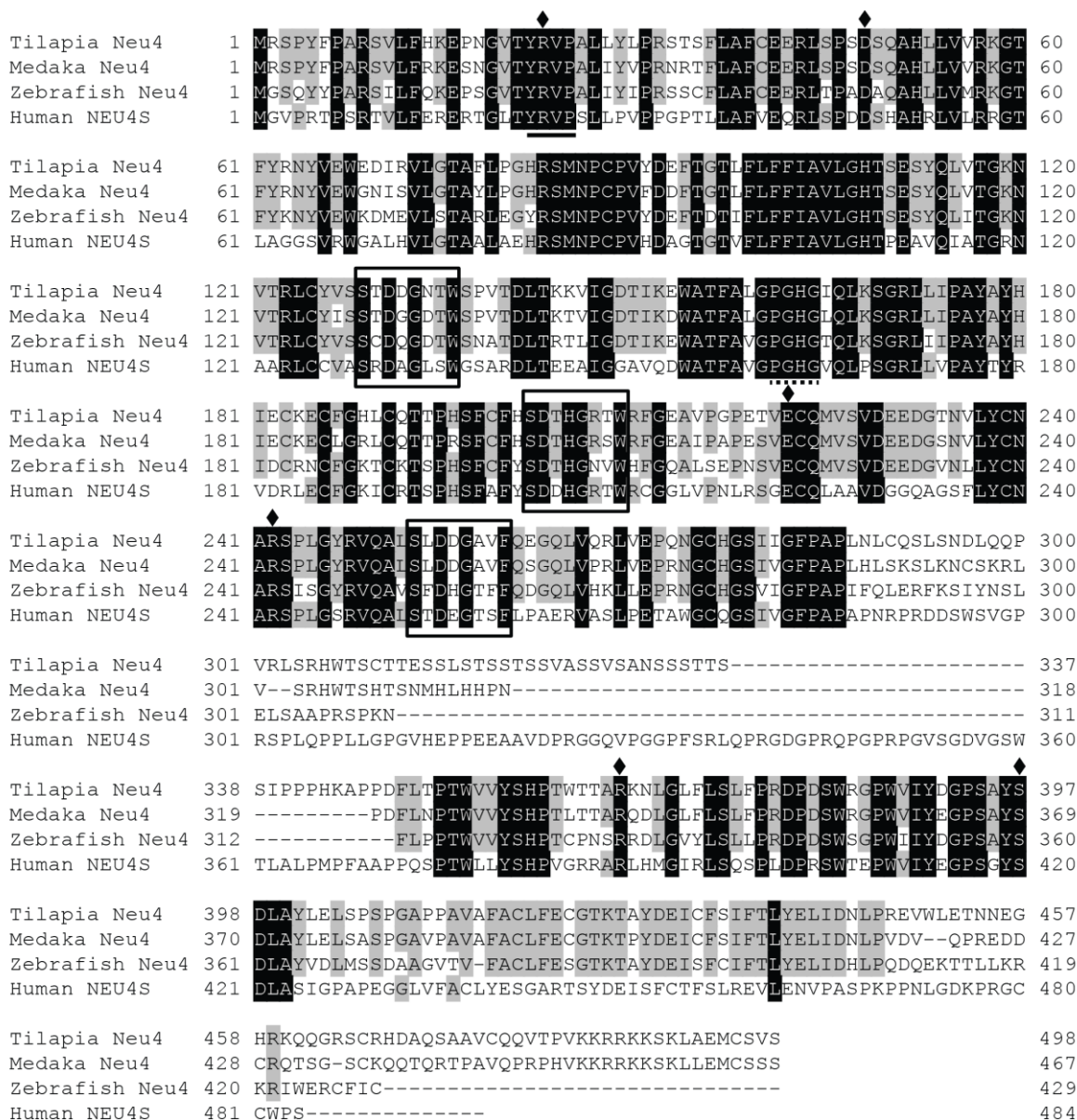
1  ATGAGGTCGCCCTATTTCCAGCCAGATCTGTGCTTTTCCACAAGGAGCCGAACGGAGTG      60
   M R S P Y F P A R S V L F H K E P N G V
61  ACGTACAGAGTCCCAGCTCTGCTCTACCTGCCCCGCTCCACATCCTTTCTGGCCTTCTGT      120
   T Y R V P A L L Y L P R S T S F L A F C
121 GAGGAGAGGCTCAGCCCGTCCGACTCTCAGGCTCACCTGCTGGTTGTGAGGAAAGGCACT      180
   E E R L S P S D S Q A H L L V V R K G T
181 TTCTACAGGAACATGTGGAGTGGGAGGACATCCGTGTTCTGGGCACTGCTTTCTTGCCA      240
   F Y R N Y V E W E D I R V L G T A F L P
241 GGCCACCGATCCATGAACCCGTGCCCGGTCTACGATGAGTTCACGGGTACTCTCTTTCTG      300
   G H R S M N P C P V Y D E F T G T L F L
301 TTCTTACATCGCTGTTCTGGGTACACCTCAGAGTCTTACCAGCTGGTGACGGGGAAGAAC      360
   F E I A V L G H T S E S Y Q L V T G K N
361 GTGACCAGACTGTGCTACGTCTCCAGCACTGATGATGGCGACACGTGGAGTCCCCGTCACT      420
   V T R L C Y V S S T D D G D T W S P V T
421 GACCTCACCAAGAAGGTCATAGGAGACACTATCAAAGTATGGGGCCACCTTCGCTCTCGGC      480
   D L T K K V I G D T I K E W A T F A L G
481 CCGGGCCACGGCATCCAGCTGAAGTCGGGCGGTTTGCTCATTCTGCTACGCCTACCAC      540
   P G H G I Q L K S G R L L I P A Y A Y H
541 ATCGAGTGCAAAGAGTGCTTCGGGCACCTCTGCCAGACCACTCCCCACTCCTTCTGCTTT      600
   I E C K E C F G H L C Q T T P H S F C F
601 CACAGCGACACTCACGGGAGAACCTGGCGCTTTGGAGAGGCGGTCCCAGGGCCAGAGACC      660
   H S D T H G R T W R F G E A V P G P E T
661 GTGGAGTGTGATGGTGTCTGTGGACGAAGAGGATGGGACTAACGTGTTGTACTGTAAT      720
   V E C Q M V S V D E E D G T N V L Y C N
721 GCTCGCAGCCCTCTCGGGTACAGAGTGCAGGCCCTCAGTCTGGACGATGGAGCCGTGTTT      780
   A R S P L G Y R V Q A L S L D D G A V F
781 CAGGAGGGGCGAGCTGGTGCAGCGGCTGGTGGAGCCTCAAATGTTGTCATGGGAGTATT      840
   Q E G Q L V Q R L V E P Q N G C H G S I
841 ATTGGATTTCTGCCCCGTTAAATCTGTGTGTCAGAGTCTTAGCAACGATTTACAGCAACCT      900
   I G F P A P L N L C Q S L S N D L Q Q P
901 GTACGGCTCTCCAGACACTGGACATCCTGCACACAGAGTCCAGTCTCTCCACATCGAGT      960
   V R L S R H W T S C T T E S S L S T S S
961 ACCAGCTCTGTAGCCTCTTTCAGTGTGAGCAACAGCTCCAGCACCAGCTCCATCCCA      1020
   T S S V A S S V S A N S S S T T S S I P
1021 CCTCCTCATAAAGCCCTCCGGATTTCCTGACTCCTACCTGGGTAGTATATTCCACCCG      1080
   P P H K A P P D F L T P T W V V Y S H P
1081 ACATGGAGACTGCACGCAAAACCTGGGCTTGTTCCTCAGCCTTTTCCCCCGAGATCCA      1140
   T W T T A R K N L G L F L S L F P R D P
1141 GACAGCTGGCGTGGCCCTGGGTGATCTATGATGGGCCGAGCGCTACTCTGACCTGGCC      1200
   D S W R G P W V I Y D G P S A Y S D L A
1201 TACCTGGAGCTGTACCCCTCACCTGGAGCCCCACCTGCTGTAGCATTCGCCTGCCTGTTT      1260
   Y L E L S P S P G A P P A V A F A C L F
1261 GAGTGTGGTACCAAAACAGCTTACGACGAAATCTGCTTCAGCATCTTCACCCTCTACGAG      1320
   E C G T K T A Y D E I C F S I F T L Y E
1321 CTCATCGATAATCTGCCCCGGAAGTTTGGCTGGAACAAACAACGAAGGGCACAGAAAA      1380
   L I D N L P R E V W L E T N N E G H R K
1381 CAGCAGGGGAGGAGCTGCAGACATGATGCTCAAAGTGCAGCAGTTTGCCAACAGGTGACT      1440
   Q Q G R S C R H D A Q S A A V C Q Q V T
1441 CCTGTGAAGAAGAGGAGGAAGAAGAGCAAGCTGGCTGAGATGTGCTCTGTGCTTAA      1497
   P V K K R R K K S K L A E M C S V S *

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**Figure 2-28.** Nucleotide and deduced amino acid sequence for *neu4*.

Single letters for the top sequence represent nucleotides of *neu4* while single letters for the lower sequence represent amino acids of Neu4. Start and stops codons are represented by solid horizontal line and asterisk, respectively. Predicted N-glycosylation site are boxed while O-glycosylation sites are circled. Vertical lines represent exon-intron boundaries

Further bioinformatic analyses, showed that the encoded Neu4 polypeptide consisted 499 amino acids with a molecular weight of 55.3 kDa. Post-translation modification analysis showed the presence of a single N-glycosylation (Asp 120) site and 18 potential O-glycosylation sites (Figure 2-28). The higher number of O-glycosylation sites was a slight deviation from other Neu4 sialidases. For instance silaidase Neu4 from medaka showed no single potential O-glycosylation site and five N-glycosylation sites (Shiozaki et al. 2014). Moreover, tilapia Neu4 showed the presence of three classical Asp-boxes, YRI(V)P and VPGP motifs similaro to other sialidases (Figure 2-29). As already reported for medaka Neue4 (Shiozaki et al., 2014) and the other members of the sialidase family, six amino acid residues that are potentially involved in the formation of the catalytic site of sialidase (Giacopuzzi et al., 2012) were also conserved in tilapia Neu4 (Figure 2-29).

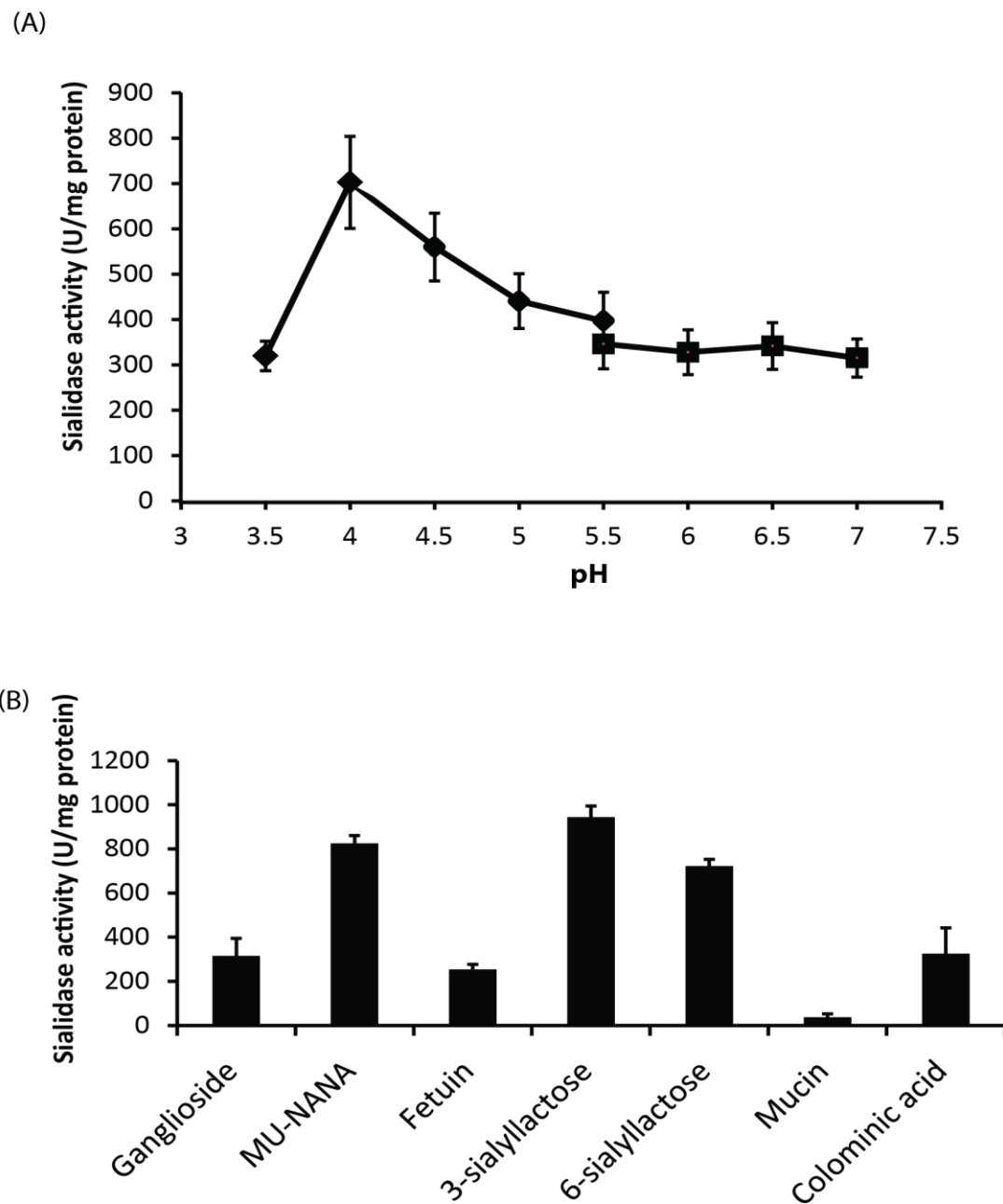


**Figure 2-29.** Multiple alignment of amino acid sequences from tilapia Neu4, medaka Neu4, zebrafish Neu4 and human NEU4.

The alignment was performed using ClustalW embedded in Genetyx software. Identical residues are shown by black background (100% identity) and gray background (partial identity). Conserved Asp-boxes are boxed with a solid line, YRIP and VPGP motifs are shown by a solid and dotted lines, respectively. Highly conserved residues involved in the formation of active sites are shown by closed diamonds.

### **2.5.3.3. Neu4 sialidase activity**

To verify that tilapia *neu4* gene does encode a sialidase, the *neu4* ORF from brain cDNA was sub-cloned into pcDNA3.1 expression vector. The pcDNA3.1 sub-cloned *neu4* gene was transfected in HEK293T cells using PEI method for the evaluation of sialidase activity. Sialidase activity was evaluated using homogenate prepared from the transfected cells towards substrate 4-MU-NANA. For the estimation of optimal sialidase activity pH, buffer at various pH were used (pH3.5-7.0). Optimal sialidase activity for Neu4 was observed at pH 4.0 (Figure 2-30A) comparable to sialidase Neu4 from medaka (Shiozaki et al., 2015) and human short form of NEU4 (NEU4S) (Yamaguchi et al., 2005). The optimal pH for tilapia Neu4 was quite different from that of zebrafish Neu4 (pH 2.6). Significant sialidase activity was recorded even at neutral pH suggesting that tilapia Neu4 possesses a slightly broader activity pH, a phenomenon also observed in medaka Neu4 (Shiozaki et al., 2014).



**Figure 2-30.** Tilapia Neu4 optimal activity pH and substrate specificity.

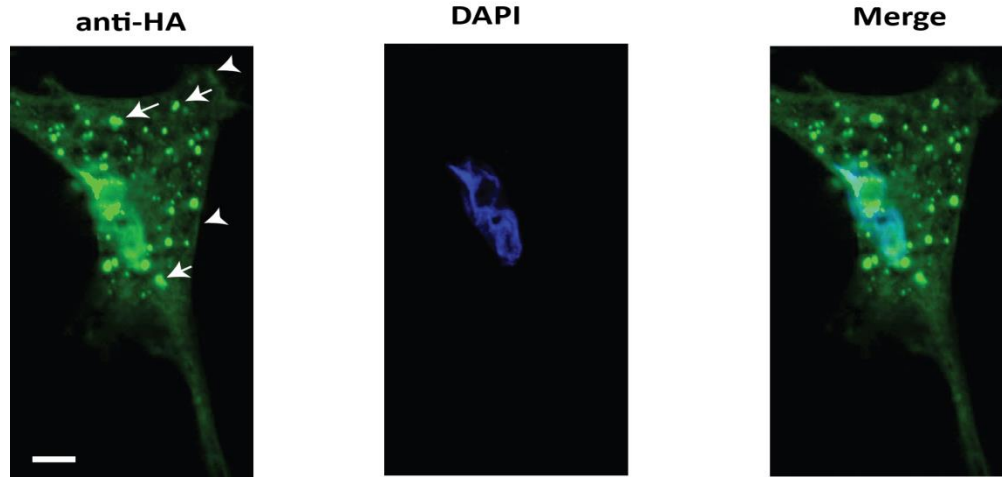
(A) Enzymatic reaction was done with *neu4*-transfected cell homogenate using two types of buffers: sodium acetate buffer for pH 3.5–5.5 (diamond) and sodium phosphate buffer for pH 5.5–7.0 (square). (B) Sialidase activity of *neu4*-transfected cell lysate was assayed towards various glycoconjugates at pH 4.0 for wild and Neu3a transfected cells.

To estimate the substrate specificity of tilapia Neu4, transfected *neu4* transfecte cell homogenate was reacted with various structurally different substrates; ganglioside mix, 3-sialyllactose, 6-sialyllactose, bovine fetuin bovine submaxillary gland mucin, colominic acid and 4-MU-NANA. Among all substrates, 3-sialyllactose, 6-sialyllactose, and 4-MU-NANA showed high Neu4 sialidase activity (Figure 2-30B). Significant Neu4 activity was also observed towards ganglioside and colomininc acid. The sialidase activities for Neu4 towards glycoproteins (fetuin and submaximally mucin) were significantly low, indicating that tilapia Neu4 hardly cleaves sialic acids from these sialoglycoconjugates (Figure 2-30B).

#### **2.5.3.4. Neu4 subcellular lozalization and tissue expression**

For the determination of Neu4 subcellular lozalization, Neu4-HA tagged transfected Hepa-T1 cells were used. Indirect immunofluorescence and sectioning fluorescent microscopy showed tilapia Neu4 primarily co-localizing with DAPI at the nucleus. Some Neu4 signals were distributed in the cells with a dotted pattern while some signals were also present at the plasma membrane (Figure 2-31).

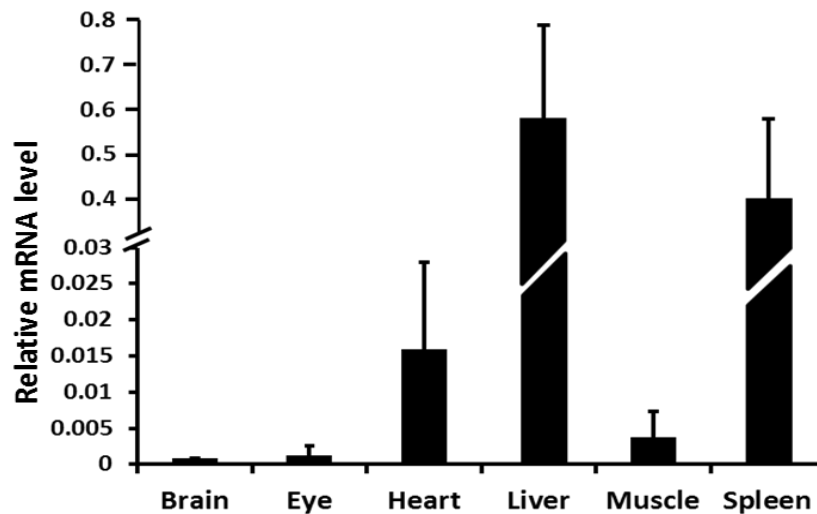




**Figure 2-31.** For subcellular localization of Neu4.

HEK293 cells grown on coverslips were transfected with each HA-tagged *neu4* gene were used. Expressed sialidase proteins were detected by anti-HA antibody and Alexa 555-anti-rat IgG. The observation was done using sectioning fluorescence microscopy.

Tilapia *neu4* tissues expression patterns were also determined in similar to *neu1s* and *neu3s*. Tilapia *neu4s* showed highest expression in the liver and spleen (Figure 2-32). Tilapia *neu4* tissue expression was low in the brain, different from medaka Neu4 (Shiozaki et al., 2014), which showed highest expression in the brain.



**Figure 2-32.** Tissue expressions of tilapia *neu4* sialidases.

Tilapia *neu4* expression levels in brain, heart, eye, muscle and spleen estimated by real-time PCR.  $\beta$ -actin isoform expression in tilapia was also examined as an internal reference. A standard curves were generated by serial dilution of the pBluescript vectors containing the entire *neu4* ORFs. The results are shown as means  $\pm$  S.D. of three independent experiments.

## 2.6. Discussion

Expansion of molecular cloning and biochemical characterisation of sialidase genes and proteins to various species continue to reveal their remarkable diversity. Recent efforts have focused on uncovering various roles of sialidases, especially in fish. Based on mammalian studies, sialidases exhibit varying properties including substrate specificity towards structurally different substrates (Miyagi et al., 1999, Monti et al., 2000). Studies of fish sialidase have lagged behind and have received attention only recently (Manzoni et al., 2007; Shiozaki et al., 2013). Available studies in fish, have demonstrated the presence of several forms of sialidase genes and their paralogues, some of which possess slightly

deviated characteristics from the usual properties reported in mammals. Nevertheless, a growing body of literature suggests the possible existence of more diversified physiological roles and functions of sialidase proteins in nature.

