การแสดงออก การทำให้บริสุทธิ์และการศึกษาลักษณะสมบัติของนิวรามินิเดสจาก เชื้อไวรัสไข้หวัดนก (H5N1) ใน *Pichia pastoris*

นางสาวปูชิตา ฤกษ์สงเคราะห์

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EXPRESSION, PURIFICATION AND CHARACTERIZATION OF NEURAMINIDASE FROM AVIAN FLU VIRUS (H5N1) IN *Pichia pastoris*

Miss Puchita Rerksongkrou

A Thesis Submitted in Partial Fulfillment of the Requirements

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ปูชิตา ฤกษ์สงเคราะห์: การแสดงออก การทำให้บริสุทธิ์และการศึกษาลักษณะสมบัติของนิวรามินิเดสจากเชื้อไวรัสไข้หวัดนก (H5N1) ใน *Pichia pastoris*. (EXPRESSION, PURIFICATION AND CHARACTERIZATION OF NEURAMINIDASE FROM AVIAN FLU VIRUS (H5N1) IN *Pichia pastoris*) อ. ที่ปรึกษาวิทยานิพนธ์: รศ. ทิฆัมพร ยงวณิชย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วันชัย อัศวลาภสกุล, 84 หน้า.

โรคไข้หวัดนกจากเชื้อไวรัสอินฟลูเอนซ่า เอสายพันธุ์ H5N1 มีการระบาดสูงในประชากรนกและฝูงสัตว์ปีกในหลายทวีป ซึ่งส่งผลเป็นภัยคุกคามต่อสุขภาพของมนุษย์ จากการศึกษาก่อนหน้านี้พบว่าไกลโคโปรตีนสำคัญบนผิวของเชื้อไวรัส คือ นิวรามินิเดสที่มีบทบาทสำคัญในการจำลองตัวเองและติดเชื้อของไวรัส ลักษณะเป็นก้านยื่นรูปเห็ด ประกอบด้วยโพลิเพปไทด์สายเดียวซึ่งแยกเป็นสามส่วนหลัก คือบริเวณส่วนหัวที่มีบริเวณเร่งของเอนไซม์ ส่วนก้านที่มีความแปรปรวนสูง และส่วน signal anchor ที่ปลายเอ็นซึ่งเอนไซม์ใช้ยึดไว้ภายในเปลือกหุ้มไวรัส หน้าที่คือสลายปลายกรดไซอาลิกของไซอะโลไกลแคนและช่วยปล่อยไวรัสออกจากเซลล์เจ้าบ้านที่ติดเชื้อ โดยการทำลายรีเซพเตอร์บนเซลล์เจ้าบ้านและไวรัส การยับยั้งการทำงานของนิวรามินิเดสจะช่วยจำกัดการแพร่ระบาดในการติดเชื้อไวรัส และยับยั้งการเกิดโรคได้ งานวิจัยนี้ได้ทำการแสดงออกของนิวรามิเดสทั้งแบบเต็มความยาวและแบบเฉพาะบริเวณส่วนหัวใน *Pichia pastoris* ภาวะการเหนี่ยวนำที่เหมาะสมในการแสดงออกที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 5 วัน ด้วยการเติมเมทานอลร้อยละ 4 จากการวิเคราะห์ผลด้วย SDS-PAGE และ western blot พบการแสดงออกของโปรตีนทั้งสองชนิดมีขนาดของโปรตีนประมาณ 47 กิโลดาลตัน ทั้งสองรีคอมบิแนนท์ผ่านกระบวนการทำให้บริสุทธิ์ อย่างไรก็ตาม รีคอมบิแนนท์ของบริเวณส่วนหัวของนิวรามินิเดสเท่านั้นที่สามารถทำให้บริสุทธิ์บางส่วนได้ 1.28 เท่า จากการศึกษาจลนศาสตร์ได้ค่า K_m และ V_{max} เท่ากับ 20.64 ± 2.13 ไมโครโมลาร์ และ 81.85 ± 2.37 ไมโครโมลต่อนาทีต่อมิลลิกรัมโปรตีนตามลำดับ ยาต้านไวรัสโอเซลทามิเวียร์ สามารถยับยั้งการทำงานของรีคอมบิแนนท์แบบแข่งขันโดยมีค่า K_i และ IC_{50} เท่ากับ 84.15 นาโนโมลาร์ และ 30.03 นาโนโมลาร์ตามลำดับ สุดท้ายเปรียบเทียบผลของสารสกัดสมุนไพรไทย 10 ชนิดต่อการทำงานของนิวรามินิเดส พบว่าหญ้าดอกขาว *Vernonia cinerea* สามารถยับยั้งได้ใกล้สมบูรณ์คือร้อยละ 99.86 ± 0.01 ใกล้เคียงกับผลของโอเซลทามิเวียร์ ในทางตรงข้าม โกงกางใบใหญ่ *Rhizophora mucronat* ให้ผลยับยั้งน้อยที่สุดคือร้อยละ 25.62 ± 2.21 จากผลการศึกษาทั้งหมดชี้ให้เห็นประโยชน์ต่อการค้นหายาต้านไวรัสชนิดใหม่สำหรับเชื้อไวรัสไข้หวัดนก H5N1 ในอนาคต

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....

ปีการศึกษา.....2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…….

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PUCHITA RERKSONGKROU: EXPRESSION, PURIFICATION AND CHARACTERIZATION OF NEURAMINIDASE FROM AVIAN FLU VIRUS (H5N1) IN *Pichia pastoris*. ADVISOR: ASSOC. PROF. TIKAMPORN YONGVANICH, CO-ADVISOR:ASST. PROF. WANCHAI ASSAVALAPSAKUL, Ph.D., 84 pp.

The avian influenza A (H5N1) virus is highly contagious in both wild bird populations and domestic poultry flocks in various continents posing a serious threat to human health. Previous studies revealed that the major surface glycoprotein of the virion namely, neuraminidase (NA) plays an important role in viral replication and infection. The NA is a mushroom-shaped spike protein composed of a single polypeptide chain with three distinct domains: head domain containing the enzyme active site, a highly variable stalk region, and *N*-terminal signal anchor domain by which the enzyme is embedded in the viral envelope. The function is to hydrolyze the terminal sialic acids of sialoglycans and promotes the release of progeny virus from an infected host cell by destroying receptors on the host cell and the virus itself. The inhibition of NA activity will limit the spread of viral infection thereby suppressing the onset of disease. In this research, the full length and globular head domain of NA were expressed in *Pichia pastoris*. The induction for the expression was optimal at 30 °C for 5 days in the presence of 4% methanol. From SDS-PAGE and western blot analysis, the sizes of both expressed proteins were approximately 47 kDa. Purification process was carried out for both recombinants. However, only the head domain could be partially purified at 1.28 folds. The kinetic studies revealed the values of K_m and V_{max} at 20.64 ± 2.13 µM and 81.85 ± 2.37 µmol/min/mg protein, respectively. The recombinant activities were competitively inhibited by the antiviral drug, oseltamivir with K_i and IC_{50} at 84.15 nM and 30.03 nM respectively. Finally, the effect from the extracts of 10 types of Thai medicinal plants was investigated in comparison. It was found that *Vernonia cinerea* exhibited nearly complete inhibition at 99.86 ± 0.01% similar to oseltamivir. On the contrary, the lowest inhibition at 25.62 ± 2.21% was obtained from *Rhizophora mucronata*. The results obtained appear advantageous towards the search for the new antiviral drug for the avian influenza virus H5N1 in the future.

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Academic Year : 2011 Advisor's Signature .....