In the present study, the existence of several forms of sialidase genes has been revealed in tilapia (*O. niloticus*), one of the most cultured finfishes in the world. We have identified a total of eight sialidase genes are present in tilapia genome; two *neu1* genes (*neu1a* and *neu1b*), five *neu3* (*neu3a*, *neu3b*, *neu3c*, *neu3d* and *neu3e*) and one *neu4* gene. In terms of Neu1, a single form of Neu1 has been reported in mammals (Yamaguchi et al., 2005), zebrafish (Manzoni et al., 2007) and medaka (Ryuzono et al., 2016). Further assessment showed that *neu1a* had a conserved exon number of six, similar to mammalian and other fish sialidase Neu1s, while *neu1b* had more exon than other reported Neu1s. The results here-in suggest that *neu1a* could possibly be the main *neu1* gene in tilapia. The enzymatic properties of its polypeptide were very similar to polypeptide properties of other Neu1s than when compared to Neu1b. The presence of two active *neu1* sialidase genes in tilapia suggest that the two genes could possibly complement each other in their functions at either the lysosome or plasma membrane. Transcripts of both *neu1a* and *neu1b* were detected after PCR from cDNA suggesting that both neu1s are expressed in tilapia. Available studies of cloned *neu1* sialidase genes in other species have reported the presence of single form of neu1 genes. For instance, in human and medaka single form of neu1 are present (Seyrantepe et al., 2003; Ryuzono et al., 2016). Despite the presence of two sialidase Neu1s in tilapia, the bioinformatic analyses and in-vitro studies suggested that Neu1a is the main

Neu1 in tilapia. Exon number, as well as substrate specificity provided strong evidence to suggest that Neu1a and not Neu1b is the immediate orthologue of human NEU1 and madaka Neu1. Consistent with human and medaka Neu1s, tilapia Neu1 showed significant activity towards 3-sialyllactose a sialoglycoconjugates with  $\alpha$  2-3 linked sialic acid, colominic acid and gangliosides, among the natural substrates. On the other hand Neu1b showed significant activity towards a synthetic substrate 4-MU-NANA. In mammals and medaka, sialidase Neu1 is present in a multienzyme complex with the protective protein/Cathepsin A and  $\beta$ -galactosidase (Bonten and d'Azzo, 2000; Ryuzono et al., 2016). These reports demonstrated the activation of Neu1 sialidase following multimerization at acidic pH in the presence of PPCA. Mechanistically, the interaction of sialidase with Cathepsin A is necessary for the lysosomal routing, correct compartmentalization and prevents the sialidase from oligomerizing prematurely. In addition, dimerization of Cathepsin A alone with sialidase Neu1, has been reported to activate the sialidase in mammals and fish (Bonten and D'Azzo, 2000; Ryuzono et al., 2016), suggesting that  $\beta$ -galactosidase is involved in other roles not related to Neu1 activation. Consequently, in this study only the Neu1s and Cathepsin A expression and properties analysis were focused. The activation of both Neu1a and Neu1b was observed, after co-overexpression of the recombinant Neu1 and Cathepsin A. Interestingly, Neu1a showed significant activity in the absence of Cathepsin A in HEK293T cells, a case that has not been observed in other fish Neu1s. Although experimental evidence is lacking to this point, it may be possible that tilapia Neu1a utilises endogenous Cathepsin A in HEK 293T cells for its activation. It is possible that Neu1a structure initiate interaction with endogenous Cathepsin A. The

structural model of Cathepsin A–NEU1 has demonstrated that some amino acids are crucial for this interaction (Bonten et al., 2009). It is plausible that some amino acid residues in tilapia Neu1a facilitate/stimulate its dimerisation with endogenous Cathepsin A. This structure may not be present or conserved in Neu1b and hence lack of Neu1b activity in the absence of Cathepsin A. A partial lysosomal targeting motif was observed in both tilapia Neu1a (Tyr-Gly-Gly-Arg ) and Neu1b (Tyr-Ser-Gly-His), similar to the partial lysosomal targeting motif present at the C-terminus of medaka neu1 (Ryuzono et al., 2016) and other lysosomal proteins such as LAMP-1, LAMP-2 and CD63 (Try-X-X-hydrophobic residue) (Guarnieri et al., 1993).

Tilapia Neu1a gene comprised six exons and possessed a more conserved lysosomal targeting motif at the C-terminus and a potential cleavage site at the N-terminus very similar to human and medaka (Ryuzono et al., 2016). Furthermore, biochemical characterization revealed that Neu1a could likely be the main orthologue of human NEU1. Neu1a was partly colocalized with lysosomal marker, suggesting that it may be a lysosomal sialidase similar to other Neu1s (Bonten et al., 2009; Ryuzono et al., 2016). In addition, sialidase activity for Neu1a was optimal at pH 4.5, within the acidic pH range for lysosomal enzymes. Sialidase Neu1b possessed seven exons and its optimal sialidase pH was more acidic (pH 4.0). Tilapia Neu1b might have evolved from Neu1a although phylogenetic analysis suggests that the two genes might have evolved at the same time from the ancestor. Although evidence is lacking, it can be speculated here that Neu1b is not a major lysosomal sialidase in tilapia. Neu1b lacked a clear lysosomal targeting motif at the

C-terminal and despite the presence of a cleavage site at the N-terminus, the exon number was higher than the the usual number of exons in the lysosomal sialidase gene family. Results from a related study in medaka Neu1, showed that introduction of *neu1* gene in tilapia Hepa-T1 cells, resulted in a a more clear lysosomal localization (Ryuzono et al., 2016), although other cell types of the transfected with the same medaka Neu1, showed a localization not matching with lysosome. The results in medaka Neu1 localisation in tilapia Hepatic cells showed no change in the presence or absence of Cathepsin A, suggesting similar case could be true with tilapia Neu1a, closely related orthologue of medaka Neu1. Neu1 lysosomal localization has been reported to dependent N-terminal signal peptide, C-terminal lysosomal targeting motif and activation of Cathepsin A (Bonten et al., 2009; Giacomuzzi et al., 2012). Tilapia Neu1a showed an almost conserved C-terminal lysosomal targeting motif and a highly conserved N-terminal signaling peptide suggesting that its properties and physiological functions could be very similar to medaka and mammalian sialidases. Based on human and medaka, Neu1a could possibly be involved in the regulation of lysosomal degradation in tissues. Related studies have also shown that Neu1 is ubiquitously expressed in medaka tissues (Ryuzono et al., 2016), which could also be the same for tilapia Neu1a, indicating that the regulation of lysosomal regulation by Neu1a could occur in all tissues. The importance of Cathepsin A in Neu1 activation has been explored (Bonten et al., 2009; Ryuzono et al., 2016). Both tilapia Neu1a and Neu1b showed significantly elevated sialidase aactivity in the presence of tilapia Cathepsin A. Interestingly, Neu1a showed some activity even in the absence of Cathepsin A, although activity was highest with Cathepsin A activation.

Previous studies have reported the presence of only a single copy of *NEU3* gene in human, other mammals and avian (Miyagi et al., 1999, Wada et al., 1999, Giacomuzzi et al., 2011). Different from mammals and birds, fishes have been shown to possess multiple copies of *neu3* genes all of which are orthologues of human *NEU3* (Manzoni et al., 2007; Shiozaki et al., 2013). Tilapia *neu3a* demonstrated significant evolutionary conservation with human *NEU3* and medaka *neu3a* based on its gene organization, subcellular distribution and substrate specificity, suggesting that *neu3a* may have played an ancestral role and might have given rise to other isoforms within the species. Moreover, the partial synteny for tilapia *neu3a* was well conserved, further supporting the orthologous nature between tilapia *neu3a*, medaka *neu3a* and human *NEU3*. Tilapia *neu3b*, present on chr LG15 was also identified as an orthologue of medaka *neu3b* based on conserved synteny. On the other hand, tilapia *neu3c*, *neu3d* and *neu3e* genes were clustered on a single chromosome (chr LG22) and no synteny could be detected with human and medaka. Although tilapia *neu3* nucleotide sequences were predicted *in silico*, conserved sialidase motifs, phylogenetic analysis and exon-intron organization provided adequate evidence suggesting that these genes are orthologues of mammalian, medaka and zebrafish *neu3*.

Although five orthologues of human *NEU3* were identified in tilapia genome, molecular cloning from cDNA was not successful for *neu3b* and *neu3c*. Tilapia *neu3b* and *neu3c* mRNA could not be detected in all tested tissues from adult fish used in the study, suggesting lack of transcription in the two genes. Surprisingly, medaka *neu3b*, an orthologue of tilapia *neu3b*, possess significant expression and encode an active sialidase

protein (Shiozaki et al., 2013). Although it has previously been suggested that medaka *neu3b* may have evolved independently in the species, the present study suggests that *neu3b* might have come from a more recent shared ancestor of the two species (tilapia and medaka) and that it might have independently lost its tissue expression due to evolutionary forces in tilapia. On the other hand, *neu3c* which might have evolved independently in tilapia also showed lack of tissues expression different from its evolutionary counterparts *neu3d* and *neu3e*. Reasons for the lack of tissue transcripts of *neu3b* and *neu3c* remains to be elucidated but it seems likely that *neu3b* and *neu3c* might be pseudogenes and not necessary in tilapia. Pseudogenes have widely been reported in vertebrates (Mighell et al., 2000 and Zhang and Gerstein, 2004) and are generally inheritable, non-functional, gene homologies, disabled at transcription level due to lack of functional promoter (Hirotsune et al., 2003).

Except in zebrafish, Neu3 sialidases in human (Wada et al., 1999 and Monti et al., 2000), rodents (Miyagi et al., 1999 and Fronda et al., 1999), chicken (Giacopuzzi et al., 2011) and medaka (Shiozaki et al., 2013) possess well conserved patterns in their subcellular localization at the plasma membrane and strict substrate specificity towards gangliosides. The expression of *neu3a*, *neu3d* and *neu3e* genes in transfected HEK293 cells showed that only tilapia *neu3a* encoded enzymatically active sialidase, which possess similar properties to human NEU3 and medaka Neu3a. Tilapia Neu3a significantly hydrolyses gangliosides, GM3, GD1a and GD3 almost equally, consistent human NEU3 (Hata et al, 1998, Monti et al, 2000) in the presence of Triton X-100. The trace Neu3a activity towards GM1 could be



attributed to the stability of NeuAc residues to the internal galactose of GM1, which has previously been reported to induce its hydrolytic resistance to microbial and mammalian sialidases (Schachter and Roseman, 1980). Mammalian sialidase Neu3 has been implicated in ganglioside degradation and shown to optimally cleave sialic acid from their terminal ends (Miyagi et al., 1999). Gangliosides, sialic acid-containing glycosphingolipids, are present in surface membranes of cells and are thought to play important functional roles in regulating a wide range of biological processes including cell surface interactions, cell differentiation, and transmembrane signaling (Watanabe et al., 2002; Sorice et al., 2004). Therefore, sialidase activity towards pure gangliosides GM3, GD1a and GD3 suggest a possibility of tilapia Neu3a playing similar physiological functions to mammalian Neu3. Tilapia Neu3a exhibited similar enzymatic profiles to that of medaka Neu3a, human NEU3 and mammalian Neu3 sialidases. Efforts to find a substrate for Neu3d and Neu3e sialidase proteins proved futile after several attempts in the presence of various substrates at pH ranging from 2 to 8 and at various temperatures. The reason for the absence of sialidase activity in Neu3d and Neu3e could not be identified experimentally. Actual Neu3d sequence showed point mutation (R328T), one of the amino acids possibly involved in the active site architecture acting as a binding site of carboxylate group common to all sialic acids (Buschiazzo and Alzari, 2008), suggesting its involvement in absence of sialidase activity in the enzyme. Six key residues have been reported to be essential for catalysis of sialidase; Arg triad, the Tyr/Glu nucleophile pair and an Asp acting as the acid/base catalyst (Giacopuzzi et al., 2012). Residue substitution at any of these catalytic sites could potentially render a sialidase enzyme inactive. Alternatively the activity of Neu3d and

Neu3e may potentially be triggered and/or enhanced by the presence of other factors or molecules *in-vivo*. Similar phenomenon has been reported in mammalian Neu1, which makes a complex with cathepsin A and  $\beta$ -galactosidase for its activation (Monti et al, 2010). Furthermore, a true substrate may be present *in-vivo* different from the assayed substrates in this study. Therefore, further studies are necessary to precisely evaluate the mechanisms that may be responsible for initiation of enzymatic activities in Neu3d and Neu3e proteins, which may help reveal their potential physiological functions.

A single form of *neu4* gene was present in tilapia similar to other species. Evidence supported by the syntenic analysis showed that the predicted *neu4* gene was flanked with similar genes to *neu4* flanking genes in humans and medaka. Furthermore, the predicted *neu4* belonged to the *neu4* subcluster in the phylogenetic analysis and exhibited higher sequence identity with human and medaka *neu4* genes, strongly suggesting that the gene was an orthologue *neu4* in other species. Different from other forms of sialidase genes in tilapia, only a single *neu4* gene is present in tilapia genome, which showed high sequence identity with *neu4* from other species. Studies of human NEU4 have shown the presence of splicing (Yamaguchi et al., 2005), a case not observed in tilapia. The genes showed to have evolved together with medaka *neu4* and could possibly exhibit similar properties to medaka *neu4*. The encoded polypeptide showed a high number of O-glycosylation sites, which were not present in medaka. The primary structure analysis of tilapia Neu4 revealed a conserved structure in tilapia Neu4, similar to human and medaka Neu4 sialidases. In general, mammalian sialidase Neu4s cloned so far show the presence of canonical Asp-

boxes, YRVP and VPGP motifs (Monti et al., 2002). In addition, most of the amino acid residues possibly involved in the active site architecture (Giacopuzzi et al., 2012) are localized in topologically equivalent positions along the protein sequence with Neu4 sialidases from other organisms. Only single transcript of *neu4* was present in tilapia same as in medaka and human genomes. In mammals, only mouse *Neu4* gene has shown the presence of two isoforms thought to be alternate splicing variant (Shiozaki et al., 2009). The multiple alignment of tilapia sialidase Neu4 amino acid sequences, performed with Clustal Omega, showed the presence of a partial long stretch of amino acids towards the C-terminus, similar to the 81 amino acid stretch reported to be unique to human NEU4 (Monti et al., 2000). Although, the roles of this extra amino acid stretch could be difficult to speculate in fish, it could play similar roles with human NEU4. In human NEU4, this long stretch amino acid has been proposed to be a separate domain that provides unique functionality to sialidase since conserved sequences are found on both sides of this ‘insertion’ and since NEU4 folds into an active enzyme (Comelli et al., 2003). In contrast to the human Neu4 stretch, which is proline-rich, tilapia Neu4 stretch is serine-rich and although the functions of this serine rich stretch could be difficult to speculate here-in due to lack of literature, the domain could confer tilapia Neu4 additional physiological roles worthy exploring.

Immunofluorescence microscopy for Neu1a showed a partial lysosomal localisation of the enzyme. In addition to the lysosomes, mammalian Neu1 has been reported to be present at the plasma membrane (Liang et al., 2006), where it is thought to influence the immune

function. Although the results here-in could not show an obvious plasma membrane localisation of tilapia Neu1a in Hepa-T1, it is possible that in other cell types, Neu1a may translocate to the plasma membrane similar to the pattern observed for medaka Neu1 in different cell types (Ryuzono et al., 2016). Neu3 transfected HEK293 cells showed that most Neu3a and Neu3d localized at the plasma membrane, with some intracellular signals. These polypeptides were mainly recovered in the pellet after centrifugation at 17,000 xg. The intracellular signals of Neu3a and Neu3d were not co-localized with markers for lysosome, endoplasmic reticulum, golgi complexes and cytosol. Yamaguchi et al (2006) and Zanchetti et al (2007) reported the co-localization of human NEU3 with endosomal compartment at the early and recycling endosomes. However, tilapia Neu3a and Neu3d co-localization with EEA1, an early endosomal marker, could not be detected in this study. These results suggest that Neu3a and Neu3d polypeptides were not concentrated at any major organelle when exhibited internal distribution, different from mammalian Neu3. Early and recycling endosomal co-localization of human NEU3 has previously been suggested to be due to trafficking from plasma membrane to intracellular compartments as a NEU3 recycling process, suggesting that tilapia Neu3 could be involved in different recycling process or mechanism from human and mammals. On the other hand, Neu3e co-localized with a cytosolic marker, a behaviour similar to human NEU2 (Monti et al, 1999). Small amount of tilapia Neu3e was detected at the plasma membrane in the indirect immunofluorescence. Tilapia Neu4 exhibited a unique localisation at the nucleus and due to lack of experimental data, the reasons and the significance of Neu4 localisation at the nucleus was difficult to suggest in this study. Furthermore, a dotted pattern and plasma

membrane distribution of Neu4 were observed. Previous studies on Neu4 localization have been controversial with others reporting localization at the plasma membrane, mitochondria and endoplasmic reticulum (Yamaguchi et al., 2005; Shiozaki et al, 2014). The prediction of Nuclear Localisation Sequence using cNLS Mapper server ([http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS\\_Mapper\\_form.cgi](http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi)) and NucPred server (<https://www.sbc.su.se/~maccallr/nucpred/cgi-bin/single.cgi>) yielded positive results. A Nuclear Localization Sequence was predicted running along VTPVKRRRKKSKLA stretch of Neu4. This stretch of amino acid might be responsible for the nuclear localisation of Neu4. The physiological significance of Neu4 in the nucleus was difficult to speculate herein, but previous studies have reported a presence of ganglioside GM1 and GD1a at the nuclear envelope (Ledeen and Wu, 2010). GM1 occurs in close association with a  $\text{Na}^+/\text{Ca}^+$  exchanger (NCX) (Xie et al., 2002). The NCX/GM1 association mediates transfer nucleoplasmic  $\text{Ca}^+$  to the nuclear envelope. Neu4 showed some activity towards ganglioside mix and could potentially desialylate GD1a, a precursor of GM1, and consequently regulate the  $\text{Ca}^+$  transfer.

Significant *neu1a* tissue expression was observed in spleen, eye, liver and brain, suggesting that tilapia *neu1a* could be crucial for lysosomal degradation of glycoconjugates in these tissues. In mammals lysosomal sialidase has been reported to have a dual physiological function; it participates in intralysosomal catabolism of sialylated glycoconjugates and is involved in cellular immune response. Tilapia Neu1a and medaka Neu1 are very closely related, therefore, it is possible that in tilapia, Neu1a and Neu1b tissues expression could

closely related to medaka tissues expression. For Neu4 tissue expressions, significant expression was observed in liver and spleen. On the other hand, tilapia Neu3s expression levels in various tissues showed that tilapia *neu3a* sialidases have significantly higher expression in brain and liver very similar to medaka (Shiozaki et al., 2013). Higher tissue expressions of *neu3a* were also observed in eye and spleen. In this study we found that Neu3a, which desialylates gangliosides and is localized at the plasma membrane, is abundantly expressed in the liver similar to human NEU3 and medaka Neu3a sialidase. Based on these results tilapia Neu3a physiological functions could be similar to human (Monti et al, 2000), mammalian and medaka Neu3s which include; apoptosis (Valaperta et al., 2006), cell proliferation and myoblast differentiation (Anastasia et al., 2008), neurite formation (Hasegawa et al., 2000 and Proshin et al., 2002), regulation and regeneration of neurons (Rodriguez et al., 2001 and Da Silva et al., 2005), improving insulin sensitivity and glucose tolerance (Yoshizumi et al., 2007).

Pathogens commonly exploit host cell surface carbohydrates or glycosylation pathways in the course of infections. As previously reported, glycoconjugates, in particular glycoproteins are among the main targets involved in bacterial and viral infections in mammals (Yanagisawa, 2007, Fishman et al., 1993 and Baum and Paulson, 1990). Glycoproteins are proteins that contain covalently attached sugar residues. Glycoproteins are frequently present at the surface of cells where they function as membrane proteins or as part of the extracellular matrix. These cell surface glycoproteins play a critical role in cell–cell interactions and the mechanisms of infection by bacteria and viruses. Tilapia

Neu1a, Neu4 showed significant activity towards various sialo-glycoproteins *in-vitro* and could, therefore, be indispensable for pathogen adhesion to tilapia and medaka (used in this study as expression model for Neu1 and Neu4) cells. Significant expression in the muscle and liver tissues, suggest that the activity of the two enzymes could modulate the infection of glycoprotein-dependent pathogens. Muscle and liver are among the main pathogen entry and processing organs in fish. As such, the presence of sialo-glycoprotein regulating enzymes in the organs confirms the importance of endogenous sialidase in infection regulation. Infact, most pathogens are known to take advantage of glycoprotein desialylation to gain binding site on the asialo-chain or enjoy the cleaved sialic acid as source of energy. Other pathogens directly bind to the sialic acid on these glycoproteins. Glycoproteins are also crucial for pathogen adhesion. Recently, GM3 reported to be vibrio receptor in fish (Matsunaga et al., 2011 and Chisada et al., 2013), a very common pathogen causing infection in cultured fishes including tilapia. Activity of Neu3a endogenous ganglioside desialylation could play crucial roles in the step and degree of infection. Although direct evidence is not available, available results suggest that desialylation of gangliosides, especially GM3, by Neu3a could potentially help inhibit bacterial infections such as vibrio in tilapia. Therefore, Neu3a could be one of the key enzymes regulating surface gangliosides as such, tilapia Neu3a exploration could be indispensable to the pathogenicity and elucidation of control mechanisms for various pathogens that posed a significant threat to aquaculture production of the species.

In conclusion, this part of the study has for the first time revealed sialidase diversity in

tilapia with a pair of *neu1*s, five *neu3* genes and a single *neu4*. Transcripts of all *neu1* genes (*neu1a* and *neu1b*), *neu4* and only three *neu3*s (*neu3a*, and *neu3d* and *neu3e*) were present in tilapia. The expressed polypeptides showed various properties *in vitro* including optimal activity pH and substrate specificities, which confirms that these enzymes possess important sialo-hydrolytic properties towards glycoconjugates, such as glycoproteins and gangliosides. Therefore tilapia sialidase could be pivotal in tilapia endogenous glycoconjugate modulation, thereby, influencing a wide range of physiological functions similar to those suggested in humans, mammals and medaka. In general, tilapia sialidase exploration is an important step towards a more comprehensive picture of sialidase family of proteins in fish.



## CHAPTER 3:

### **Functional sialidase roles in modulating cell surface glycoconjugates: Roles in bacterial infection using sialidase NanA (rNanA) as a model in *Edwardsiella tarda* infection**

#### **3.1. Abstract**

Sialidases are known to regulate various cellular processes in nature, including infection where they regulate attack by pathogens through their action on cellular glycoconjugates. Infection cases, especially bacterial infections, are very common in fish. Sialidase roles in fish infection is still not well understood because of the limited sialidase studies in fish. In the first part of the studies here-in, sialidase from tilapia have been determined and evaluated. To understand the roles of revealed tilapia sialidases in infection, *Edwardsiella tarda*, one of the major pathogenic bacteria affecting both marine and freshwater fish species was employed. Tilapia sialidases were transiently expressed in GAKS cells, subsequently infected with *E. tarda* and its infection rate was determined in the cells. *E. tarda* infection rate showed an increase with tilapia sialidase Neu1a and Neu4 overexpression in Goldfish scale fibroblasts (GAKS) cells, suggesting that tilapia sialidases exhibit desialylation ability towards cells surface glycoconjugates. On the other hand, Neu3a overexpression showed attenuated *E. tarda* infection and GM3 up-regulated it suggesting that cell surface GM3 could be involved in regulation of *E. tarda* infection through action of Neu3 of the host cells. The bacterium also possessed endogenous sialidase NanA recently reported to promote its infection with an unknown mechanism. Therefore, to clarify the mechanism with which sialidase NanA aids *E. tarda* infection and provide

insights on the possible mechanism of tilapia sialidase roles in infection, comprehensive biochemical properties of NanA was carried out *in vitro* towards various substrates to establish the potential NanA target molecule at the host cell. GAKS cell pretreated with recombinant NanA showed up-regulation of *E. tarda* infection. Moreover, sialidase inhibitor- treated *E. tarda* showed a significantly reduced ability to infect GAKS cells. These results indicated that NanA-induced desialylation of cell surface glycoconjugates is essential for the initial step of *E. tarda* infection. Among the natural substrates, NanA exhibited highest activity towards 3-sialyllactose, a  $\alpha$ 2-3-linked sialic acid carrying sialoglycoconjugates. Supporting this finding, intact GAKS cell membrane exposed to recombinant NanA showed alteration of glycoconjugates only in  $\alpha$ 2-3 sialo-linked glycoprotein, but not in glycolipids and  $\alpha$ 2-6 sialo-linked glycoproteins. Lectin staining of cell surface glycoprotein provided further evidence that  $\alpha$ 2-3 sialo-linkage of the N-linked glycoproteins was target of NanA sialidase. To confirm the significance of  $\alpha$ 2-3 sialo-linkage desialylation for *E. tarda* infection, HeLa cell which possessed lower amount of  $\alpha$ 2-3 sialo-linkage glycoprotein were used for infection experiment along with GAKS cells. As a result, *E. tarda* infection was significantly low in HeLa cells compared to GAKS cells. Furthermore, *E. tarda* infection was significantly inhibited by mannose pretreatment suggesting that the bacterium potentially recognize and bind to mannose or mannose containing chain following desialylation. Together, these results suggest that *E. tarda* may employ endogenous NanA to desialylate  $\alpha$ 2-3 glycoproteins revealing the penultimate molecules which it potentially recognizes and binds to during infection. Tilapia sialidase

overexpressing cells also showed changed in  $\alpha$ 2-3 glycoproteins after lectin analysis, suggesting that tilapia sialidases, Neu1a and Neu4 could employ similar pathway to up-regulate *E. tarda* infection.

### 3.2. Introduction

Vetebrate sialidases are known to regulate various physiological roles in nature. Mammalian studies have proposed that sialidases are involved in the initial phase of the infections process and during intracellular multiplication (Seto et al.,1967). In fish, the role of sialidase in infection is not well understood due to limited sialidase studies, yet infection pose great threat to both marine and freshwater species. Bacteria are among the most critical infection agent in fish. It has previously been reported that attachment of bacteria to the epithelial cells of host animals is the initial step in the infection. The attachment process facilitates bacterial colonization and penetration of the cells (Rotner et al., 2005). In the initial part of this study, presence of tilapia sialidase has been reported and to reveal their roles in bacteria infection, *Edwardsiella tarda* was used as a model. *E. tarda*, a Gram-negative bacterium belonging to the family Enterobacteriaceae, is one of the most well characterized species in the family. The bacterium causes Edwardsiellosis, a disease reported in both freshwater and marine fish species (Mohanty and Sahoo, 2007; Xu and Zhang, 2014). Cases of *E. tarda* have been reported in tilapia (*Oreochromis niloticus*) (Muratori et al., 2000; Park and Jeong, 1996), channel catfish (*Ictalurus punctatus*) (Meyer and Bullock, 1973; Thune et al., 1993), eels (*Anguilla japonica*) (Matsuyama et al.,2005), seabass (*Lates calcarifer*) (Nadirah, et al., 2012), Japanese flounder (*Paralichthys olivaceus*) (Thune et al., 1993) and turbot (*Scophthalmus maximus*) (Xiao et al., 2008).

A number of virulence factors have been described to-date in various *E. tarda* pathogenic strains including flagellar genes (Xu et al., 2014), lysozyme inhibitor (Wang et al., 2013),

type III secretion system (T3SS) (Li et al., 2011) and NanA sialidase (Jin et al., 2012). Sialidases cleave N-acetylneuraminic acid from carbohydrate chains of glycolipids, glycoproteins and other glycoconjugates (Shiozaki et al., 2013). Sialidase are widely distributed in nature, from mammals to micro-organisms including bacteria. In bacteria, endogenously expressed sialidases have been shown to enhance their virulence advantages by promoting biofilm formation (Soong et al., 2006), inducing chemokine release from epithelial cells (Kuroiwa et al., 2009) and promoting bacterial adhesion to sialic acid and/or glycoconjugate epitopes present at the plasma membrane of host cells (Corfield, 1992; Ciavaglia et al., 1993). In general, sialic acids are present at the non-reducing end of sugar chain, where they cap glycoconjugates at the cell surface and the modulation of their content has been implicated in different bacterial binding abilities during infection (Beachey, 1981). Furthermore, bacterial binding affinity to the host cells is also dependent on the structure of glycoconjugates (Fantini et al., 2000), which in-turn determines the content and ease with which sialidases liberate sialic acid. For instance, different bacterial sialidase have been reported to show varying sialic acid cleavage rate depending on linkage pattern (Corfield et al., 1983; King et al., 2006). Therefore, glycoconjugates desialylation by sialidases seemed plausible as one of the critical factors in bacterial infection. Direct evidence of the involvement of glycoconjugates and desialylation during pathogenic bacterial infection common to fish is limited. Available results suggest that some pathogenic bacteria in fish target and bind to glycoconjugates during infection. For instance, *Vibrio* has been shown to adhere to gangliosides GM3 and GM4 in intestinal tract of fish (Chisada et al., 2013; Matsunaga et al., 2011).

*E. tarda* has received significant attention recently and a great deal of vaccines candidates has been developed. Unfortunately, the bacterium still poses significant threat to fish production due to the ineffectiveness of these vaccines and of other remedial mechanisms. Recent research advances have shown that an endogenous *E. tarda* sialidase NanA is involved in its ability to infect and colonize fish tissues after a mutant of NanA showed a reduction in colonization and infectivity in Japanese flounder (Jin et al., 2012), although the actual mechanism still remains elusive. Understanding the mechanism NanA promotes of *E. tarda* infection could help elucidate the involvement of fish sialidase in infection. It is possible that the sialidase enzymes, regardless of the sources could utilize similar pathways in regulating bacteria infection in fish. According to previous studies in other types of bacteria (Honma et al., 2011; Soong et al., 2006), it was hypothesized that *E. tarda* NanA is required to liberate sialic acid thereby exposing the binding epitopes on the host cell surface, crucial for *E. tarda* recognition and adhesion. More recently varying sialidase activities and biochemical properties have been observed in bacterial sialidases according to sialic acid linkage type in glycoconjugates residues (Li et al., 2014). Indeed, different cell types possess different sugar linkage types depending on their glycoconjugates compositions. In *E. tarda* data ascertaining glycoconjugate family targeted by sialidase NanA has been lacking. Furthermore, the molecular basis of sialidase NanA involvement in pathogenicity has not been characterized in details. Therefore, it was crucial to further explore sialidase NanA properties, nature of preferred glycoconjugates substrates and linkage patterns. This would be instrumental in providing the possible mechanism of fish sialidase involvement of in regulating bacteria infection. To understand the role of tilapia sialidase in infection and

its mechanisms, tilapia sialidase transfected cells were infected with *E. tarda* and compared with the mock and to understand the mechanism of their involvement, endogenous *E. tarda* NanA sialidase was used. The recombinant NanA was prepared and its properties analyzed towards various glycoconjugates substrates. Furthermore, the recombinant NanA treated fish cell lines were assessed for infectionability by *E. tarda*.

### **3.3. Materials and Methods**

#### **3.3.1. Materials**

##### **3.3.1.1. Growth medium**

Liquid and solid media were used for bacterial culture. For liquid medium, bacteria were aerobically grown in Lysogeny broth (LB) containing 10 g Bacto-tryptone, 5 g yeast extract and 5 g NaCl dissolved in 1 L water and autoclaved at 121°C for 30 minutes. Two types of solid media culture plates were prepared, LB agar plates and Tryptone Soy Agar (TSA) (Nissui). For LB agar plates, the liquid composition of LB medium was supplemented with 13 g agar and autoclaved. The sterile molten agar was left to cool to 50°C prior to addition of 50 µg/mL ampicillin. Approximately 20 ml of molten agar supplemented with ampicillin was poured into each Petri dish and allowed to solidify. The plates were dried for 20 minutes and stored at 4°C until use for at least four weeks. For TSA agar plates, 40 g of the TSA premix was dissolved in 1 L water and autoclaved. The agar plates were prepared same way as LB agar plates, without supplementation of any antibiotics.

#### **3.3.1.2. Antibiotics**

Ampicillin was added from freshly made stock solution of 100 mg/ml. The ampicillin was used to supplement liquid or solid media at a final concentration of 100 µg/ml. Gentamycin, streptomycin and penicillin were used each time from 10,000 µg/ml for gentamycin and 10,000 U/ml for penicillin and streptomycin.

#### **3.3.1.3. Inducer**

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce protein expression from bacteria. IPTG was added from a freshly made 0.1 M stock solution that had been filter sterilised. The final concentration of IPTG used was varied according to the experiment.

#### **3.3.2. Buffer and solutions**

##### **3.3.2.1. Buffer and solutions for SDS-PAGE**

For the preparation of both stacking and resolving gels (contents summarized in Table 2-8), 30% (w/v) acrylamide and 0.8% (w/v) N,N' Methylenebisacrylamide (BIS) was used. The resolving gel buffer was 1.875 M Tris-HCl (pH 8.8) and the stacking gel buffer was 1.25 M Tris-HCl (pH 6.8). Both membranes were polymerized with 10% ammonium persulfate (APS) and a catalyst acting as an oxygen scavenger (-N,N,N',N'-tetramethylethylene diamine (TEMED)). The gels were also incorporated with 10% SDS (sodium dodecyl sulfate). Sample buffer was prepared as 3x stock solution contained 500 µl 1.25M Tris-HCl (pH 6.8), 500 µl β-mercaptoethanol 2 mL 10% SDS, 1 mL glycerol and 10 µl of 1% bromophenol blue and water was added to a final volume of 5 mL.



#### **3.3.2.2. Buffers and solution for gel staining**

Coomassie blue stain solution contained 0.2% (w/v) Coomassie brilliant blue in 50% (v/v) methanol and 10% (v/v) acetic acid in distilled water. This was filtered before use to remove any undissolved solid.

#### **3.3.3. Solutions for western blotting**

Tris –Glycine-SDS (TGS) was prepared as a 1 × stock solution, which contained 30 g Tris, 144 g glycine and 10 g SDS in 10 L of water. For blotting, a blotting buffer containing 17.46 g Tris base, 8.79 g glycine, 600 mL methanol and 11.25 mL of 10% SDS was used.

PBST (wash buffer) was composed of 1 × PBS, 0.1% Tween-20. Blocking buffer contained 1 × PBS, 0.1 % Tween-20 with 1% (w/v) bovine Serum albumin (BSA).

#### **3.3.4. Bacterial strains and plasmids**

The FPC498 strain of *Edwardsiella tarda* was used in this study for both sialidase gene preparation and all infection experiments. The BL21 strain (Nippon Gene) of *E. coli* was the main strain used in the expression experiments on recombinant NanA sialidase. Bacteria were stored on nutrient agar plates at 4°C. For long-term storage, bacteria were frozen at -80°C either in 15% glycerol-LB or as competent cells in a 15% glycerol 0.1 M CaCl<sub>2</sub> solution.

#### **3.3.5. Bacteria strain and culture conditions**

The *E. tarda* used in this study was type FPC498 strain, isolated from the ascites of Japanese flounder (*Paralichthys olivaceus*) in Nagasaki, Japan (Yamasaki et al., 2013). The

bacteria strain was anaerobically grown on Tryptic Soy Agar (TSA, Nissui Pharmaceutical Co., Japan) and maintained at 28°C.

### **3.3.6. Cell culture**

Goldfish scale fibroblast (GAKS) cells and Human cervical carcinoma (HeLa) cells were obtained from RIKEN CELLBank (Japan). The cells were cultured in Dulbecco modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum at 37°C in a 5% CO<sub>2</sub> incubator.

### **3.3.7. Selection of suitable cell line for *E. tarda* infection studies**

To determine the suitable cell line model suitable for *E. tarda* infection *in vitro*, two cell lines, HeLa and GAKS, were tested. GAKS or HeLa cells were grown on Cellmatrix type-I C (Nitta Gelatin Inc, Japan) coated 12-well plate. A known weight of *E. tarda* used in infection was collected from a TSA plate grown for 48 hours. The *E. tarda* cell pellet was suspended with RPMI medium to a 1 mg/mL concentration. The suspension was serially diluted and used for infection. For infection, an appropriate volume of *E. tarda* suspension was added to an adequate volume of fresh RPMI medium and inoculated directly to the monolayers at MOI of 100 and the cells were incubated at 28°C for 2 hours. After 2-hour incubation (infection period), the monolayers were washed three times with sterilized PBS and re-incubated for 1 hour with medium containing 200 µg/ml gentamycin, 200 µg/ml of streptomycin and 200 U/ml penicillin to kill external bacteria. The monolayers were washed again for three times with PBS and subsequently cultured in fresh medium containing 100 µg/ml gentamycin and 100 µg/ml streptomycin and 100 U/ml penicillin for

2 hours (multiplication period). After three washes with PBS, the monolayers were lysed by 0.5% Triton X-100/PBS and to aid the lysis, the plates were shaken for 5 minutes. The lysate was serially diluted in PBS, plated on TSA and cultured for 48 hours at 28°C to allow bacterial growth and colony counting.

### **3.3.8. Effect of tilapia sialidase overexpression on *E. tarda* infection**

GAKS cells were transiently transfected with each of the main tilapia sialidase genes sub-cloned into pcDNA3.1 expression vector using PEI transfection as described in section 2.4.14. One day before transfection,  $4 \times 10^5$  GAKS cells were seeded onto 12-well plate in triplicates and cultured for 24 hours. After 48 hours of culture, the cells overexpressing Neu1a, Neu3a and Neu4 were infected with *E. tarda* at MOI of 100. The effect of each sialidase gene was determined in respect to promotion or suppression of *E. tarda* infection using CFU count. Furthermore, based on the initial study that Neu3a significantly desialylates GM3 *in vitro*, GM3 was overexpressed in GAKS by transient transfection with GM3 synthase (kindly provided by Dr. Taeko Miyagi). The GM3 overexpressing cells were also infected with *E. tarda* at MOI of 100 and the infection rate was compared with the mock.

### **3.3.9. Cloning of *E. tarda* sialidase *nanA***

Genomic DNA from *E. tarda* was extracted from the cell suspension by boiling the sample for 3 minutes, followed by centrifugation at 12,000 xg. The supernatant was used as template for PCR reaction. For amplification of *nanA* gene, PCR was carried out using

sequence specific primers (Table 3-1). The cloning primers were designed by incorporating endonuclease HindIII for the 5' end and BamHI for the 3' end, Kozak sequence and part of the ORF's 5' and 3' up to 30 nucleotides. These primers were designed according to *nanA* nucleotide sequence of TX01 strain (accession No: JX122859).

**Table 3-1.** *E. tarda* *nanA* cloning and GST-tagging primers

<i>nanA</i> Primer	Sequence
Cloning primers	5'-GTAAGCTTCCACCATGCTGATTTTGGCCGA-3' (Sense)
	5'-GTGGATCCCTAAAAGGTGTAGGTGAAGCTG-3' (Antisense)
GST-tag primers	5' – GTGGATCCCTGATTTTGGCCGAACACGATC -3' (Sense)
	5'-GTCTCGAGCTAAAAGGTGTAGGTGAAGCTG-3' (Antisense)
$\Delta$ autotransporter domain	5'-GTAAGCTTCCACCATGCTGATTTTGGCCGA-3' (Sense)
	5'-GTGGATCCTTAATACACAAATTCGGCATCT-3' (Antisense)

The ORF of *nanA* were amplified using KOD-Plus-Neo DNA polymerase (TOYOBO, Japan) using the 25  $\mu$ l reaction mixture (Table 2-2). The following PCR conditions were used; an initial denaturation of 94°C for 2 minutes followed by 30 cycles of two-step cycle denaturation at 94°C for 10 seconds, annealing and extension for 2 minutes at 68°C. The PCR product was electrophoresed in 1% agarose gel for band size checking and purification as described in section 2.4.2. The PCR products were digested with appropriate restriction enzymes, cloned into pBluescript SK (+) vector (TAKARA) and sequenced using ABI3130xl Genetic Analyzer (Applied Biosystems). The predicted sequence was analysed for conserved domains using

<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi> to confirm the previous reported presence of an autotransporter domain at the C-terminal region (Jin et al., 2012). To determine the effect of removal of autotransporter domain on the sialidase activity, a mutant was prepared using PCR and a C-terminal end primer designed based on the sialidase domain while incorporating stop codon. PCR conditions were as follows; an initial denaturation of 94°C for 2 minutes followed by 30 cycles of two-step cycle denaturation at 94°C for 10 seconds, annealing and extension for 1 minute at 68°C. The PCR was done using pBluescript sub-cloned NanA gene and the PCR product was confirmed using 1% agarose gel. The band was eventually extracted, purified, ligated into linearized pBluescript and sequenced.

### **3.3.10. Expression of recombinant NanA protein in mammalian cells**

After sequencing, the pBluescript-*nanA* and pBluescripts-  $\Delta$ autotransporter *nanA* were excised to collect the *nanA* ORF. For the expression of NanA in mammalian cell lines, both *nanA* (full-size and domain) were subcloned into the HindIII and BamHI sites of the expression vector pcDNA3.1 (+) (Invitrogen). Expression vectors were transiently transfected into HeLa cells using Polyethylenimine (PEI) method following the protocol outlined here-in. After 48-hour culture, the cells were harvested and cell homogenate containing overexpressed protein harvest. The homogenate was used to determine sialidase activity using 50  $\mu$ l reaction mixture as described in section 2.4.12. The optimal pH for mammalian expressed sialidase NanA was determined from pH 3.5-7.0 using sodium acetate buffer (pH 3.5 to 5.5) and sodium phosphate buffer (pH 5.5 to 7) using 4-

methylumbelliferyl-N-acetyl- $\alpha$ -D-neuraminic acid (4-MU-NANA) (Nacalai Tesque) (Worthington, NJ) as substrate. *E. tarda* NanA substrate specificity was assessed at the determined optimal pH towards various structurally different sialyloglycoconjugates; ganglioside mix (IsoSep, Sweden) for glycolipid preference, 3-sialyllactose and 6-sialyllactose for  $\alpha$ 2-3 and  $\alpha$ 2-6 linked sialic acids, bovine fetuin for N- and O-glycosylated protein with  $\alpha$  2-3 and 2-6 sialosyl linkages glycoproteins (Bovogen, Australia) and bovine submaxillary gland mucin for O-glycosylated protein with  $\alpha$  2-3 and 2-6 sialosyl linkages).

### **3.3.11. Preparation and expression of recombinant NanA polypeptide**

For the expression of recombinant NanA protein (rNanA), plasmid construct for the glutathione-S-transferase (GST)-fused NanA was prepared. DNA fragments coding for *nanA* were constructed by PCR using pBluescript subcloned transcripts as template. Primers were designed by incorporating the endonuclease sites BamHI at the 5' end and XhoI at the 3' end, and removing the start codon (ATG) of the *nanA* ORF at the 5' end. The templates were PCR amplified using KOD-Plus-Neo DNA polymerase (TOYOBO, Japan) under the following PCR conditions; an initial denaturation at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 68°C for 5 minutes. The PCR product was electrophoresed using 1% agarose gel and purified using Genopure PCR/Gel purification kit as describe previously. The PCR product was excised from the pBluescript vector and ligated into into BamHI/XhoI double digested pGEX-6P (GE Healthcare), a vector containing Glutathione-S-Transferase, a naturally occurring 26 kDa protein. The vector is designed in such a way to prevent protein

expression before induction with IPTG under the control of IPTG-inducible tac promoter. The pGEX vector produced recombinant proteins with an N-terminal GST tag that is normally included in the vector.