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
°C	degree celcius
et al	et alia (latin)
hr.	hour
kDa	kilodalton
L	liter
µg	microgram
µl	microliter
µM	micromolar
mA	milliampere
mg	milligram
mM	millimolar
min	minute
M	Molar
mol	mole
nm	nanometer
PBS	phosphate buffer saline
PBST	phosphate buffer saline-Tween20
PVDF	polyvinylidene difluoride
$1b/in^2$	pound (force) per square inch
v/v	volume by volume
w/w, wt	weight by weight

CHAPTER I

INTRODUCTION

1.1 Statement of purpose

The avian influenza A (H5N1) virus is highly contagious among birds including poultry in several continents and poses a serious threat to human health owing to its virulence with high lethality, endemic presence, increasingly large host reservoir, and significant ongoing mutation (Yuthavong *et al*., 2009). Human can be infected with such virus by close contact with infected poultry or objects contaminated by their feces. The occurrence of this kind of virus has been recorded in 2004 in Southeast Asia. Recently, the total number of confirmed human cases caused from such virus in five countries amounts to 358 (Thailand: 25; Cambodia: 20; Indonesia: 188, Lao: 2 and Viet Nam: 123), of which 254 were fatal. To date, 602 human cases in 15 countries around the world have been documented while 355 cases died. In Thailand, 17 deaths from 25 infected cases have been reported (WHO, 2012). The problems and the possible reassortments of this virus depend on mixing vessels which can generate new viral strains highly infectious for human. This is the most concern for the public health. (Jong and Hien, 2006; Pappaioanou, 2009; Boyce *et al*., 2009; Medina and Sastre, 2011)

Influenza viruses are enveloped RNA viruses belonging to the family *Orthomyxoviridae*. There are three types of influenza viruses: influenza A, influenza B and influenza C classified from the different antigenic determinants among their nucleocapsids (NP) and matrix (M) proteins (Lee and Saif, 2009). The genome of influenza virus consists of eight segments of single stranded RNAs, namely

polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acid (PA), hemagglutinin (HA), nucleocapsid protein (NP), neuraminidase (NA), matrix (M) and nonstructural proteins (NS). Four of these segments involved in the composing of viral envelope are NP, PA, PB1 and PB2. The assembly of last three RNA fragments is called polymerase complex responsible for the replication and transcription of RNA. In addition, the proteins contain the virion such as Matrix 2 protein (M2) and viral nuclear export protein (NEP). The prior protein works in assembly and budding process whereas another one functions in the export of RNA from the nucleus (Jong and Hien, 2006). The influenza A viruses have been classified into subtypes based on two surface glycoproteins called hemagglutinin (HA) and neuraminidase (NA). There are 16 subtypes of HA (H1-H16) and 9 for NA (N1-N9), respectively and most of these subtypes have been recently identified (Fouchier *et al*., 2005)

At present, the new strain of influenza virus, H1N1 (swine influenza) appears more severely contagious. The increased numbers of the infected patients have been reported and the widespread outbreak has been in great concern that the endemics might also spread to the countries which have never been reported before. It is evident that neuraminidase, subtype N1 is the same surface glycoprotein present in both swine (H1N1) and avian influenza (H5N1). The enzyme neuraminidase (NA) is therefore the attractive target for antiviral strategy because of its essential role in pathogenicity of many respiratory viruses (Kitazato *et al.*, 2007)

Neuraminidase (NA) is coded from the sixth RNA segment, an integral membrane glycoprotein, mushroom like structure with 3 distinct domains: globular head domain containing the active site of enzyme, a highly variable stalk region, and N-terminal signal anchor domain by which enzyme is embedded in viral envelope

(Wu *et al*., 2009). The function of NA has been known to hydrolyze the terminal sialic acids of sialoglycans. In addition, it is widely accepted that NA promotes the release of progeny virus from an infected host cell by destroying receptors on the host cell and the virus itself (Ohuchi *et al*., 2006). Therefore, the inhibition of NA activity will obviously limit the spread of viral infection, thereby suppressing the onset of disease (Moscona, 2005). The control and treatment of influenza depends mainly on chemical and biochemical agent. There are two classes of anti-influenza drugs currently available for influenza therapy, which target either the influenza A M2 ion channel or neuraminidase (NA). However, several previous studies have shown that some particular strains of viruses can develop resistance to antiviral drugs. Hence, it is of utmost importance to prepare for the emergence of antiviral drug-resistant H5N1 viruses and the advent of a pandemic by developing novel NA inhibitors. Previous studies have shown that the alterations of amino acids on the NA gene result in the adaptation and the evolution of the virus causing the contagion in living organisms (Suzuki, 2005). On the other hand, the alterations of the amino acids in the active sites result in the structure of the enzyme. The viruses will then show resistance to the antiviral drug, oseltamiver, which is used for the treatment of the disease. The amino acid alterations are at the positions of 119 (Glu 119 Val), 292 (Arg 292 Lys), 294 (Asn 294 Ser) and 275 (His 275 Tyr) (Kiso *et al.*, 2004). Therefore, the search for the new antiviral drug is in progress and requires the sufficient quantities of purified NA. The development and the improvement of the production of NA can be accomplished by the recombinant NA to avoid the direct usage of virus which poses danger and avoid the high cost. Since the screening for novel inhibitors of H5N1 NA requires an adequate quantities of pure NA**,** the heterologous expression system in various

recombinant proteins has been provided as an alternative way for viral protein production. The heterologous recombinants of viral proteins have been performed in mammalian or insect cell lines and in yeast *Pichia pastoris.* However, expression in *Pichia pastoris* is more popularly used for viral protein production because it is safe and potentially adaptable for inexpensive large-scale fermentation (Yuthavong *et al*., 2009). From the previous research of Miss Kanidta Niwasabutra, both of the full length and head domain neuraminidases from avian flu (H5N1) virus in *Pichia pastoris* were investigated. Both domains were successfully cloned and expressed in *Pichia pastoris* (Niwasabutra, 2009). Therefore, this research was attempted to express, purify and characterize the neuraminidase from avian flu virus (H5N1) in *Pichia pastoris*. In addition, the search for the neuraminidase inhibitors from Thai medicinal plants were also attempted.

1.2 Objectives of this research

To express, purify and characterize neuraminidase from avian flu virus (H5N1) in *Pichia pastoris*

1.3 Scopes of the investigation

1.3.1 To express the recombinant full length and head domain of neuraminidase

1.3.2 To determine the optimal conditions for expression of neuraminidase

1.3.3 To purify the full length and head domain of neuraminidase

1.3.4 To characterize the full length and head domain of neuraminidase

1.3.5 To screen for the neuraminidase inhibitor from the Thai medicinal plant extracts

1.4 Expected results

The recombinant neuraminidase can be used as the model for screening the antiviral drugs to develop the testing kit and used as the vaccine in the future.

1.5 Thesis organization

This thesis comprises five chapters as follows: Chapter 1 is the introduction. Chapter 2 gives the theoretical background and literature reviews. In Chapter 3, materials and methods are provided. The results can be found in Chapter 4. Chapter 5 is the discussion and conclusion.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Avian influenza virus

Influenza virus was discovered in animal diseases and influenza pandemics have occurred every 10-50 years throughout recorded history. There were three pandemics in the 20th century. Firstly, in 1918, the influenza virus called Spanish flu that killed about forty million people worldwide. Secondly, in 1957, the influenza virus called Asian flu that killed more than two million people. Thirdly, in 1968, the influenza virus called Hong Kong flu that killed about one million people. The pandemics suddenly increase illness and deaths that overcome health service and can cause social disruption and economic losses. The concern at the moment is a lethal avian influenza virus which killed millions of domestic poultry, some wild birds and some animals, including humans. At present, infection of influenza A (H5N1) virus is primarily disease of birds. Human can be infected with highly pathogenic avian influenza virus by close contact with infected poultry or objects contaminated their waste.

In 1977, the first reported human infection with H5N1 avian influenza in Hong Kong, the virus caused respiratory disease in eighteen people. Between December 2003 and October 2005, more than 100 human cases of avian influenza and more than 60 deaths, caused by the H5N1 strain, had been detected in Cambodia, China, Indonesia, Thailand and Vietnam (Cheng, 2005). In January 2004, highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype was first confirmed in poultry and humans in Thailand. The outbreaks were concentrated in the central, the southern part of the northern, and eastern regions of Thailand, which are

wetlands, water reservoirs, and dense poultry areas. More than 62 million birds were either killed by HPAI virus or culled. H5N1 virus from poultry caused 17 human cases and 12 deaths in Thailand; a number of domestic cats, captive tigers and leopards also died of the H5N1 virus (Tiensin *et al.,* 2005). Avian influenza virus and the problem of its possible reassortment in suitable "mixing vessels" generating new viral strains highly infectious for humans is a main concern for the public health. Although so far the cumulative number of confirmed patients is incomparable to the recently emerged H1N1 "swine influenza", the scientific community agrees that the emergence of new highly pathogenic avian influenza pandemic is more a real concern (Jong and Hien, 2006; Eichelberger *et al.,* 2008; Pappaioanou, 2009).