### **3.3.12. GST tagged *nanA* expression**

GST fusion proteins were expressed in *E. coli* BL21 (Nippon Gene, Japan). For expression, BL21 competent cells were transformed with pGEX-*nanA* plasmid by heat shock at 42°C and grown on plates containing 50 µg/ml ampicillin. Individual colonies were picked and cultured overnight in 4 mL of LB medium with 50 µg/ml ampicillin at 37°C for 16 hours. After 16 hours, 100 µl of the previous culture was transferred to fresh LB medium and cultured at 23°C until mid-log phase ( $OD_{600} = 0.55$ ), at which IPTG was added to a final concentration of 0.1 mM. After induction, the bacteria were cultured for an additional 3 hours after which the bacteria were harvested by centrifugation at 1,450 xg for 10 minutes and pellets were suspended in sonication buffer (1% Triton X-100/PBS). The suspension was sonicated for 30 seconds and centrifuged at 20,630 xg for 10 minutes at 4°C. To check the expression, intact bacterial cells, the supernatant (soluble fraction) and pellet (insoluble fraction) were resolved in 10% SDS-PAGE (Table 2-7). The resulting gel was fixed effectively by soaking for 30 minutes in 40% methanol, 50% water, and 10% acetic acid and subsequently incubated with CBB for 16 hours at room temperature. The gel was destained with several washes in water and visualized using naked eyes or under a light box to confirm the expression and solubilization of rNanA.

### **3.3.13. Purification of GST-NanA from inclusion bodies**

After several attempts and conditions, solubilisation of rNanA could not be achieved and the expressed protein was subsequently purified from inclusion bodies. Various conditions were tested in an effort to solubilize GST-NanA including; temperature, IPTG concentration and post IPTG culture duration. To solubilize the rNanA protein from inclusion bodies, denature and refolding procedures were employed. The expression of foreign proteins at high level in *E. coli* often results in the formation of inclusion bodies composed of insoluble aggregates of the expressed protein. For Isolation of rNanA from inclusion bodies, the pGEX-*nanA* transformed BL21 were culture in LB medium overnight. The following morning, 100 µl of the bacterial suspension was transferred to the new flask containing 100 mL fresh medium. The cells were grown until OD<sub>600</sub> reached 0.55 where ther cells were induced with 0.5 mM IPTG and culture for another 2-3 hours. The bacterial cells, supposedly containing inclusion bodies, were harvested by centrifugation at 2,850 xg for 10 minutes. The pellet containing inclusion body was thoroughly re-suspended in buffer (10 mM KH<sub>2</sub>PO<sub>4</sub> and 1 mM ethylenediaminetetraacetic acid (EDTA) and adjusted to pH 7) and disrupted by sonication 60 times with a 1-second pulse. The disruption procedure was repeated three times followed by centrifugation at 13,200 xg for 20 min. The pellet containing inclusion bodies was washed four times with 4% Triton X-100 in lysis buffer. In addition, to eliminate genomic DNA, samples were treated with 20 U/mL DNase and incubated at 37°C for 30 minutes. The final washing was done by suspending the pellet in 5 ml of water and centrifuged at 20,630 xg for 2 minutes. The resultant pellet containing



purified inclusion bodies was weighed, denatured in urea buffer (8 M urea, 10 mM dithiothreitol and 50 mM Tris-HCl, pH 8.0) to a final concentration of 10 mg/ml by incubation at 37°C for 1 hour while shaking. The mixture was subsequently centrifuged at 20, 630 xg for 20 minutes and resulting supernatant was exhaustively dialyzed overnight at 4°C in against cold PBS, to remove the urea and facilitate refolding of the protein. The resulting dialysate was resolved on a 10% SDS-PAGE gel and visualized with CBB-staining. To confirm that the isolated protein was GST-NanA the protein was blotted onto a PVDF (GE Healthcare) membrane after electrophoresis. The membrane was subsequently blocked with 1% BSA/PBST and treated with monoclonal primary GST antibody for 1 hour at 37°C followed by washing with 10 minutes-shake in PBST for three times. As a final process, the membrane was incubated with horse radish peroxidase conjugated anti-rat IgG secondary antibody for another 1 hour at 37°C. Detection of immunocomplexes was done using Peroxidase Stain DAB Kit (Nacalai Tesque). The dialysate was used subsequently for sialidase activity assay and its protein concentration was determined by the Dye binding assay (Nacalai, Japan).

#### **3.3.14. NanA sialidase activity assay**

To confirm the refolding of rNanA, the dialysate was used for determination of sialidase activity as described previously (Shiozaki et al., 2013) with a slight modification using a 50 µl reactions mixture containing 50 mM acetate buffer (pH 5.5) and 4-MU-NANA as a substrate. The amount of free 4-methylumbelliferone was measured with an F-2000 spectrofluorometer (Hitachi) with excitation at 365 nm and emission at 448 nm. The

amount of 4-methylumbelliferone that was liberated from 4-MU-NANA during the 1 hour reaction was determined by comparison to a standard curve of increasing amounts of 4-methylumbelliferone. In this assay, 1 nmol of released 4-methylumbelliferone represented the release of 1 nmol of sialic acid, and a unit of sialidase activity was defined as the amount of enzyme that released 1 nmol of sialic acid per hour. Protein concentration was measured by the Bradford method using a protein assay kit as described above and the amount of activity measured in each sample was corrected based on protein concentration to represent activity per milligram of protein

The nature of the preferred glycoconjugates by rNanA sialidase were investigated at pH 5.5 toward structurally different glycoconjugates as follows; ganglioside mix (IsoSep, Sweden), 3-sialyllactose, 6-sialyllactose and MU-NANA, bovine fetuin (N-and O-glycosylated protein with  $\alpha$ 2-3 and  $\alpha$ 2-6 sialosyl linkages) (Bovogen, Australia), bovine submaxillary gland mucin (O-glycosylated protein with  $\alpha$ 2-3 and  $\alpha$ 2-6 sialosyl linkages) (Worthington, NJ). Sialidase activity assay was carried out using 10  $\mu$ l of rNanA in triplicates. The sialidase activity was further examined using pure gangliosides GM1, GM3 and GD1a as substrates. The reaction mixtures were incubated at 40°C for 1 hour. The amount of free sialic acid was determined using Thiobarbituric acid method as described previously in section 2.12.2 (Warren, 1959). One unit (U) of sialidase activity was defined as the desialylation of 1 nmol of sialic acid/h from each substrate. To confirm the endogenous expression of NanA sialidase, *E. tarda* FPC498 were inoculated on TSA plate and cultured

at 28°C for 48 hours. The resultant colonies were collected by scraping and prepared into lysate, and then its sialidase activity was estimated using 4MU-NANA as a substrate.

#### **3.3.15. Effect of rNanA exposure to *E. tarda* infection**

To determine the involvement of cell surface glycoconjugates in *E. tarda* infection, GAKS cells were incubated with rNanA to facilitate the desialylation of cell surface glycoconjugates. For the experiment, GAKS monolayers were exposed to 50 U/ml of rNanA and cultured at 37°C for 1 hour. The cells were washed three times with PBS to remove the rNanA and infected with *E. tarda* at MOI of 100 for 1 hour at 28°C. As outlined above, the cells were treated with 200 µg/ml gentamycin, 200 µg/ml of streptomycin and 200 U/ml penicillin to kill external bacteria for 1 hour at 28°C. The monolayers were washed again for three times with PBS and subsequently cultured in fresh medium containing half-concentration of the antibiotics for 2 hours. The cells were lysed by 0.5% Triton X-100/PBS followed by the serial dilution and plating of the lysates. Wild cells without rNanA exposure were used as control.

#### **3.3.16. Effect of sialidase inhibitor on *E. tarda* infection**

*E. tarda* were treated with 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA) a known sialidase inhibitor (Meindl et al., 1974) and used for infection. To inhibit endogenous NanA sialidase, *E. tarda* was cultured on TSA plate for 48 hours and collected by scraping and weighed. The bacteria were suspended by PBS containing 2mM DANA to a final concentration of  $10^9$  bacterial cells per mL and incubated at 37°C for 30 minutes

with mild shaking. The cells were washed 3 times in PBS using centrifugation at 4000 rpm and after the final wash, the bacteria were suspended with RPMI and used to infect wild GAKs cells at MOI of 100. The infection procedure was carried as outlined in section 2.10.1. *E. tarda* without DANA treatment was used as control. The RPMI suspended bacteria were also serially diluted to and plated to confirm the input CFU.

### **3.3.17. Lectin blotting**

To determine the cell surface glycoproteins alterations and sialo-linkage patterns preferred by NanA, GAKS cell treated with recombinant NanA were harvested and homogenized in 9-volumes of lectin buffer containing 50 mM Tris-HCl (pH 7.5), 10 mg/ml leupeptin and 0.5 mM phenylmethanesulfonyl fluoride in PBS using Handy Sonic UR-21P ultrasonic disruptor (TOMY, Japan). The sonicated mixture was centrifuged at 600 xg for 5 minutes. The resulting supernatant was resolved using 8% SDS-PAGE and blotted onto PVDF membrane. *E. tarda* adhesion to the host cell is thought to be through cell surface glycoconjugates. Recombinant NanA is thought to alter the cell surface glycoconjugates on the host cell surface before being internalized. For a more detailed lectin analysis, cell membrane was isolated by fractionation using a protocol described previously (Shiozaki et al., 2014) and treated with rNanA. For this protocol, homogenate from wild GAKS cells was prepared by Dounce homogenizer in lectin buffer described above. The initial homogenate was centrifuged at 600 xg for 5 minutes to remove nuclear and other unwanted material. The supernatant was centrifuged again at 20,000 xg for 15 minutes to collect the subsequent pellet fractions containing cell membrane components. The pellet was

suspended with equal volume of the above buffer containing 0.1% Triton X-100. The lysate containing membrane fraction was exposed to 50 U/ml of rNanA and incubated at 37°C for 1 hour. Since, rNanA used in this study was still bound to GST tag, the lysate was also exposed to 50 U/ml of rGST to determine if GST tagging had no effect on the results. The cell membrane lysate reacted with PBS was used as a control. The three reactions (lysate + rNanA, lysate + rGST and lysate + PBS) were resolved using 8% SDS-PAGE and the protein electrotransferred onto PVDF membrane followed by blocking using 1% BSA. The membranes were treated with the following lectins; *Maackia amurensis* lectin II (MALII) ( $\alpha$ 2-3 linked sialic acid specific lectin in O-linked glycoproteins) (Vector laboratories, CA), *Maackia amurens* lectin (MAM) ( $\alpha$ 2-3 linked sialic acid specific lectin in N-linked glycoproteins) and *Sambucus siebaldiana* agglutinin (SSA) ( $\alpha$ 2-6 linked sialic acid specific lectin in both N- and O-linked glycoproteins) (Honen, Japan). After washing, lectin-binding glycoproteins were visualized with VECTASTAIN ABC standard kit (Vector laboratories) and Chemiluminescence. The band intensities of the blots were analyzed using ImageJ software (National Institutes of Health). Lectin blotting and analysis was also done for wild GAKS and HeLa cells to determine the composition of  $\alpha$ 2-3 linked sialo-glycoprotein. GAKS and HeLa cells were grown seeded into 6 cm dishes and grown to about 70% confluence. The cells were harvested and homogenate prepared and protein resolved using SDS-PAGE as described above. The membrane containing the blots were treated with  $\alpha$ 2-3 linked specific lectins (MAM and MALII) and visualized as described herein. Furthermore, glycoprotein alterations were also determined using lectin analysis for the transiently transfected cells. HeLa cells were transiently transfected with each

tilapia sialidase gene (*neu1a*, *neu3a*, and *neu4*). The homogenate containing overspressed sialidase proteins were resolved onto 8% SDS-PAGE and blotted onto PVDF membrane. The mebrane were exposed to MAM and SSA lectins and visualized as outlined above.

### **3.3.18. Thin layer chromatography**

GAKS cell glycolipids were extracted as described previously (Ueno et al., 2006) with slight modifications. Briefly, cells were treated with 2 ml of isopropanol/hexane/water (55:25:20, v/v/v) and hydrolyzed with 0.1 M NaOH/methanol. The resulting glycolipid extract was desalted using Sep-Pak C18 cartridge. The samples were then applied to high-performance TLC plates (Baker, Phillipsburg, NJ) and glycolipids were separated by chromatography in a solvent system of chloroform/methanol/0.5% aqueous  $\text{CaCl}_2$  (60:40:9, v/v/v). The resulting resolved glycolipids were visualized with orcinol- $\text{H}_2\text{SO}_4$ .

### **3.3.19. Flourescence lectin staining for cell surface glycoprotein**

To confirm the results of lectin blotting, intact cells were stained with biotinylated lectins after *E. tarda* exposure for fluorecence. For this part of experiment, cells were grown on cover slip for 24 hours and exposed to 1 U/ml of rNanA for 1 hour at 37°C. The cells were then washed three times with PBS, fixed with 4% paraformaldehyde for 15 minutes and blocked with 1% BSA for 15 minutes. Intact GAKS cells without rNanA treatment were used as control. To eliminate any background signal from endogenous biotin, cells were also sequentially treated with avidin and biotin blocking kit (GBI labs, WA). Biotinaylated MAM and SSA were incubated with the cells at room temperature for 2 hours. The cells were then washed with PBS and treated with Streptavidin-Fluorescein Isothiocyanate

(FITC) Conjugate (Immunobioscience, WA) at room temperature for 15 minutes. Fluorescence was evaluated on the mounted cover slips by observation under Apotome sectioning fluorescence microscopy system (Carl-zeiss, Germany).

### **3.3.20. Sugar-mediated inhibition of *E. tarda* adherence to GAKS cells**

To determine the preferred monosaccharide for *E. tarda* binding after glycoprotein desialylation, bacterial binding inhibition assay was carried out as described previously (Ryan et al., 2001). Monosaccharides representing individual residues present in glycoproteins, were used to provide insights on the oligosaccharide residues that *E. tarda* may bind to during infection. Individual monosaccharides; Glucose, Fucose, Galactose, Mannose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) were prepared as 200 mM solutions in PBS. *E. tarda* suspensions containing  $1 \times 10^9$  CFU/ml were prepared in PBS. For monosaccharide pretreatment, 1ml of *E. tarda* suspensions were mixed with the individual sugar solutions to yield  $5 \times 10^8$  CFU/ml in 100 mM monosaccharide solutions and incubated at 37°C for 30 min. As a control, an *E. tarda* suspension was added to PBS without monosaccharide. After incubation, the bacteria were washed three times with PBS and resuspended with medium to the 1ml. The bacteria suspension was added to GAKS monolayers grown in 12-well plate at MOI of 100 and incubated for 1 h at 28°C. The infections and invasion assay was further carried-out as described above.

### 3.4. Data analysis

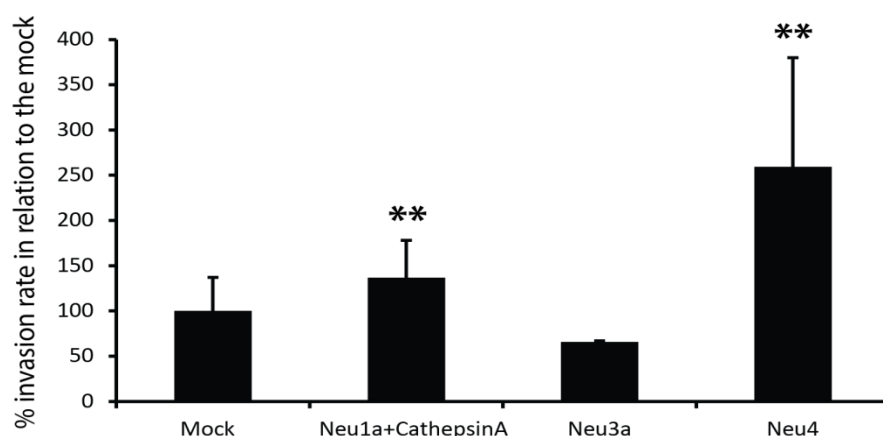
Results were expressed as mean  $\pm$  S.D. All values were compared using the Welch's t-test and Turkey's test.

### 3.5. Results

#### 3.5.1. Effect of tilapia sialidase overexpression on *E. tarda* infection

*E. tarda* infection was determined towards GAKS cells overexpressing tilapia sialidase Neu1a, Neu3a and Neu4. Due to the significance of Cathepsin A expression for sialidase Neu1a activity, *neu1a* gene was cotransfected with *ctsa*. When compared with the vector transfected cells (mock), Neu1a+Cathepsin A and Neu4 overexpressed cells showed increased *E. tarda* invasion rate than mock cells, while Neu3a overexpressing cells showed a significantly lower infection rate, approximately 65% to that of the mock cells (Figure 3-1). The effect of Neu1a+Cathepsin A was only slightly higher compared to the mock although not significantly different. A significantly enhanced *E. tarda* infection was only observed in Neu4 overexpressed cells compared to the mock. Neu4 transfection promoted *E. tarda* infection by about 1.5 times when compared to the mock (Figure 3-1). These results suggest that tilapia sialidase overexpression could modulate cell surface sialoglycoconjugates and eventually modulating the bacterial adhesion to the GAKS cells.



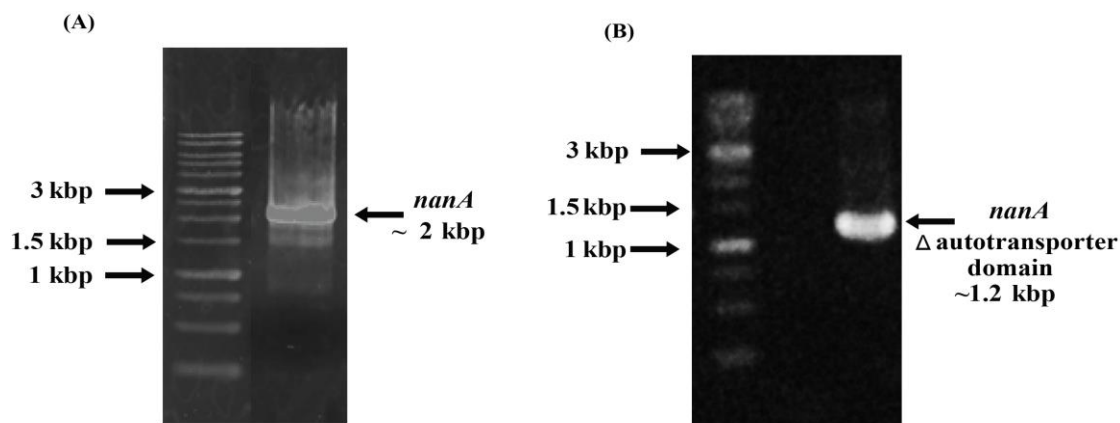


**Figure 3-1.** Infection of tilapia sialidase overexpressing GAKS cells.

GAKS cells were transiently transfected with *neu1a+ctsa*, *neu3a* and *neu4* sub-cloned into pcDNA3.1 expression vector. The cells were infected with *E. tarda* at MOI of 100, lysed and with 0.5% Triton X-100/PBS and plated for CFU count. The CFU was expressed as percentage with the mock CFU designated as 100%. \*\*,  $p < 0.01$  compared with mock cells.

### 3.5.2. Sequence analysis of *nana* sialidase gene from *E. tarda* FPC498 strain

Alteration of sialoglycoconjugates is known to play a role in bacterial infection. Bacteria are known to utilize their endogenous sialidase to modulate these sialoglycoconjugates. Therefore, in this study endogenous *E. tardas* sialidase NanA was used to determine its mechanism in modulating *E. tarda* infection. It was hypothesized that tilapia sialidases could potentially modulate *E. tarda* infection through a similar mechanism. Estimation of endogenous sialidase activities in *E. tarda* FPC498 yielded significant activity (5 U/mg protein) towards all MU-NANA, confirming the expression of NanA in *E. tarda*. After PCR amplification using primers designed from TX01 NanA sialidase gene, a single PCR band was observed around 2000 bp (Figure 3.2A), corresponding to the predicted size of NanA.



**Figure 3-2.** Electrophoresis of the *nanA* PCR product.

(A) Full length *nanA* PCR product resolved using a 1% agarose gel electrophoresis. (B) PCR product electrophoresis for the mutant *nanA* lacking autotransporter region ( $\Delta$  autotransporter domain). The autotransporter domain was removed by designing a primer targeting the C-terminal end of sialidase domain.

The PCR products were sub-cloned in the BamHI and HindIII site of pBluescript sk (+) and sequenced as described in section 2.4.5. Sialidase *nanA* gene from FPC498 strain was found to have an open reading frame (ORF) of 2013 bp same as TX01 strain (Jin et al., 2012). Assembled consensus sequences for *nanA* revealed the presence of a synonymous single nucleotide polymorphism at 594nt (A→G) compared *nanA* gene from TX01. The deduced amino acid sequence was 100% conserved with TX01 and comprised 670 amino acids. *E. tarda* FPC498 DNA and amino acid sequences were deposited in the DNA Databank of Japan under the accession numbers LC011908. Analysis of conserved domain showed the presence in FPC498 strain's NanA of conserved motifs (RIP and several Asp-Boxes) contained within the sialidase domain (residues 1–397) (Figure 3-3A, B). An autotransporter domain was found at the C-terminal region of NanA.