2.2 Structure of avian influenza virus

Influenza viruses are spherical or filamentous in shape, with the spherical forms on the order of 100 nm in diameter and the filamentous forms often in excess of 300 nm in length (Bouvier and Palese, 2008). They are RNA viruses of three genera from the family Orthomyxoviridae: influenza A, influenza B and influenza C. The influenza A, B and C are classified from the different antigenic determinants among their nucleocapsid (NP) and matrix (M) proteins. Influenza A viruses are further divided into subtypes based on the antigenic relationships of their hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. A total of 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) have been identified. All avian influenza viruses are classified as type A influenza virus (Lee and Saif, 2009).

The influenza A virus particle has a lipid envelope. Three envelope proteins-hemagglutinin (HA), neuraminidase (NA) and an ion channel protein (matrix protein 2, M2) are embedded in the lipid bilayer of the viral envelope. HA (rod-shaped) and NA (mushroom shaped) are the main surface glycoproteins of influenza A viruses. The ratio of HA to NA molecules in the viral envelope usually ranges from 4-5 to 1. The HA glycoprotein is responsible for binding of the virus to sialic acid residues on the host cell surface and for fusion of the viral envelope with the endosomal membrane during virus uncoating. The NA glycoprotein cleaves sialic acid receptors from the cell membrane and thereby releases new virions from cell surface. M2 functions as a pH-activated ion channel that enables acidification of the interior of the virion, leading to uncoating of the virion. Matrix protein 1 (M1), which is the most abundant protein in the virion, underlies the viral envelope and associates with the ribonucleoprotein (RNP) complex (Subbarao and Joseph, 2007).

The genome of influenza viruses is segmented, consisting of eight single-stranded, negative sense RNA, which are named hemagglutinin (HA), neuraminidase (NA), matrix (M), nucleoprotein (NP), nonstructural (NS) genes, and three polymerase segments named PB1, PB2, and PA (Fig. 2-1). The eight segments of influenza A virus encode 10 proteins. The RNA segments are contained within the viral envelope in association with the nucleoprotein (NP) and three subunits of viral polymerase (PA, PB1, and PB2), which together form ribonucleoprotein (RNP) complex responsible for RNA replication and transcription. Additional proteins contained within the virion include M2 and the viral nuclear export protein (NEP), which function in assembly and budding, and export of RNP from the nucleus (Jong and

Hien, 2006). The genomic segment of influenza A virus and protein function were shown in Table 2-1.