(A)

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1  ATGCTGATTTTTCGCCAACACGATCGAACGGCAAATAATGGCTGGCGTATTCCGGCGATTACGGTCACGCGAGGGTGGGGCGCTTATTGCCGTCAGCGATGCCCGGGCGGTCCGTACCAGC 120
M L I F A E H D R T A N N G W R I P A I T V T Q G G A L I A V S D A R A V G T S
121 GATATAGGTCAGAATCAGGATGTGCATTTGGCTATAGAATATCCTACGATCGGGGGGACACCTGGGGTGAGATCCAGCATATCGCCCCAGTTTAAATGGTAAGCGACAAATCTCCGAT 240
D I G Q N Q D V H F G Y R I S Y D R G D T W G E I H D I A P S F N G K R Q I S D
241 CCGGCCATCGTGTACAACCCAGACTCGGGGAGTACGTTTCTGTTCCGCTATTACAACGATCGCTTATCACCAGTAAACCGCTCAGCCGCGTTTCTGATTCTTTATGTTTACCTCTGAC 360
F A I V Y N P D S G S T F L F G Y Y N D R F I T S K P L S R V S D F F M F T S D
361 GATGGCGAAAAACATGGGATCGGGGCACCGTCTGTACGATCTGGCGCCGACGGGCTACAAATATATACTGCAGGGCCCGGGCTCCGGGATGTATCAGACGGCAGCGCTCTATCTCGCC 480
D G G K T W D R G T S L Y D L A P T G Y K Y I L Q G P G S G M Y H D G T L Y L A
481 GCTCAGGCGTGGCATCAGATAGGGACAGTACCTGGTGGGAAGCGGTGCGACGGTAACCTCAGGATATCTCTACTCATCGGATAATGGCGTAACGTGGAATTCAGCCTGGCTACGCCCC 600
A Q A W H H D R D S S L V G S G A T V T S G Y L Y S S D N G V T W N S A W L R P
601 GATGATAAAATGTGGTGGCCCGGGGACGGAGTGAGCATCTCCCGACATCACCTCCGAGAGCAGCGTATTCCACCAGATGGTCATATCTATTTGGCCGCAAGTCCGAGACATCG 720
D D K I V G A P G T G V D D L P D I T S E S S V F H H D G H I Y L A A K S E T S
721 CGGGAGAGCAAGTCGCGGGTCTGTATCGGACAAAGGACAACGGCCAGCGCTGGGAGCGGGTAGAGGAAGCATTCTGCCGAGAAATATTTCCCGCGCGAGACGAGCTCGCTCTCTCTG 840
R E S K S R V V Y R T K D N G Q R W E R V E E A F L P E N I S R A E T S S L S L
841 GATGACAGCGTTTATCTGGTTGGCTACAGCACGGCGACGGAGCGGGCTAACCGTGTGGTATCTATATTACCACCAATACCGGGCGCACCATCCAGGTGTATGACTCACCTCCTATGGC 960
D D S V Y L V G Y S T A T E R A N R D G I Y I T T N T G R T I Q V Y D S P S Y G
961 TATACCTCAATGACAAAGGATGAGGATAACCTCTATATCTGTTGAAGGCGAAGGGGATATCTATCTTCAGCGGTATGACATCGCCGCGCGAGACTATGCCAATATCAGCGCCAGCATC 1080
Y T S M T K D E D N L Y I L F E G E G D I Y L Q R Y D I A A R D Y A N I S A T I
1081 TTAATCGCAGCAACGATTTATTCGATATCGCCACGAGCTCAGGACGGCGTCTTATATTAAGGGGGGCTATGGTAGCCATCGCTCGTCAGATGCCGAATTTGTGTATCCCGACGAC 1200
L N R S N D L F D I A T K L R T A S S Y I K G G Y G S H A S S D A E F V Y A D D
1201 TGGGGGAAGCTGGGGGTGTTTTTCGCGAGGCAATCCGAGAAATAGTCAGCGTGTGCCCGGGACTATCCAATATACCAATAGCGATGIGTCGCTGACCTTGGCCAAAGATGATATTTATTC 1320
W G K L G V F F A R Q S E N S Q R V P G T I Q Y T N S D V S L T L A K D D I L F
1321 TCTGGCGATAATGTCTTTGTCGGCTATCAGCATAGTGATATCAATACGCAATGACGCTGCTAATAAAGTTAAGTCGGTGTGGCTGGCTATTCATATCAACATGACTTTGGCGTCTTA 1440
S G D N V F V G Y Q H S D I Q Y D N D A A N K V K S V L A G Y S Y Q H D F G V L
1441 TCCTATCACCTGATGGTCAATACGATTTATGGTGAAAACAGATTCTCAGCAATCGTCGAGAGGGGGTTAGGGCGTAAGGCCGATTTTACGCCTATAGCGTGGCTATAAAAAATATGCTG 1560
S Y H L M V N T I Y G E N R F S R N R E G L G R K A D F N A Y S V A I K N M L
1561 GCCAAAGACATTTACCTTGCCGATGGTTTTACGCCGGCGTCGCTCGGGGCTTCACTCCAGCTTTTTTCAGTCATAGCGCGATTACAGGAGAGGGGAGGCAACGGTTGGAATAATGCGAGC 1680
A K D I Y L A D G F Y A G V A S G L H S T F F S H S A I Q E R G G N G W N N A S
1681 GTGGACTCCGCGCATCAGCCTCGAACCAGCTTTTGGCTCGATGGACAGCTCAGCAAGCGGGTTCATATCATGCAATCGAAGAGCTGACGCTGACGGCCAAAGCCAGCTACAGTATGAG 1800
V D S A D H A S N Q L L L D G Q L S K R L H I M Q S N E L T L T A K A S Y Q Y E
1801 CTGATGGACACGGCAAGTGGGCGGAGGCGTACCGAGTGCCTCGATGCCCGGCGTCTGATGCCAGCGCGGTAAAGCGCTATCCGCATGGCTTGGCAACTGGCTATCTGGAGGCGGCGCTG 1920
L M D T A K W A E A Y R V L D A R R L M P A P V K R Y P H G L A T G Y L E A A L
1921 AGCCTTCATCCGACGGCCGTTATCGCCGCGGGGCGATCGCTCAATACCGATGGTACGGGCTGGTTCGGCGCAGCTTACCTACACCTTTTAG 2013
S L H P Q A V I A G A S L N T D G D G L V A G S F T Y T F *

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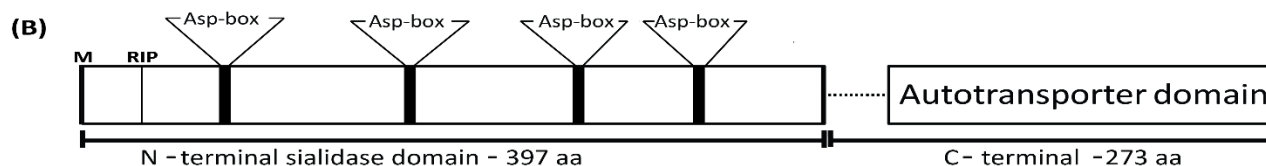
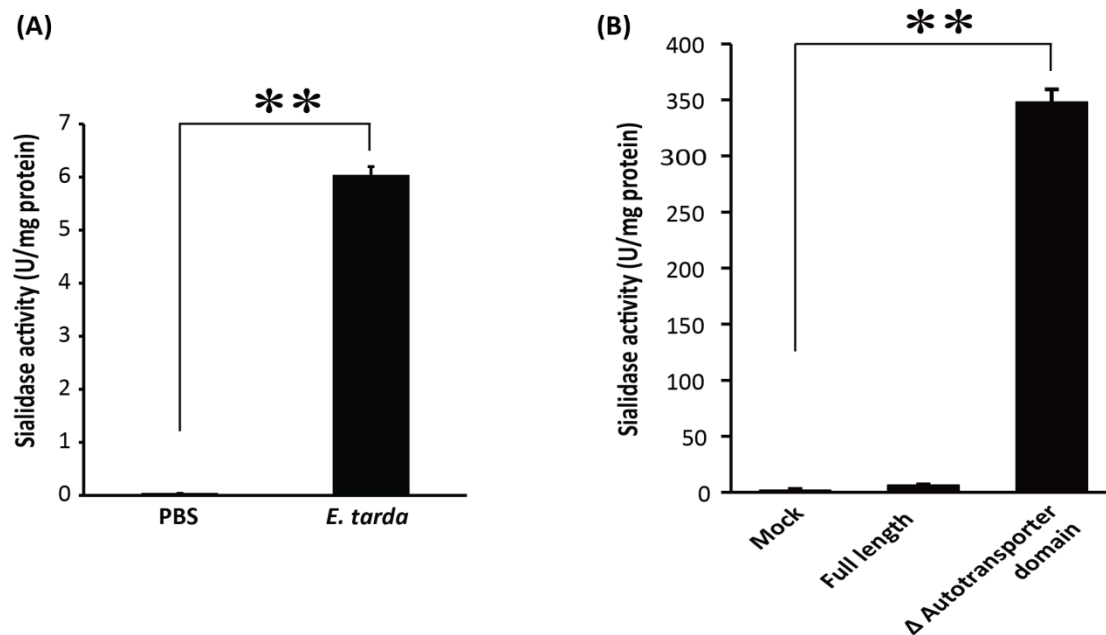


Figure 3-3. NanA nucleotide and deduced amino acid sequences.

(A) The consensus sequence were deduced using Genetyx software version 1.0. The conserved typical sialidase motifs Asp-boxes are underscored with a solid line and RIP motif is boxed. The start codon is shown in bold and the stop codon is shown by an asterisk. The end of sialidase domain predicted by SignalP 4.1 server is represented by a solid vertical line. (B) The diagrammatic sketch of sialidase NanA showing the N and termini structure. The conserved motifs are shown by solid vertical lines with their corresponding names. M represent the start amino acid.

### **3.5.3. Endogenous NanA and mammalian cell NanA expressed sialidase activity**

To confirm the endogenous expression of *E. tarda* NanA sialidase, FPC498 strain were inoculated on TSA plate and cultured at 28°C for 48 hours. The resultant colonies were collected by scraping and prepared into lysate. The lysate was used for sialidase activity assay. Estimation of endogenous sialidase activities in *E. tarda* FPC498 yielded activity of 5 U/mg protein towards all 4MU-NANA (Figure 3-4A), showing that *nanA* gene encodes an active sialidase NanA in the strain. Furthermore, sialidase activity estimation for the NanA expressed yielded higher sialidase activity compared to mock (Figure 3-4B), although the sialidase activity for the full-length NanA was not significantly different from mock. To determine if this low sialidase activity was due to the autotransporter domain on sialidase activity, a mutant NanA polypeptide lacking autotransporter domain was prepared and expressed in mammalian cells and its sialidase activity was estimated and compared to that of full-length rNanA and mock. Mutant NanA showed significantly higher sialidase activity (348 U/mg protein) when compared with the mock (2 U/mg protein), while full length NanA polypeptide showed slight sialidase activity (Figure 3-4B), suggesting that the removal of the autotransporter domain is necessary for the sialidase activity.



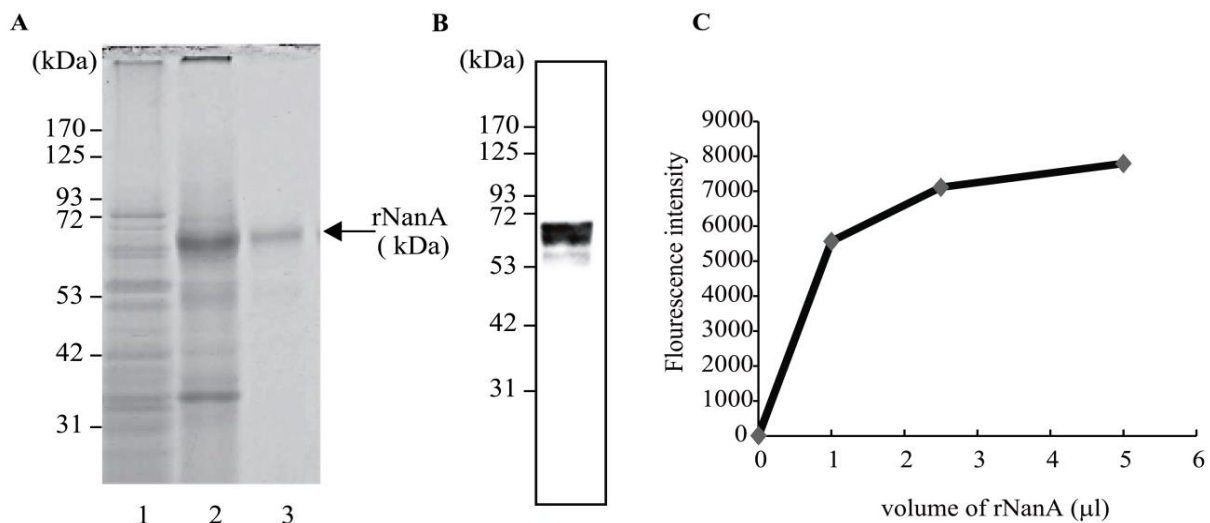
**Figure 3-4.** Endogenous and mammalian-cell-expressed NanA sialidase activity.

(A) *E. tarda* was collected from TSA plate and lysed by sonication and used for estimation of sialidase activity towards MU-NANA at pH 5.5. (B) Full length NanA and its mutant lacking autotransporter domain ( $\Delta$  autotransporter domain) were introduced into HeLa cells. Cell homogenates were prepared and used for sialidase activity assay toward MU-NANA at pH 5.5. \*\*,  $p < 0.01$  compared with mock cells (empty vector). The results are shown as means  $\pm$  standard deviation from three independent experiments.

#### 3.5.4. Expression of recombinant NanA sialidase.

To assess the significance of NanA in *E. tarda* infection, rNanA was expressed as fusion protein with GST by subcloning of *nanA* gene into pGEX-6P plasmid. The pGEX-*nanA* was then introduced into *E. coli* BL21, and recombinant polypeptide expression was induced by IPTG treatment. The lysed *E. coli* were centrifuged, and then separated into soluble and insoluble fractions. Each fraction was resolved using SDS-PAGE. Bioinformatic analysis of NanA using NCBI Conserved Domain database

(<http://www.ncbi.nlm.nih.gov/cdd>) showed the presence of highly conserved sialidase domain at the N-terminal (residues 1-345) and autotransporter domain at the C-terminal (residues 429-668) (Figure 3-3A) similar to previous report (Jin et al., 2012), which could be cleaved during processing resulting into a relatively shorter mature NanA. According to this analysis, premature NanA was predicted as a 74 kDa protein that could be processed to form about 39 kDa mature protein containing sialidase domain. In this study, the mature GST-fused protein yielded about 65 kDa, consistent with predicted size ( $\approx$  39kDa for mature NanA and 26 kDa from the GST tag). The rNanA expression was only observed in the insoluble fraction yielding band consistent with the predicted molecular sizes of GST-fused mature NanA (Figure 3-5A), indicating the presence of rNanA in inclusion body. Therefore, rNanA protein was purified from insoluble fraction under denaturing conditions and renatured by dialysis against decreased concentrations of urea. The purified rNanA was also resolved using SDS-PAGE to confirm purification. Only a single major band corresponding to rNanA was observed for purified protein (Figure 3-5A, lane 3). Moreover, immunoblotting analysis showed rNanA band as predicted size. Furthermore, the purified rNanA showed high sialidase activity, which was dose dependent (Figure 3-5B-C). These results indicated that purification of rNanA from inclusion body was successful.



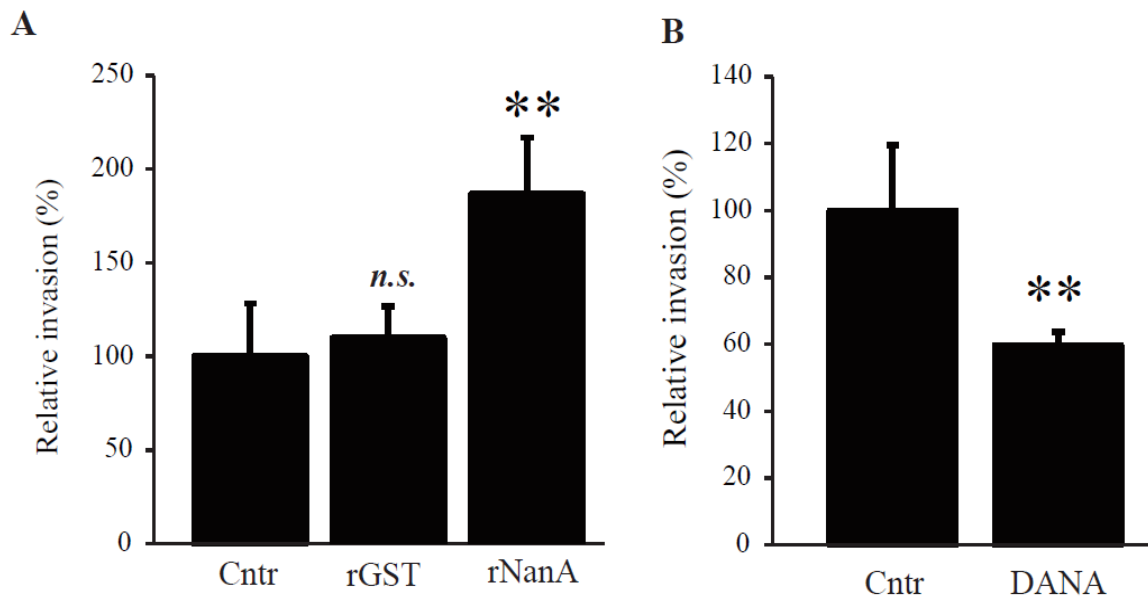
**Figure 3-5.** Recombinant NanA properties.

(A) SDS-PAGE analysis of supernatant (soluble fraction, lane 1) and precipitate (insoluble fraction, lane 2) were obtained after centrifuging the BL21 transformed with *nanA* gene at 17,000 xg for 15 min, while purified rNanA (lane 3) was obtained from inclusion bodies. The protein was separated using 8% SDS -PAGE and visualized using coomassie staining. (B) The purified rNanA protein was also resolved using SDS-PAGE, blotted onto PVDF membrane and treated with anti-GST antibody. (C) For the determination of sialidase activity of purified rNanA, activity was assayed towards MU-NANA at pH 5.5 using various enzyme volumes.

### 3.5.5. Exposure of GAKS cells to rNanA increases *E. tarda* invasion and infection

Sialidase are known to impart pathogenic advantages to bacteria in various ways. It was hypothesized herein that *E. tarda* sialidase NanA might be involved in unmasking the sialic acid bound glycoconjugates on host cells, exposing penultimate molecules, which are epitopes for bacteria interactions. GAKS cells were pre-treated with 50 U/mL of rNanA before *E. tarda* exposure to examine the role of NanA in the interaction with fish host cells.

Our results showed that rNanA pre-treatment significantly enhanced the bacterial infection compared with control (no NanA-treated GAKS cells) and rGST (Figure 3-6A). This result indicated that cell surface glycoconjugates desialylation enhances the *E. tarda* interaction in GAKS cells, consequently, enhancing their ability to invade the cells.



**Figure 3-6.** *E. tarda* Infection and invasion rates to GAKS monolayers.

(A-B) *E. tarda* invasion to GAKS cells were evaluated after cell exposure to rNanA and pretreatment of the *E. tarda* with DANA. (A) GAKS monolayers were preincubated with rNanA, rGST and medium only as control for 1 hour. The monolayers were infected with *E. tarda* at MOI 100, lysed and plated on TSA plates to determine the CFU. (B) *E. tarda* was treated with DANA for 1 hour to inhibit endogenous sialidase and used for infection of GAKS monolayers. The cells were lysed and the lysate was plated on TSA plates cultured for 48 hours followed by CFU counting. Invasion rates were expressed as percentage of observed CFU relative to the total input bacteria. \*\*,  $p < 0.01$  compared with control. The results are shown as means  $\pm$  standard deviation from three independent experiments.

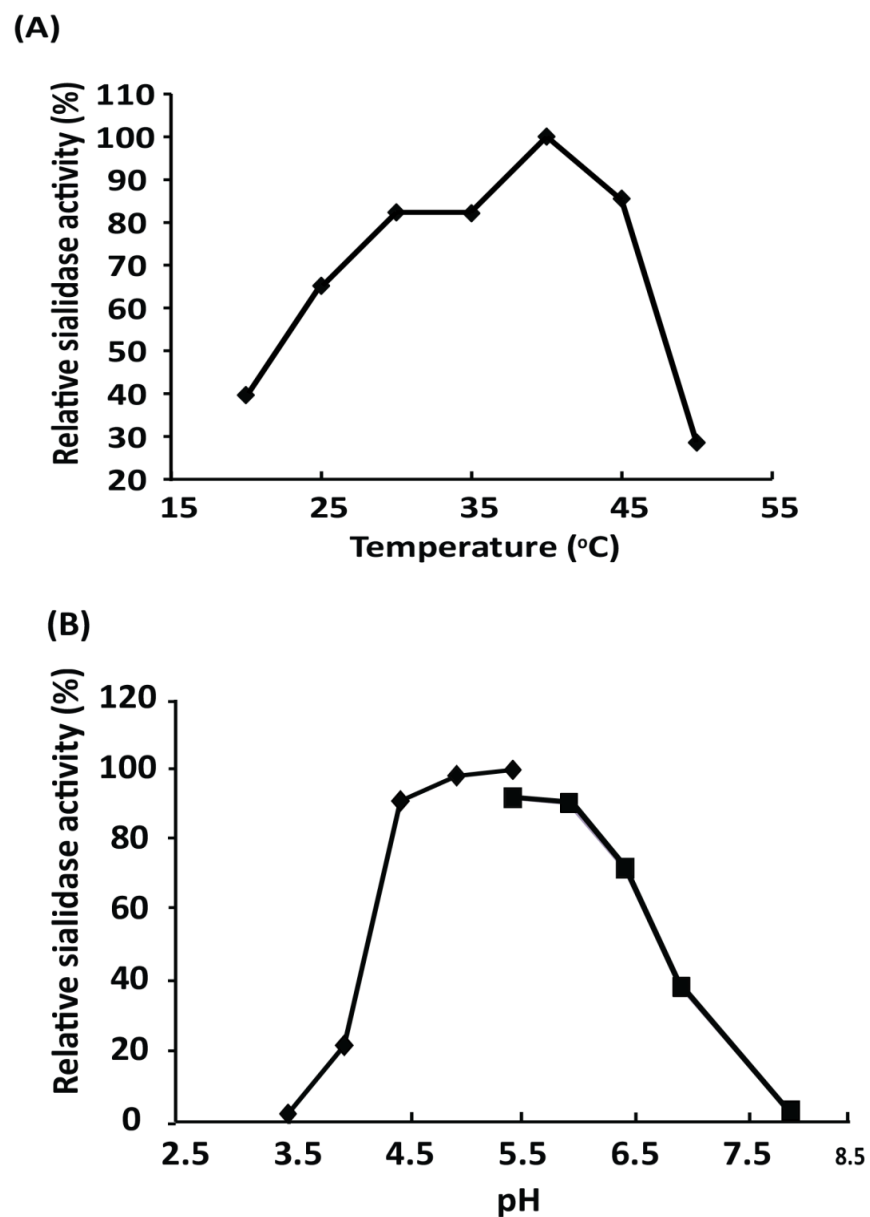
Furthermore, DANA inhibition of endogenous NanA was used to confirm the role of NanA as observed herein. During infection and invasion periods (2 hours), cell death was not



observed in both experiments. The results demonstrated that pretreating the bacteria with sialidase inhibitor significantly reduced their ability to invade GAKS cell (Figure 3-6B). Taken together, these results suggest that NanA desialylation of cell surface glycoconjugates is essential in initial step of *E. tarda*'s infection to fish cells *in vitro*.