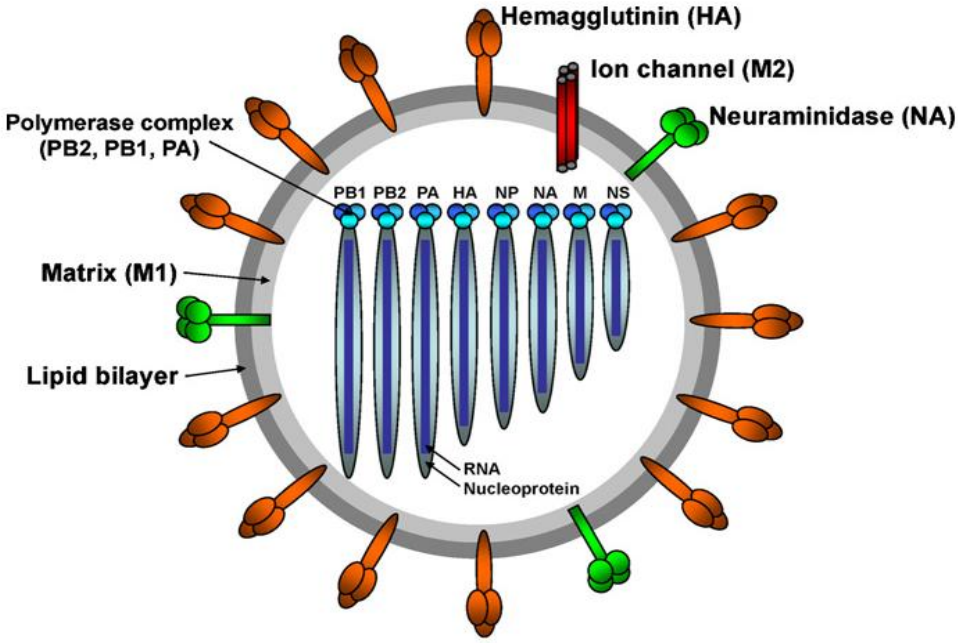

Figure 2-1 A schematic diagram of the influenza A virus (Lee and Saif, 2009)

Table 2-1 The genomic segment of influenza A virus and protein function

Segment	Encode protein (s)	Protein function
1	PB2	Polymerase subunit; mRNA cap recognition
2	PB1 PB1-F2	Polymerase subunit; RNA elongation, endonuclease activity Pro-apoptotic activity
3	PA	Polymerase subunit; protease activity
4	HA	Surface glycoprotein; major antigen, receptor binding and fusion activities
5	NP	RNA binding protein; nuclear import regulation
6	NA	Surface glycoprotein; sialidase activity, virus release
7	M1 M2	Matrix protein; vRNP interaction, RNA nuclear export regulation, viral budding Ion channel; virus uncoating and assembly
8	NS1 NEP/NS2	Interferon antagonist protein; regulation of host gene expression Nuclear export of RNA

From Bouvier and Palese, 2008

2.3 The replication cycle of avian influenza virus

The replication cycle of avian influenza virus starts from HA binding to a receptor (sialic acid) on the target cell surface and then the virus is internalized into an endosome through receptor-mediated endocytosis. HA mediates fusion of the viral envelope with the endosomal membrane when endosomal pH becomes acidic. The acidity of the endosomal compartment is crucial to influenza virus uncoating in two ways. Firstly, low pH triggers a conformational change in the HA, exposing a fusion peptide that mediates the merging of the viral envelope with the endosomal

membrane, thus opening a pore though which the viral ribonucleoproteins are released in to the host cell cytoplasm. Secondly, hydrogen ions from the endosome are pumped into the virus particle via the M2 ion channel. The M2 protein, a transmembrane ion channel found only in influenza A virus, has portions external to the viral envelope, along with the HA and NA. The viral genome (in the form of viral ribonucleoprotein; vRNPs) is then delivered into the cytoplasm of the target cell and subsequently transported to the nucleus, where replication and transcription take place. Messenger RNAs are exported to the cytoplasm for translation. Early viral proteins, that is, those required for replication and transcription, are transported back to the nucleus. Late in the infection cycle, the M1 and NS2 proteins facilitate the nuclear export of newly synthesized vRNPs. PB1-F2 associates with mitochondria. The assembly and budding of progeny virions occur at the plasma membrane, initiated by an accumulation of M1 matrix protein at the cytoplasmic side of the lipid bilayer. When the budding is complete, HA spikes continue to bind the virions to the sialic acid on the cell surface until virus articples are actively released by sialidase activity of the NA protein. NA hydrolyzes terminal sialic acids residues and it is generally accepted that NA promotes the release of progeny virus from infected host cell by destroying receptors on the host cell and the virus itself (Ohuchi *et al.*, 2006; Bouvier and Palese, 2008; Neumann *et al.*, 2009; Rossman and Lamb, 2011). Figure 2-2 illustrates the replication cycle of avian influenza virus.