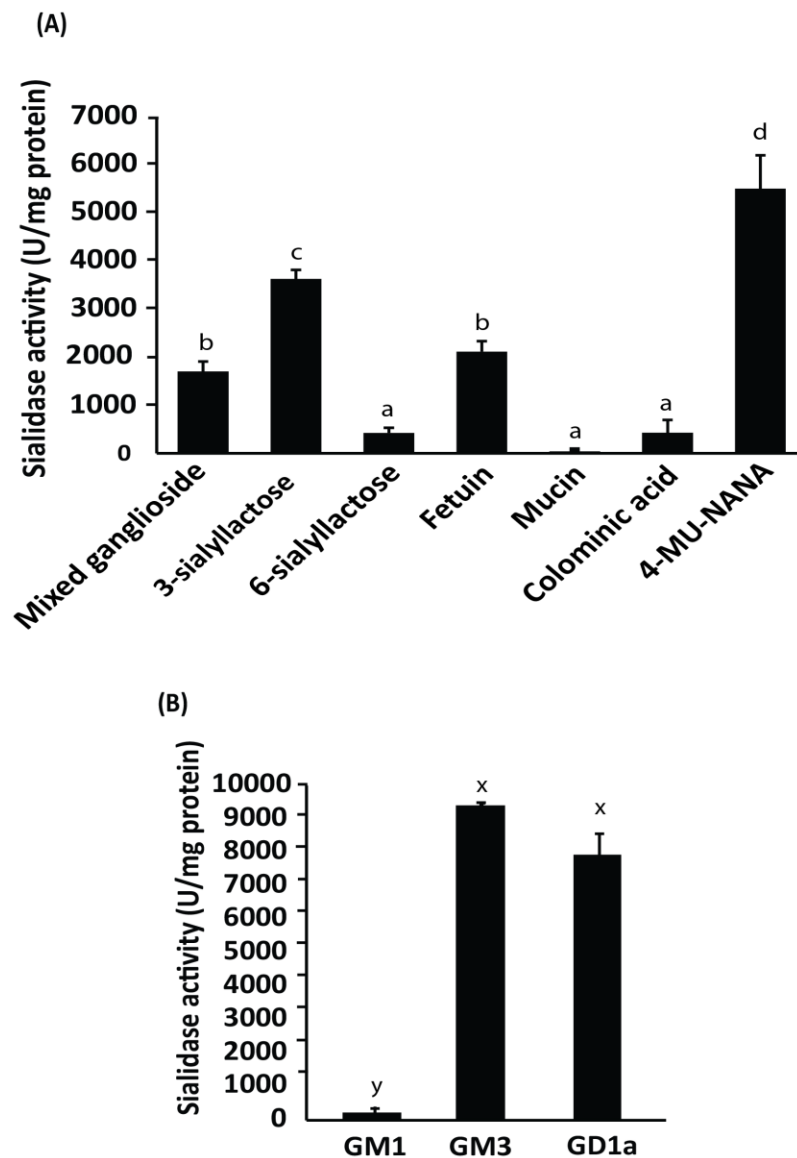
### **3.5.6. Enzymatic properties of NanA**

To clarify the mechanism of desialylation by NanA and give more insights on its roles during *E. tarda* infection, NanA sialidase activity was determined towards various commonly used sialoglycoconjugates in substrate specificity assays. Determination of sialidase activity for rNanA, yielded sialidase activity optimally at pH 5.5 and at temperature of 40°C (Figure 3-7A, B), similar to what has been reported previously (Jin et al., 2012). Sialidase activity assay towards various substrates showed that the rNanA exhibited highest activity towards MU-NANA and 3-siallyllactose followed by bovine fetuin and gangliosides (Figure 3-8A). On the other hand, significantly low sialidase activity was observed towards submaxillary mucin, 6-siallyllactose and colominic acid. Furthermore, NanA sialidase activities towards pure gangliosides yielded significantly higher activity in GM3 and GD1a, with only trace activity in GM1 (Figure 3-8B). These results revealed that sialidase NanA could preferentially cleave sialic acids that are bound through  $\alpha$ 2-3 linkage to glycoconjugates.



**Figure 3-7.** Effect of pH and temperature on *E. tarda* NanA sialidase activity.

Enzymatic reaction was done using types of buffers: sodium acetate buffer for pH 3.5–5.5 (diamond) and sodium phosphate buffer for pH 5.5–8.0 (square). Results are plotted as means  $\pm$  standard deviation from three independent experiments.

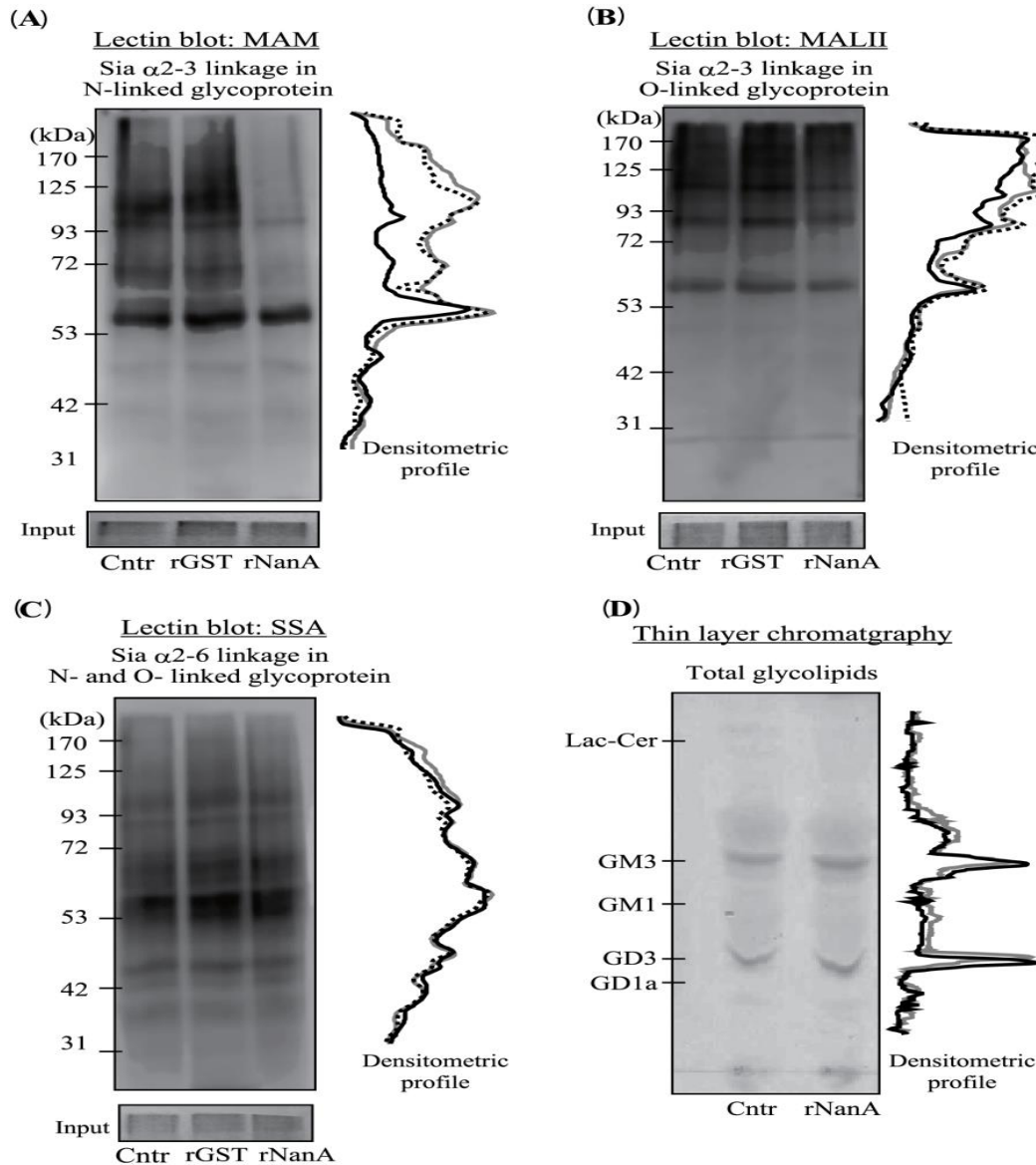


**Figure 3-8.** Substrate specificity of NanA sialidase.

Sialidase activity was assayed towards various glycoconjugate substrates at pH 5.5 for purified rNanA using a 50  $\mu$ l reactions mixture containing 0.1% Triton X-100, 10 mM sodium acetate buffer (pH5.5), 2.5  $\mu$ l substrate and 10  $\mu$ l rNanA. (B) Sialidase activity was also assayed towards pure gangliosides, GM1, GM3 and GD1a. The results are shown as means  $\pm$  standard deviation from three independent experiments. The differences in means were compared using ANOVA and statistical differences are shown by letters presented on each substrate. Substrates having the same letter were not statistically different, vice versa.

### **3.5.7. Sialidase NanA predominantly desialylates $\alpha$ 2-3 linked glycoconjugates on GAKS cell surface.**

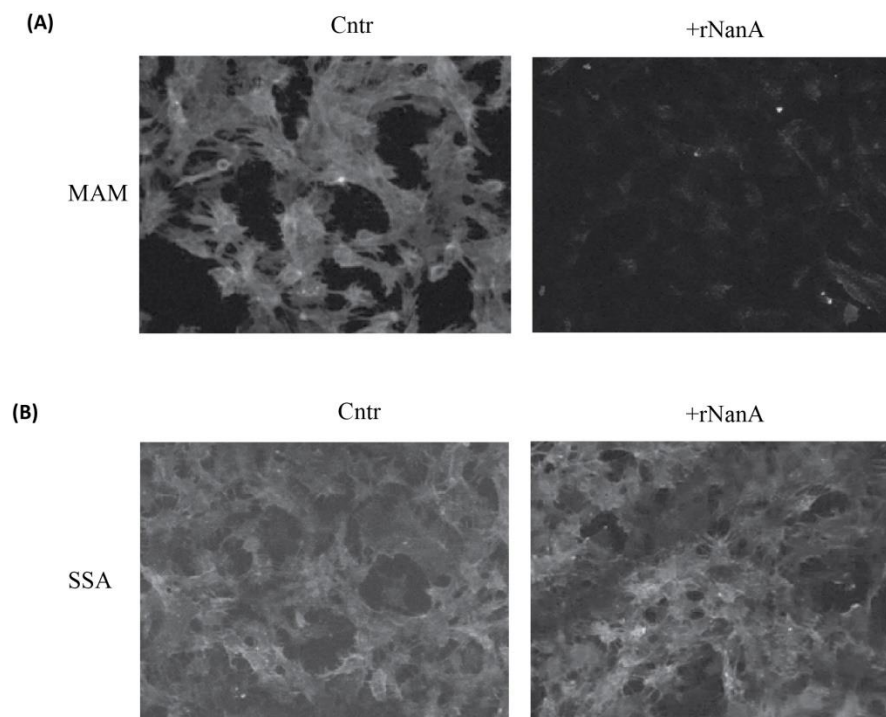
Based on earlier results reported herein, sialidase NanA preferentially cleaves sialic acid from  $\alpha$ 2-3 glycosidic linkage of glycoconjugates *in vitro* (Figure 3-8A). To determine the nature of glycoconjugate and confirm the linkage type significantly affected by sialidase rNanA, lectin blotting for glycoprotein analysis and TLC for glycolipid analysis were conducted for wild and rNanA-treated GAKS cells. There was a significant decrease of MAM lectin binding, which recognizes  $\alpha$ 2-3 linked sialic acid of sialylated N-linked glycoprotein, in NanA-treated GAKS cells compared to the untreated cells (Figure 3-9A). No differences in lectin binding between rNanA-treated cells and control were observed in MALII and SSA lectin which recognizes  $\alpha$ 2-3 linked sialic acid in O-linked glycoproteins and  $\alpha$ 2-6 linked sialic acids in N- and O-linked glycoproteins, respectively (Figure 3-9B-C). Glycolipid alterations did not differ in rNanA-treated and control after analysis using TLC (Figure 3-9D). These results demonstrated that NanA preferentially cleave  $\alpha$ 2-3 linked sialic acid than  $\alpha$ 2-6 linkage on glycoprotein, similar to the results of rNanA substrate specificity. Furthermore, the results demonstrated strict preference of the  $\alpha$ 2-3 linked sialic acids, which only towards N-linked glycoprotein and not O-linked glycoprotein.



**Figure 3-9.** GAKS cell surface glycoconjugates alterations after exposure to rNanA. (A-C)

Glycoproteins and glycolipids of GAKS were analyzed by lectin blotting and thin layer chromatography, respectively, after exposure of GAKS cell membrane lysate to 50 U of rNanA for 1 hour. (A) Lysate was resolved on 8% SDS-PAGE, transferred onto PVDF membrane and reacted with bionylated MAM (A), MALII (B) and SSA (C) lectins. Lectin blots were visualized by staining with HRP-conjugated streptavidin and the corresponding band densitometric profiles analysed using ImageJ software. The Control, rGST and rNanA are represented by dotted line, grey solid line and black solid line, respectively, for each lectin. After the blotting the protein onto PVDF membrane, the gel was stained with CBB by washing it in CBB solution for 24 hours. The gel was washed and visualized to confirm the amount of protein applied in each well. (D) Glycolipids were extracted from rNanA-exposed and wild GAKS cells, resolved by thin layer chromatography and visualized with orcinol-H<sub>2</sub>SO<sub>4</sub>. Control and rNanA were represented by black solid line and grey solid line, respectively, in TLC

To validate these results, alteration of sialoglycoproteins on cells surface induced by rNanA treatments were estimated using MAM and SSA fluorescence staining. As a result, rNanA treatment caused a significant loss of signal in MAM lectin compared to the untreated cells (Figure 3-10A), while the signal was similar in the rNanA-treated and control cells in SSA lectin (Figure 3-10B), confirming  $\alpha$ 2-3 linked sialic acids on glycoprotein were target molecule of *E. tarda* NanA. These results together suggest that NanA preferentially cleaves sialic acid from  $\alpha$ 2-3 linked glycoprotein and that this desialylation potentially stimulates the interactions of *E. tarda* with GAKS cells during infection.

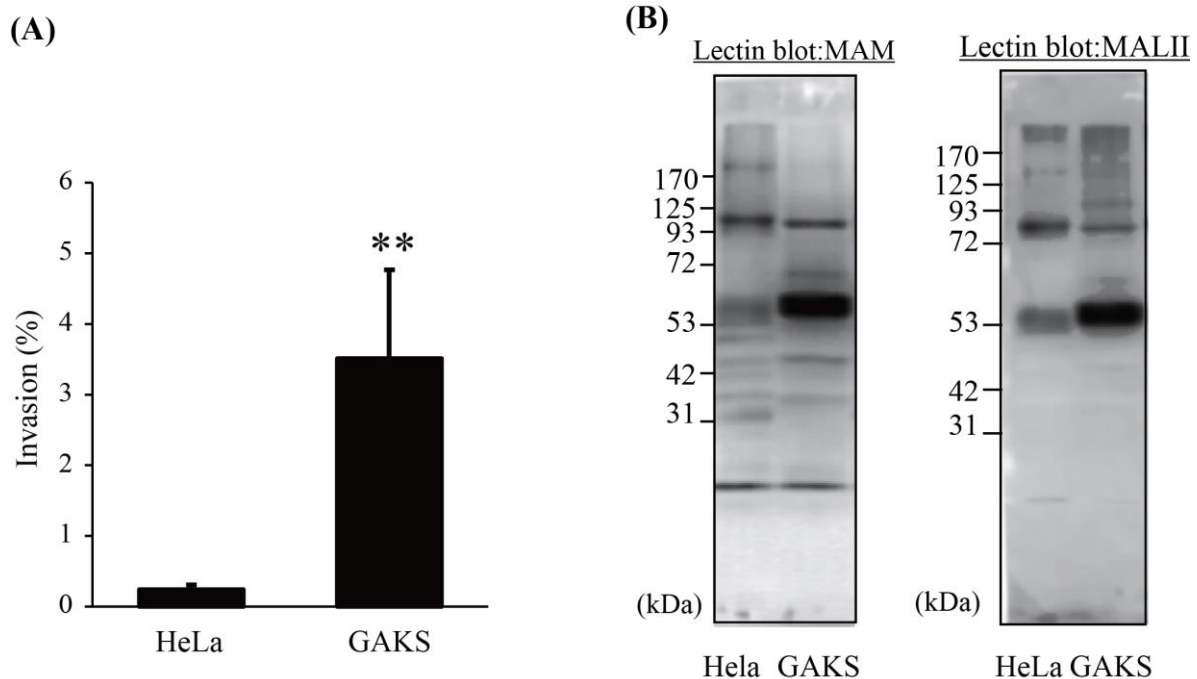


**Figure 3-10.** Lectin binding analysis of GAKS cell surface.

Surface glycoproteins of intact GAKS cells were analysed by lectin binding after 1 hour exposure to rNanA. Cells were fixed with 4% paraformaldehyde for 15 minutes and reacted with bionylated MAM (A) and SSA (B). Lectin staining was visualized with Fluorescein Isothiocyanate (FITC) conjugated streptavidin.

### **3.5.8. *E. tarda* preferentially infects cells well decorated with $\alpha$ 2-3 linked sialoglycoproteins**

Various host cells are decorated with a variety of sialic acid containing glycoconjugates and their manipulation by bacteria sialidases have been reported to play various pathogenic roles during or after infection (Honma et al., 2011; Soong et al., 2006). Firstly, to choose an appropriate cell line for *E. tarda* infection, HeLa and GAKS cells were tested for *E. tarda*. As a result, *E. tarda* exhibited a significant ability to invade GAKS cells compared to HeLa cells. *E. tarda* cell invasion was about 14 times higher in GAKS cells when compared with HeLa cells (Figure 3-11A). To determine the possible reason for this difference, profiles of  $\alpha$ 2-3 sialoglycoproteins were estimated in GAKS and HeLa cells by lectin blotting analysis in the two cell types. Lectin analysis showed significant difference in band intensity in MAM and MALII lectin between two cells. GAKS showed higher intensities for both MAM and MALII lectins than HeLa cells (Figure 3-11B), indicating an abundance of  $\alpha$ 2-3 sialoglycoproteins in these cells. . These results thus demonstrate that GAKS cells are decorated with a significant higher amount of  $\alpha$ 2-3 linked sialylo-glycoprotein compared with HeLa cells, and this phenotype might be responsible for an increased rate of *E. tarda* infection.



**Figure 3-11.** Comparison of *E. tarda* invasion in HeLa and GAKS cells.

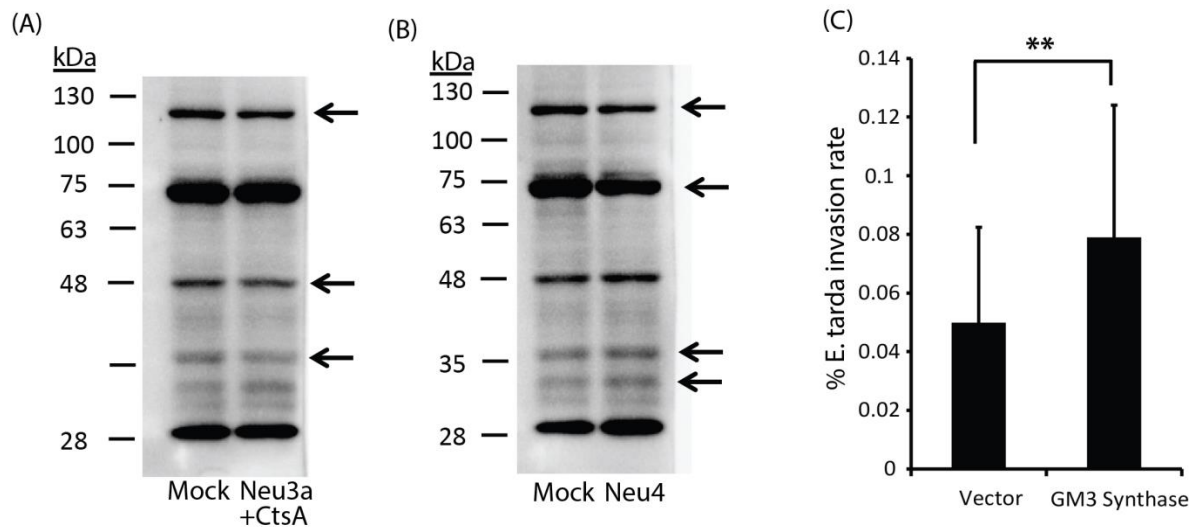
(A) Glycoprotein composition in wild GAKS and HeLa cells were analyzed using lectin blotting. Lectin-binding glycoproteins were visualized with VECTASTAIN ABC standard kit. (B) HeLa and GAKS cells were seeded into a 12-well plate and cultured for 24 h followed by exposure to *E. tarda* at MOI of 100 for 1 hour. The cells were washed and treated with gentamycin, streptomycin and penicillin for another 1 hour. The monolayers were culture for another 2 hours, lysed with 0.5% Triton X-100 in PBS and plated on TSA plates in serial dilutions. The CFU were counted after 48-hour culture and expressed as a percentage relative to the input bacteria. \*\*,  $p < 0.01$  comparing the two cell lines. The results are shown as means  $\pm$  standard deviation from three independent experiments.

### 3.5.9. Potential mechanism for effect of tilapia sialidase on *E. tarda* infection

The results here-in suggest that *E. tarda* infection is modulated by NanA through the modulation of cell surface  $\alpha 2$ -3 linked sialo-glycoproteins. The effect of tilapia sialidase overexpression on *E. tarda* infection was also hypothesized to be through modulation of these cells surface glycoproteins. To confirm this hypothesis, tilapia sialidase



overexpressing cells were homogenized and the resulting homogenates were electrophoresed using 8% SDS-PAGE. After protein electro-transfer onto a PVDF membrane, the membrane were treated with  $\alpha$ 2-3 sialo-specific MAM lectin.



**Figure 3-12.** Glycoprotein profiles by lectin analysis.

(A-B) Tilapia Neu1a and Neu4 sialidase transiently overexpressing cells were homogenized, resolved using 8% SDS-PAGE and blotted onto a PVDF membrane. The membrane were treated with MAM lectin and visualized using VECTASTAIN. (C) GM3 synthase transiently transfected and mock GAKS infected with *E. tarda* at MOI of 100. The CFU were counted after 48-hour culture and expressed as a percentage relative to the input bacteria. \*\*,  $p < 0.01$  compared with mock (vector) cells. The results are shown as means  $\pm$  standard deviation from three independent experiments.

As a result, small changes in MAM lectin binding were observed in a cotransfectant of Neu1a and Cathepsin A, compared with the mock (Figure 3-12A). Similar results were observed for Neu4 transfected cells with bands around 120 kDa, 73 kDa, 36 kDa and 30 kDa showing decrease in MAM lectin binding (Figure 3-12B), suggesting that tilapia Neu4 overexpression induce desialylation of cell surface  $\alpha$ 2-3 sialo-glycoprotein, resulting in the

increase in *E. tarda* adhesion to GAKS cells. Biochemical properties of tilapia Neu3a revealed that Neu3a has strict substrate specificity towards gangliosides, as such lectin analysis was not done for the Neu3a overexpressing GAKS cells. As GM3 is major substrate for vertebrate Neu3 sialidase (Monti et al., 2000; Shiozaki et al., 2013). It was hypothesized in this study that GM3 could be among the molecules involved in the *E. tarda* attenuation in Neu3a overexpressing GAKS cells. Therefore, GM3 synthase genes (ST3GAL5) was transfected into GAKS and infected with *E. tarda*. GM3 overexpression showed an up-regulated *E. tarda* infection when compared to the infection in mock cells (Figure 3-12C).

#### **3.5.10. Effects of monosaccharides on *E. tarda* invasion of GAKS cells**

To determine the potential monosaccharide receptor component of glycoprotein, inhibition assays were carried out using sugars generally present in glycoproteins. Monosaccharides, Glucose, Fucose, Galactose, Mannose, GalNAc and GlcNAc were exposed to *E. tarda* prior to infection. Preincubating *E. tarda* with 100 mM of mannose solution significantly reduced invasion to GAKS monolayers by about 74% compared to the control (Table 3-2) suggesting that *E. tarda* potentially binds to mannose of the desialylated  $\alpha 2-3$  sialo-linked glycoproteins during infection. GlcNAc also inhibited *E. tarda* infection by 50%, although the effect was less pronounced compared to mannose inhibition (Table 3-2). These results suggested that mannose and GlcNAc could be constituents of the binding sites on host cells of GAKS cells for *E. tarda*.

**Table 3-2.** The effects of monosaccharide pretreatment on *E. tarda* infection to GAKS monolayers

	Glc	Gal	Fuc	Man	GlcNAc	GalNac
<b>Inhibition of <i>E. tarda</i> invasion (%)</b>	0	0	0	74.1±22.4**	0	50±15.7

\*\*,  $p < 0.01$  compared to the monosaccharide non-treated cells (control)

### 3.6. Discussion

Much research in microbial sialidases has focused on uncovering their presence in various microbial species and development of their inhibitors. Microorganisms still remain among the most diverse groups of living things and extensive research on the roles of microbial sialidases has a potential of providing clues on the general involvement of sialidase family, be it from microorganisms or from vertebrates, on the development of various diseases in the vertebrates. For instance, bacterial sialidase have been confirmed to aid bacterial colonization of host cells through various mechanisms in mammals as outlined above. The role of host cell's endogenous sialidases in potentially regulating bacterial infection is unknown. Direct studies to understand the importance of vertebrate sialidases in bacterial infection are almost impossible in the absence of an insightful model. Therefore, understanding the involvement and mechanisms of bacterial sialidases in infection, could significantly inform the process for vertebrate sialidase involvement. Because of common role of bacterial or vertebrate sialidases, which is the cleavage of terminal sialic acid, it is possible that they all modulate bacterial infection through a similar process. Furthermore,

despite the availability of extensive literature on microbial sialidases roles in mammalian pathogenesis, their roles in pathogenic microbes important in fish have remained elusive.

Adherence, invasion and intracellular replication in host cells are important for pathogenesis by intracellular pathogens (Finlay and Falkow, 1997). Bacteria have been reported to employ various glycohydases to promote their interaction with host cells (Corfield, 1992; Honma et al., 2011; Vimr et al., 2004). *E. tarda*, an intracellular bacterium common in fish, is thought to adhere and invade the host cells as initial steps of its infection similar with other intracellular pathogenic bacteria (Karlsson, 1989). Cell surface glycoconjugates, including glycoproteins and glycolipids have been considered to serve important roles during this initial step in several pathogenic bacteria (Beachey, 1981; Matsunaga et al., 2011; Karlsson, 1989). Sialoglycoconjugates are modulated by sialidase enzymes (Miyagi and Yamaguchi, 2007; Shiozaki et al., 2013), which are distributed in both prokaryotic and eukaryotic members. Bacterial sialidases have been reported to enhance pathogenic ability of most bacteria by their glycohydrolytic action towards host cell glycoconjugates (Kuroiwa et al., 2009; Corfield, 1992; Tong et al., 2000). It has previously been reported that *E. tarda* possesses endogenous sialidase NanA, and that *E. tarda* mutant lacking sialidase activity exhibited limited ability to invade and infect fish cells (Jin et al., 2012). However, there has been no definitive effort to clarify the mechanism of sialidase NanA's involvement during invasion of host cells, as such, roles of sialidase in *E. tarda* virulence have remained unclear. We, herein, report for the first time that involvement of glycoconjugates modulation by sialidase NanA is crucial for the initial

step of infection through desialylation of  $\alpha$ 2-3 linked sialic acids from N-linked glycoproteins.

Sialidase genes play various roles in various organisms. The role of bacteria sialidases in modulating infection have been reported (King et al., 2006; Parker et al., 2009). The significance of host cell endogenous sialidase in bacterial infection has not been extensively examined. This study provides an initial insight into the role of host cell sialidases in modulating bacteria infection. Neu1a and Neu4 from tilapia promoted *E. tarda* infection after their overexpression in fish cell line. *E. tarda* infection was significantly up-regulated in GAKS cells in response to Neu4 expression, while Neu1a overexpressing cells exhibited a statistically non-significant increase in *E. tarda* infection. These results suggest that tilapia sialidase Neu1a and Neu4 have a regulatory role on the possible binding moieties of the GAKS cells surface. Strangely, the overexpression of tilapia sialidase Neu3a inhibited *E. tarda* infection. The tilapia sialidase exploration studies showed that Neu1a and Neu4 cleave sialic acid from  $\alpha$ 2-3 sialyllactose *in vitro*, suggesting that similar nature of glycoconjugates on GAKS cells surface could be desialylated and be responsible for promotion of *E. tarda* binding. On the other hand, Neu3a inhibition of *E. tarda* infection was rather puzzling.

To gain more insights and understand potential roles of tilapia sialidases in bacterial infection, endogenous *E. tarda* sialidases NanA was cloned, purified and its properties comprehensively evaluated. Based on the previous finding that *E. tarda* sialidase induces virulence and infection advantages (Jin et al., 2012), we hypothesized that the *E. tarda* may

employ NanA enzyme during infection to modify host cell surface's sialo-glycoconjugates. Firstly, intact GAKS cells exposed to rNanA exhibited higher *E. tarda* infection and invasion rates than wild GAKS cells. Action of sialidase rNanA potentially altered the glycoconjugates profiles on GAKS cell surface, mediating the infection. Furthermore, sialidase inhibition by DANA significantly attenuated *E. tarda* infection confirming sialidase's crucial role in this process. The importance of sialidase in bacterial pathogenesis has previously been reported in various bacteria including *S. pneumoniae* (King et al., 2006; Camara et al., 2004; Barthelson et al., 1998), which is thought to be through the modification of the glycosylation state of cell surface glycoconjugates (Buschiazzi and Alzari, 2008). These results suggest that *E. tarda* utilizes its endogenous NanA to desialylate and modify host cell glycoconjugates facilitating its interaction with host cells and infection. The treatment of cells with sialidase has allowed the evaluation and conclusions about pathogen entry into host cells (Sayeed et al., 2011). Several previous studies have supported the role of bacterial sialidase, where sialidase treatment of tissues or cells showed reduced or stimulated adhesion of *E. coli*, *V. cholerae* and *P. aeruginosa* and *S. pneumoniae* (Krivan et al., 1988; Tsai et al., 2003; Parkkinen et al., 1983).

The surfaces of most host cells are decorated by wide variety of glycoconjugates possessing different structural characteristics and these are desialylated differently by various bacterial sialidase (King et al., 2006; Krivan et al., 1988). Therefore, we were prompted to examine the target sialo-glycoconjugate by analyzing the properties of rNanA *in vitro* towards various substrates. The results that rNanA showed significant desialylation towards  $\alpha$ 2-3

sialo-oligosaccharide and bovine fetuin, an N- and O-glycosylated protein with  $\alpha 2-3$  and  $\alpha 2-6$  sialosyl linkages, suggest that *E. tarda* potentially deploys NanA targeting and modifying  $\alpha 2-3$  linked sialo-glycoconjugates. In contrast, relatively small activity towards  $\alpha 2-6$  sialo-oligosaccharide excluded this group of molecules as target for NanA. These results suggesting that rNanA mediated *E. tarda* infection in GAKS cells is through desialylation of  $\alpha 2-3$  sialo-glycoconjugates. The preference of  $\alpha 2-3$  sialyl linkage in bacterial sialidase have been reported, for instance sialidase from *S. pneumoniae* and *S. typhimurium* and *C. perfringens* released sialic acid more rapidly from  $\alpha 2-3$  linked glycoproteins than the  $\alpha 2-6$  linkage pattern (Corfield et al., 1983; Minami et al., 2013; Crennell et al., 1993). The reasons for rNanA substrate specificity maybe difficult to suggest based on the results here-in but could be explained by the crystal structure of the enzyme and the recognition sites for glycoconjugate substrates. In other bacterial sialidases, reasons for substrate specificity have been deduced based on the crystal structural studies. For example, the strict preference of sialidase towards  $\alpha 2-3$  in *S. pneumonia* and *S. typhimurium* have been attributed to amino acid pairs Tyr589/Trp674 (Gut et al., 2008) and Tyr307/Arg309 (Crennell et al., 1993), respectively, present in each sialidase. These residue pairs have been implicated in the recognition of the difference between sialyl  $\alpha 2-3$  and sialyl  $\alpha 2-6$ -linkages. The reasons for rNanA substrate specificity here-in could be in-line with previous reports on other bacterial sialidase possessing similar properties. Therefore, studies of the crystal structure and active site architecture are required to clarify the mechanism for NanA preference towards  $\alpha 2-3$  linkage type. In addition, the differential

cleavage pattern of sialidase could also be attributed to the nature of core oligosaccharide structure and the linkage of the sialic acid residues in terminal position to galactose or as a branch to GalNAc in the sialylated glycoprotein derivatives (King et al., 2006). In general, substrate selectivity have largely been attributed to sialyl linkage (Kodama et al., 1991; Baum and Paulson, 1990; Chokhawala et al., 2007) and penultimate monosaccharide (Chokhawala et al., 2007). Therefore, it was not surprising that rNanA exhibited differential glycohydrolysis rates *in vitro* towards the tested sialoglycoconjugates.

Based on the *in vitro* data presented here, it was likely that NanA desialylation of  $\alpha$ 2-3 linked glycoproteins mediated *E. tarda* infection. However, at this point, it was still premature to conclusively suggest the nature of target glycoconjugate, as significant rNanA activity was also observed in sialo-glycolipids. We could not rule-out the possibility of glycolipid desialylation involvement in *E. tarda* infection. To clarify the nature of glycoconjugates involved in sialidase mediated infection of *E. tarda*, cell membrane fractions were exposed to rNanA for glycoconjugates alteration analysis. As expected, GAKS cell membrane fraction exposed to rNanA showed significant desialylation only in  $\alpha$ 2-3 sialo-glycoprotein using lectin analysis, but not in  $\alpha$ 2-6 sialoglycoproteins. In contrast, changes in glycolipid profiles were not observed, suggesting that sialidase NanA involvement in *E. tarda* infection is strictly through its deglycosylation of  $\alpha$ 2-3 linked glycoproteins. Although MAM and MALII lectin binding in wild cell types revealed higher  $\alpha$ 2-3 linked sialo-glycoproteins in GAKS cells, the glycosylation linkage pattern preferred by NanA was still not clear. Based on literature, two major patterns of  $\alpha$ 2-3 linked sialo-



glycoproteins exist; N- and O-linked. Interestingly, close examination of the nature of linkage pattern using lectin analysis further revealed that only  $\alpha$ 2-3 linked sialic acid in N-glycosylated proteins are preferred in GAKS cells membranes by NanA, evidenced by the clear lectin differences in MAM and not MALII lectin. Both MAM and MALII lectins are specific to  $\alpha$ 2-3 linked sialic acid of glycoproteins, but differ in the glycosylation type with MAM recognizing N-glycosylation and MALII recognizing O-glycosylation. Our study thus revealed that sialidase NanA initiated a deglycosylation process of  $\alpha$ 2-3 linked sialic acid from N-linked glycoproteins for bacterial interaction with host cells. Suggesting that during infections, *E. tarda* is able to utilize its endogenous sialidase NanA to desialylate adequate amount of  $\alpha$ 2-3 linked N-glycoprotein of the host cells necessary for a stimulated interaction. Sialic acids cap sugar chains in N- and O-linked glycoproteins, potentially masking the bacterial binding moieties on eukaryotic cell surfaces. This is especially true for those bacteria recognizing and possessing an affinity towards penultimate residues or oligosaccharide on the glycoconjugates. So far, we could suggest that NanA removes sialic acid from  $\alpha$ 2-3 N-linked glycoproteins potentially exposing binding moieties for *E. tarda*'s adherence through microbial lectins complementary to glycoconjugate receptors on the host cells.

These results together suggest that  $\alpha$ 2-3 sialo-glycoproteins are critical for *E. tarda* binding. Lectin analysis of the Neu1a and Neu4 overexpressing cells showed alterations in the lectin profiles, when compared with the mock transfected GAKS cells. This was plausible considering that both Neu1a and Neu4 show significant activity towards  $\alpha$ 2-3 sialol-

glycoconjugates *in vitro*. These results suggest that Neu1a and Neu4 desialylate cell surface  $\alpha$ 2-3 sialo-glycoproteins, revealing the binding epitopes suitable for *E. tarda* binding, similar to the sialidase NanA action. In addition, both tilapia Neu1a and Neu4 showed partial distribution at the plasma membrane similar to medaka Neu4 (Ryuzono et al., 2016; Shiozaki et al., 2014). Therefore, it is conceivable that Neu1a and Neu4 also regulate cells surface glycoprotein by their partial localization at the plasma membrane. This could also be the reason for the significant *E. tarda* invasion compared to the mock and Neu1a. Despite *E. tarda* expressing sialidases, enzymes that hydrolyse sialic acids from host sialo-glycoproteins to facilitate its adhesion, the expression of host cell sialidases is also crucial component contributing to host–microbe interactions at the fish cell surface.

Despite the localization of the Neu3a at the plasma membrane, significant sialidase activity was observed towards gangliosides and only minimal activity was present towards glycoprotein. This suggested that overexpression of Neu3a significantly affects cell surface glycolipids based on substrate specificity *in vitro*, which based on the current findings should exhibit no effect on *E. tarda* infection rate. The action of NanA on glycolipids did not show any significant changes in ganglioside profile and NanA sialidase activity towards gangliosides, *in vitro*, yielded mild activity results. On the other hand, Neu3a overexpressing cells showed a significantly attenuated *E. tarda* invasion, suggesting that the significance of gangliosides in *E. tarda* infection could be different from that of glycoproteins. While the mechanism underlining this inhibition remains unknown, it is noteworthy that Neu3a activity is involved in several signaling pathways. Interestingly,

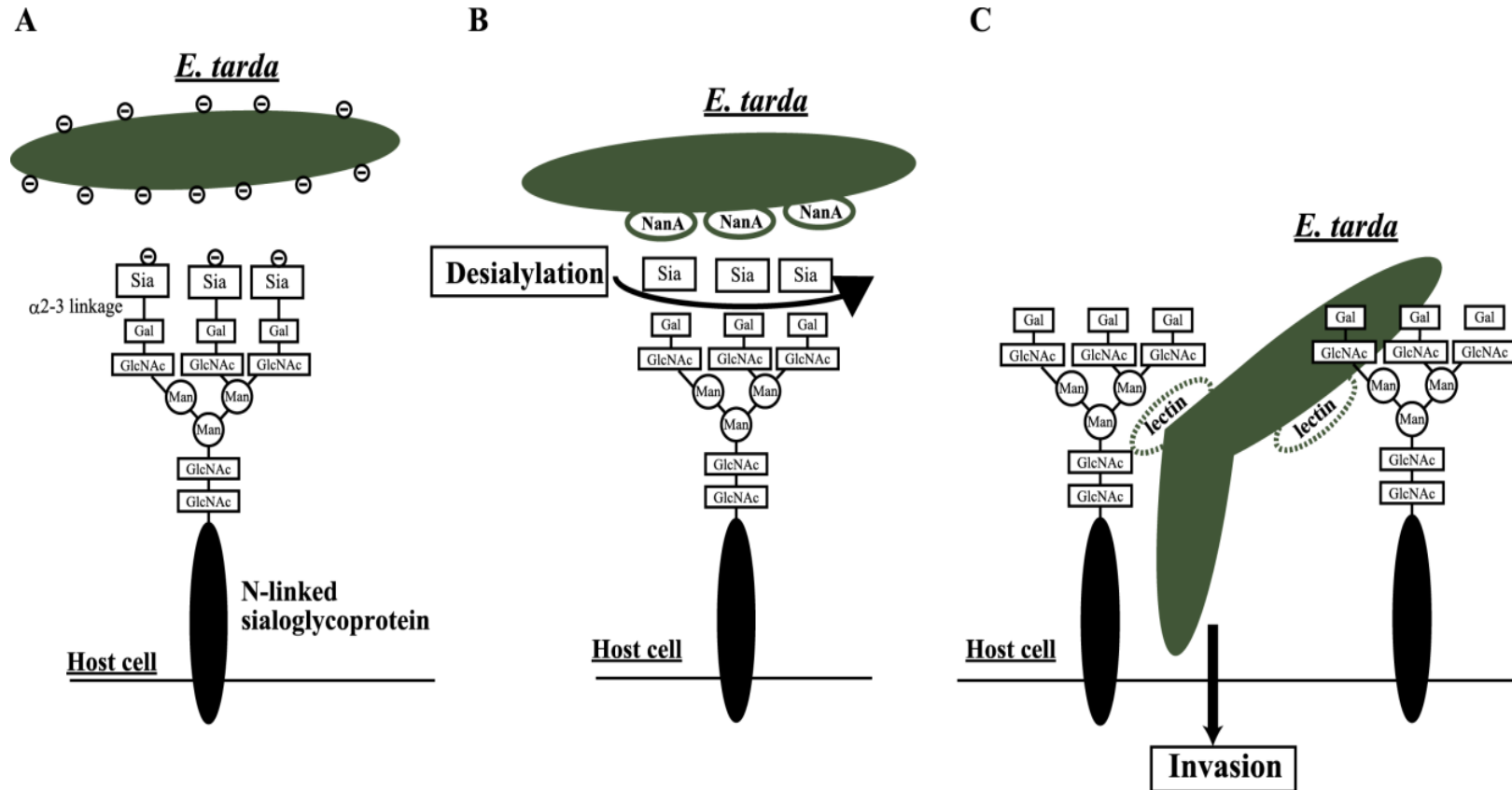
GM3 overexpression in GAKS cells up-regulated *E. tarda* infection suggesting that GM3 could be one of the key molecules involved in the observed Neu3a associated up-regulated *E. tarda* infection. Furthermore, Neu3a activity towards cell surface gangliosides could potentially trigger innate immunity response and enable cells initiate a defense mechanism against the *E. tarda*. For instance, Interleukin-6 (IL-6), a pleiotropic cytokine produced by various cells to regulate various processes including immune responses (Kishimoto, 2005), has been cloned in several fish species (Bird et al., 2005, Nam et al., 2007 and Castellana et al., 2008) i In mammals, the elevation of Neu3 has been reported to correlate to IL-6 levels (Ueno et al., 2006). Therefore, it could be speculated here-in that the increased Neu3a might have led to increased contribution of Neu3 to the IL-6-induced effects on immune response.

N-linked glycoproteins comprise various monosaccharides residues. To provide more insights into the specific epitope in the glycoprotein to which *E. tarda* binds, inhibition assay was done using binding to various monosaccharide candidates of glycoproteins. Mannose was found to be more effective in inhibiting *E. tarda* invasion followed by GlcNAc. Our data strongly suggest that *E. tarda* binding sites in N-glycoprotein of GAKS cells contain mannose and GlcNAc to a less extent. The differential inhibition where mannose showed to be more effective than GlcNAc, further suggests a common receptor in which either mannose is more important in the binding or that mannose is more accessible by mannose specific-lectins. Similar results have been reported previously in other bacteria groups (Chokhawala et al., 2007). Mannose is a predominant monosaccharide present in

numerous N-linked glycoproteins where it is attached to the core. Previously, the binding of Gram negative bacteria to mannose of various host cells (Parker et al., 2010; Ofek et al., 1977) and the production of mannose-specific lectin by many members of Enterobacteriaceae has been reported (Ofek et al., 1977). Furthermore, Enterobacteriaceae family, including *E. tarda*, has been shown to possess fimbriae or pili, in particular Type I pili at their surface (Wang et al 2013b) and that the lectin domains in these structures exhibit affinity for mannose (Pratt and Kolter, 1999; Westerlund-Wilson and Korhonen, 2005). Therefore, we hypothesize that *E. tarda* employs these mannose-specific fimbrial lectins to facilitate its binding to mannose. Thus our findings suggest that *E. tarda* possesses mannose-specific, lectin-like structures on the surface that mediate binding to GAKS cells, further suggesting that several kind of N-linked glycoproteins could be targets for actual *E. tarda* infection.

Taking these results together, a mechanistic hypothesis of molecular basis of sialidase NanA, involvement in host cell infection (Figure 3-13). Like most bacteria (Rosenberg and Kjelleberg, 1986), *E. tarda* potentially possess negative surface charge under physiological conditions. Furthermore, sialic acids are also negatively charged (Varki and Gagneux, 2012) and because of its terminal positioning in glycoconjugates, are responsible for the negative charge of the glycoconjugates. This potentially creates repulsion between the glycoconjugates and *E. tarda* (Figure 3-13A). Therefore, *E. tarda* potentially utilize sialidase NanA to cleave the terminal sialic acid (Figure 3-13B), nullifying the charge consequently stimulating the interaction with the host cell. The stimulated interaction

facilitates the binding of mostly mannose-specific lectin to the mannose containing residues (Figure 3-13C) in the adhesion process. Tilapia sialidase Neu1a and Neu4 potentially regulated *E. tarda* infection following the same model, therefore, modulation of sialidase expression of Neu1a and Neu4 could be instrumental in the control of *E. tarda* infection in tilapia.



**Figure 3-13.** Schematic diagram for the proposed mechanism of sialidase NanA involvement in *E. tarda* infection.

(A) The presence of negative charge on both *E. tarda* and host cell surface creates repulsive force. (B) *E. tarda* employs its endogenous sialidase NanA to cleave sialic acids, diminishing this negative charge and in-turn, promoting interaction of between *E. tarda* and host cell (C). In particular, *E. tarda* possibly takes advantage of this interaction to bind to mannose containing receptors using its lectins.

In conclusion, we have revealed for the first time that *E. tarda* utilizes NanA sialidase to cleave sialic acid from glycoconjugates, potentially revealing the binding epitopes on the cell surface of host cells. The study has further demonstrated that  $\alpha$ 2-3 linked sialic acid on the N-linked glycoproteins, are preferentially cleaved by NanA, resulting into stimulated interaction of the *E. tarda* with host cell. It seems likely that NanA might expose the mannose containing chain, next to the cleaved sialic acid and that *E. tarda* may utilize unidentified mannose-specific lectins facilitating its binding to GAKS cells during invasion. Therefore, *E. tarda* preferentially invade and infect host cell well decorated with  $\alpha$ 2-3 linked N-glycoproteins and utilize endogenous NanA to cleave the sialic acid and facilitate its binding. In addition, tilapia sialidases could also be crucial in regulating *E. tarda* infection after determining that Nue1a and Neu4 stimulate its infection towards overexpressing GAKS cells while Neu3a attenuated the infection rate. Further studies are necessary to clarify the mechanism of Neu3a observed attenuation of *E. tarda* infection and additional *in vivo* or *in vitro* systems investigations are necessary using other cell line or primary fish cells. This study provides indispensable insights into sialidase mediated infection and receptors macromolecules for *E. tarda* on the host cell and could subsequently help in the formulation of an effective remedial mechanism for this very fish pathogen.

## **CHAPTER 4:**

### **GENERAL DISCUSSION**

Intensification of fish production in aquaculture has led to fish being cultured in conditions that are fragile, therefore, prone to various physiological disarrays. Therefore, exploration of biochemical, genetic, or molecular characteristic or substance that could indicate a particular biological condition or process is critical. As such, more effort has now been focused towards determining and establishing more sensitive biochemical markers for monitoring the fish wellbeing in the changing environment (Pretti and CognettiVarriale, 2001). Sialo-glycoconjugates and their related enzymes are known to exhibit drastic changes in relation to the physiological conditions of mammals. The alteration of these molecules exhibit regulatory roles on various cellular processes, suggesting a possibility of their use as biological indicator of the affected physiological changes. To have a full understanding sialo-glycoconjugates regulatory roles, extensive basic biochemical studies are necessary. Sialidase, distributed in most organisms from viruses to higher animals, are among the critical regulator glycoconjugates. They are important for the initial step of catabolism of sialylglycoconjugates by hydrolyzing the terminal non-reducing sialic acid residues from the oligosaccharides attached to glycoproteins and glycolipids, and have marked effects on the biological functions of these glycoconjugates (Miyagi and Yamaguchi, 2012; Monti et al., 2010). Sialo-glycoconjugates and sialidase studies are extensive in mammals including human and very limited in fish. Therefore, the



investigations outlined in this paper are the first on sialidase explorations in tilapia, expanding the understanding on sialidase regulatory roles in fish.

Although all mammalian sialidases share some degree of their primary structure similarity (ranging from 42 to 70%), they show different biochemical properties in terms of localization and suitable activity conditions. Apparently, only few structural differences have been identified to account for their different properties including subcellular localizations and effective activity profiles. With time, biochemical isolation and characterization of sialidases from mammals have provided evidence for the existence of four types of sialidases. Molecular cloning of sialidases validated these four forms and facilitated elucidation of their different subcellular localization, enzymatic properties, functional roles and tissues expression under different physiological conditions (Miyagi et al., 2009; Seyrantepe et al., 2004; Bigi et al., 2010). Mammalian sialidase are now useful markers for various physiological conditions such as malignant phenotypes and differentiation and achieving the level of reliability as markers required their exploration and extensive property analysis. Nonetheless, mammalian sialidase have been attributed to wide range of physiological functions including lysosomal exocytosis, a cellular process for the recruitment of lysosomes to the plasma membrane, resulting in an increase in extracellular proteolytic activity (Yogalingam et al. 2008), cellular signaling for immune responses (Pshezhetsky and Hinek 2011), muscle cell and neuronal differentiation (Sato and Miyagi 1995; Sorice et al, 2004), regulation of cell apoptosis, proliferation and differentiation (Hasegawa et al. 2007), regulation of neurite formation (Shiozaki et al.

2009). The revealed significance of sialidase in mammals has helped shape-up major cellular pathways in nature. On the other hand, biochemical characterisations of sialidases in fish are limited and the full picture of sialidase roles in vertebrates is still not clear. Fishes constitute the most diversified group of vertebrates. Therefore, it is possible through this diversified nature, fish may possess more complex systems that may not be present in mammals and other groups of other vertebrates. Sialidase may thus be involved in these processes and their evaluation in fish has a potential of unfolding other functions of sialidase. The studies here-in, therefore, provided further insights into the biological diversity and roles of sialidases by focusing on sialidase explorative studies in fish. Much as the study could not provide direct evidence of sialidase usage as biomarkers in tilapia, it provides foundation for their potential use as biomarkers by comprehensively exploring different aspects of tilapia sialidases that are critical when selecting biomarkers.

In this study, the presence of mammalian sialidase orthologues in tilapia (*O. niloticus*) genome was determined *in silico*. Tilapia was considered a suitable species for this exploration based on several factors. Firstly, tilapia genome sequence information is available online (Lee et al., 2005). Furthermore, Nile tilapia is one of the most important species of fish in tropical and sub-tropical aquaculture. It is currently the second most farmed fish in the world (FAO, 2013). In general, tilapia are a valuable source of protein for most developing countries and now also an important fish commodity in developed countries. There is also a wealth of studies on different aspects of tilapia biology including their physiology, endocrinology, immunology, toxicology and genetics. Furthermore,

tilapia have a short generation period, grow to large sizes for physiological studies and are easy to handle making them a perfect model system. They survive under a wide environmental conditions and because of their versatile adaptability to different environmental conditions, they constitute exquisite models for environmental genomics, to analyse the interactions between the genome and the environment, and the adaptive responses to environmental stresses. Tilapia grows and reproduces in a wide range of environmental conditions and tolerates stress induced by handling (Tsadik and Bart, 2007). However, intensity of production has resulted into increases cases of infection and timely indicators of diseases and infection are important. In fish, some genes and proteins, especially those related to immunity and infection show varying expression pattern under different pathological conditions (Purcell et al., 2006; Moyner et al., 1993; Ewart et al., 2005; Marsden et al., 1996). Other genes are up-regulated while others show an opposite pattern during infection. Such genes provide useful infection monitoring tool by routinely determining their expression levels. In mammals sialidases are among such proteins but their limited exploration in fish limits their usage. The exploration of sialidase in tilapia has potential of developing sialidases as useful monitoring tool not just for infection, but also other physiological anomalies. Therefore, tilapia has potential of becoming a model animal for advancing the knowledge on sialidase roles in infection.

The first part of this study explored the diversity and properties of sialidase protein family in tilapia. Although four sialidase genes have been reported in mammals (Miyagi et al., 1990; Monti et al., 2002; Miyagi et al., 2004), only three sialidase orthologues were

identified in tilapia. A more complex sialidase gene organization was identified in tilapia constituting eight sialidase genes with paralogues in Neu1 and Neu3. Presence of two forms of Neu1 sialidases in tilapia revealed a significant disparity from the usual single lysosome sialidase reported in mammals (Bonten and d'Azzo, 2000; Miyagi et al, 2012), medaka (Ryzuno et al., 2015) and zebrafish (Manzoni et al., 2007). This is the first time, two forms of Neu1 have been identified in vertebrates. The reason for the duplicated *neu1* genes in tilapia may be difficult to speculate here-in, but based on phylogenetic analysis, *neu1b* might have evolved from *neu1a*. Different theories have previously been suggested on the existence of duplicated genes. A more plausible theory in relation to *neu1a* and *neu1b* is that each duplicated copy will retain only some of their original functions, complementing each other to fulfill the original ancestral gene activities (subfunctionalization). Less frequently, one of the duplicated copies may acquire a new function, whereas the other copy retains the original one (neofunctionalization) (Prince and Pickett 2002). This study suggest that Neu1a could be the main lysosomal gene in tilapia. Therefore, further studies are required to determine if the physiological role of Neu1b are independent or complementary to Neu1a.

Cathepsin A activated-tilapia-Neu1a showed substrate specificity significantly towards oligosaccharides while Neu1b showed a narrow substrate specificity only towards 4-MU-NANA. The results for Neu1a substrate specificity was similar to the substrate specificities reported in mammalian and medaka Neu1s with sialyloligosaccharides serving as good substrates (Seyrantepe et al., 2003; Ryuzono et al., 2016). In addition to

glycoconjugate catabolism in lysosomes, mammalian Neu1 is involved in cellular signaling for immune responses (Pshezhetsky and Hinek 2011). As is the case of the current production intensity in tilapia, where infections and diseases are a common occurrence, Neu1 could be explored for potential remedial strategy development in control of emerging tilapia diseases. The results from a related study showed Neu1 alterations after starvation in medaka, a closely related species to tilapia. One week starvation in medaka showed a significant drop in sialidase *neu1* expression in various tissues of medaka (data not shown). It was also observed that tilapia Neu1a potentially regulated cell surface glycoprotein and subsequently promoted *E. tarda* infection. The increase in *neu1* expression due to starvation suggest that exposing the fish to inadequate feed supply could potentially trigger *E. tarda* invasion. Therefore, maintaining constant food supply to fish is paramount and could prevent undesirable sialidase *neu1* gene expression. Further studies are necessary to establish the nutrition element that trigger the elevated *neu1* expression. Tilapia Neu1a showed a lysosomal localization similar to medaka Neu1 (Ryuzono et al., 2016). Interestingly, despite its localisation at the lysosome, Neu1a was able to promote *E. tarda* through cell surface glycoprotein desialylation. Previous studies have reported the move of human NEU1 and other lysosomal proteins such as Cathepsin A and LAMP-1 from lysosomes to the plasma membrane under conditions of cell stimulation (Miyagi and Yamaguchi, 2012; Lukong et al. 2001; Liang et al. 2006). Therefore, tilapia Neu1a may be trafficked to the plasma membrane through the lysosome-to-plasma membrane transport pathway and regulate cell surface glycoproteins.

The occurrence of multiple forms of ganglioside specific Neu3 sialidases in fish was first reported in zebrafish (Manzoni et al., 2007) and medaka (Shiozaki et al., 2013) where it was suggested that the multiple genes were as a result of independent duplication event after the study determined a single form in fugu (*T. nigroviridis*). The present study demonstrates that the occurrence of multiple forms of sialidase genes including *neu3* might be quite common among fish species. Moreover as suggested by the sialidase characterization in zebrafish, which posses five orthologs of human NEU3 gene, evolution of this protein family has been complex, probably following the increasing complexity of sialoglycoconjugates from microorganisms to higher vertebrates (Giacopuzzi et al., 2011). It was tempting in this study to hypothesise that these multiple forms of sialidase gene play roles beyond those previously reported in mammals, but two of them lacked transcripts (*neu3b* and *neu3c*) while the other two, Neu3d and Neu3e, despite possessing transcripts, possessed no sialidase activity against all the substrate examined in this study. Therefore, Neu3a was the main sialidase in this family explored in tilapia. The Biochemical characterization of Neu3a revealed a highly conserved enzymatic profiles to those reported in mammalian and medaka Neu3 (Miyagi et al., 2012; Shiozaki et al., 2013) characterized by a clear membrane localization and narrow substrate specificity towards gangliosides. The results here-in confirm that plasma membrane-associated sialidase Neu3a in tilapia is a key enzyme in the degradation of gangliosides, for which it exhibits substrate preference. Different from lysosomes, plasma membranes lack glycosidases for glycoproteins and glycolipids catabolism (Miyagi and Tsuiki, 1986), suggesting that Neu3 family of proteins is involved in cell surface events other than catabolism of glycoconjugates. Based on

mammalian studies, ganglioside degradation by tilapia Neu3a could regulate various physiological functions including cell differentiation, apoptosis and growth. Neu3a could be instrumental in the control transmembrane signalling for many cellular processes. Overexpression of sialidase Neu3a caused a significant attenuation of *E. tarda* infection. Although direct reason for the reduced *E. tarda* infection in Neu3a overexpressing cells is difficult to suggest based on the current results, ganglioside alteration could be central. In this study, increase in GM3 through overexpression of GM3 synthase up-regulated *E. tarda* infection. This reveals that GM3 could be one of the binding moieties for *E. tarda* and that Neu3a overexpression results in mass degradation of cell surface GM3, diminishing the binding entities for *E. tarda*. Recently, studies in mammals have reported dynamic changes in Neu3 localisation through action stimuli (Shiozaki et al., 2015), which could be similar to tilapia Neu3a due to its conserved nature with mammalian Neu3. Therefore, amount of Neu3a present at the plasma membrane at any point in time could be critical for *E. tarda* infection ability. For instance, dynamic internalization of Neu3a could possibly reduce plasma membrane Neu3a and consequently, promote *E. tarda* infection.

Sialidase Neu4 studies described here show the presence of a single form of *neu4* genes in tilapia and the encoded Neu4 enzyme shares the typical sequence features of the mammalian and medaka sialidase Neu4. The enzymatic profiles were partially conserved with mammalian and medaka. The results in this study suggest that tilapia Neu4 could share similar physiological attributes to its counterpart in human and medaka (Seyrantepe et al., 2008; Shiozaki et al., 2014). In mammals, Neu4 shows activity towards a majority of

endogenous substrates of Neu1 and as a result, Neu4 has also been implicated in lysosomal catabolism of glycoconjugates (Seyrantepe et al., 2008). The results here-in show Neu4 localisation at the nucleus. Furthermore, tilapia Neu4 significantly up-regulated *E. tarda* infection despite the observed localization pattern. Therefore, a possible trafficking to the plasma membrane of Neu4 could also be present, resulting in a significant amount being dispatched to the membrane and acting on glycoproteins. It is difficult to suggest the possible reason for the Neu4 trafficking here-in, but the results suggest a possibility of Neu4 regulation of *E. tarda* infection by the regulation of plasma membrane Neu4 amount. The localization of Neu4 at the nucleus and the significance of this localization required further studies.

The present results have shown that sialidase action on glycoprotein could be critical for the regulation of bacterial infection. In spite of the sialidase localization, plasma membrane trafficking and other mechanisms facilitate the presence of significant sialidase amount at the plasma membrane enabling desialylation of glycoproteins. Adherence to host cell surfaces is an essential first step in the pathogenesis of many pathogenic bacteria. Most bacteria express adherence factor or “adhesin” (Beachey, 1981). Large fraction of microbial adhesins is lectins that bind directly to host cell surface glycoproteins. Adhesion may be mediated through terminal sugars including sialic acids or internal carbohydrate motifs, which may be exposed after the removal of the terminal sugar. In the latter scenario, host cell endogenous and/or bacterial sialidase action is central by their cleavage of sialic acid to reveal the binding epitopes. Bacteria express adhesins or lectins that bind matrix



glycoproteins of the host cell surface. The results that tilapia sialidases overexpression was able to modulate *E. tarda* infection suggest that endogenous sialidases could be a potential target for control of *E. tarda* infection in tilapia. Suppression of Neu1a and Neu4 could also suppress *E. tarda* infection reducing infection rate in the species. On the other hand, any conditions that induce an elevation in Neu3a overexpression have a potential of significantly reducing *E. tarda* infection in fish. It is also worthy to note that elevation or suppression of tilapia endogenous sialidase could also trigger pathways and potentially nullifying the effect *in vivo*. Therefore, a carefully trade-off needs to be established before tilapia and other sialidase could be utilized as remedy for bacterial infection. Studies that are more comprehensive are necessary to establish thresholds and tilapia sialidase expressions that may be effective for the control of *E. tarda*.

In conclusion, this is the first study to explore sialidases in *O. niloticus*, an important fish species worldwide. Three sialidase sub-families, Neu1, Neu3 and Neu4 are present in tilapia. The study revealed the existence of a novel duplication of *neu1* genes, *neu1a* and *neu1b* both encoding active sialidase polypeptides. Five *neu3* genes were present in tilapia with *neu3a* as the main sialidase, *neu3b*, *neu3c* lacking transcripts while *neu3d* and *neu3e* possessed transcripts that encoded inactive polypeptides. A single *neu4* gene with highly conserved properties was present in tilapia. The encoded polypeptides showed varying biochemical properties. Neu1a and Neu1b showed optimal sialidase pH at 4.5 and 4.0, respectively, with both sialidase depending on activation by Cathepsin A. Neu1a showed significant sialidase activity towards 3-sialyllactose, ganglioside mix and colominic acid,

while Neu1b showed narrow substrate specificity significantly only towards 4-MU-NANA. Neu3a and Neu4 showed optimal pH at 4.6 and 4.0, respectively. Neu3a showed narrow substrate specificity towards gangliosides while Neu4 showed activity towards 3-sialyllactose, 4MU-NANA and 6-sialyllactose. In terms of distribution, Neu1, Neu3a and Neu4 were localized at the lysosome, plasma membrane and nucleus, respectively. Analysis of the roles of tilapia sialidase towards *E. tarda* infection, showed that Neu1a and Neu4 promoted *E. tarda* infection, while Neu3a suppressed it. To understand the mechanisms for the role of sialidase in *E. tarda* infection, sialidase NanA was analyzed. Fish cells exposed to recombinant NanA showed up-regulated *E. tarda* infection while *E. tarda* treated with sialidase inhibitor showed attenuated infection ability. NanA activity *in vitro* and cell surface glycoconjugates analysis showed significant NanA desialylation of  $\alpha$ 2-3 sialoglycoproteins, suggesting sialidase NanA from *E. tarda* removes sialic acid from  $\alpha$ 2-3 glycoproteins to facilitate its binding to host cells. Sialic acid and *E. tarda* bacteria are negatively charged, as such, removal of sialic acid by action of sialidase diminishes the negative charge on the host cell surface improving the bacteria-host cell interaction. Tilapia sialidase Neu1a and Neu4 potentially utilized a similar principle to promote *E. tarda* infection.

This study unequivocally showed that tilapia sialidases could have regulatory roles on bacterial infection and their expression profile could potentially be used as a cellular and tissue-based biomarker of various physiological functions, possibly offering reliable and more sensitive physiological tests for fish in future. This could in future offer new approach

to the aquaculture management. Furthermore, given the fact that tilapia sialidases are more diversified than for mammals, it would be of great interest for future studies to expand the scope of their physiological roles of in nature.

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