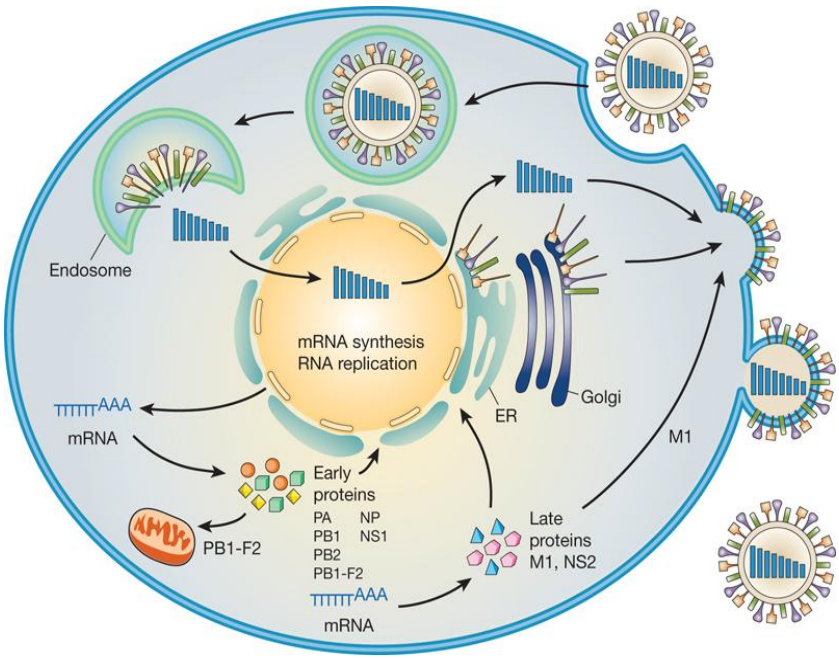

Figure 2-2 Replication cycle of the influenza A virus (Neumann *et al.*, 2009)

2.4 Neuraminidase

In 1957, Alfred Gottschalk, realized that if the receptor-destroying activity was an enzyme, this might remove something from the surface of the cells. He therefore set about looking for the "split product". This was eventually identified as *N*-acetylneuraminic acid (sialic acid) and the enzyme was called sialidase or neuraminidase, now designated acylneuraminyl hydrolase (EC 3.2.1.18) (Air and Laver, 1989). Subsequently, it was discovered that sialidases are quite widespread in nature. Other viruses, bacteria, mammalian cell, and some parasites all have their own sialidase enzymes (Garman and Laver, 2005) Influenza virus neuraminidase is a major surface glycoprotein of the influenza A and B viruses. The NA is a mushroom-shaped spike on the surface of the virion (Air and Laver, 1989). It is a tetramer of four identical subunits, each composed of six 4-stranded anti-parallel β-sheets (Bouvier

and Palese, 2008). The neuraminidase molecule is composed of a single polypeptide chain, coded by RNA segment 6 and consisted of three distinct domains: globular head domain containing the enzyme active site, a highly variable stalk region, and *N*- terminal signal anchor domain by which the enzyme is embedded in the viral envelope (Ohuchi *et al*., 2006). The role of influenza virus NA has been shown to facilitate mobility of the virus both to and from the site of infection. This enzyme is responsible for catalyzing the cleavages of (α-2-3) or (α-2-6)-ketosidic linkage that exists between a terminal sialic acid and an adjacent sugar residue. The breaking of this bond has several important effects that facilitate spread of the virus in the respiratory tract. Firstly, it permits transport of the virus through mucin and destroys the HA receptor on the host cell, thus allowing elution of progeny virus particles from infected cells. Secondly, the removal of sialic acid from the carbohydrate moiety of newly synthesized HA and NA is necessary to prevent self-aggregation of the virus after the release from host cells. Thirdly, this enzyme, by cleaving the sialic acid found in respiratory tract mucus, may prevent viral inactivation and promote viral penetration into respiratory epithelial cells (Gong *et al*., 2007). In viruses with inactive or absent NA, virus particles clump at the cell surface and infectivity is consequently reduced (Bouvier and Palese, 2008). Figure 2-3 and 2-4 show the structure of neuraminidase (mushroom-shaped spike) and the features of neuraminidase polypeptide respectively.

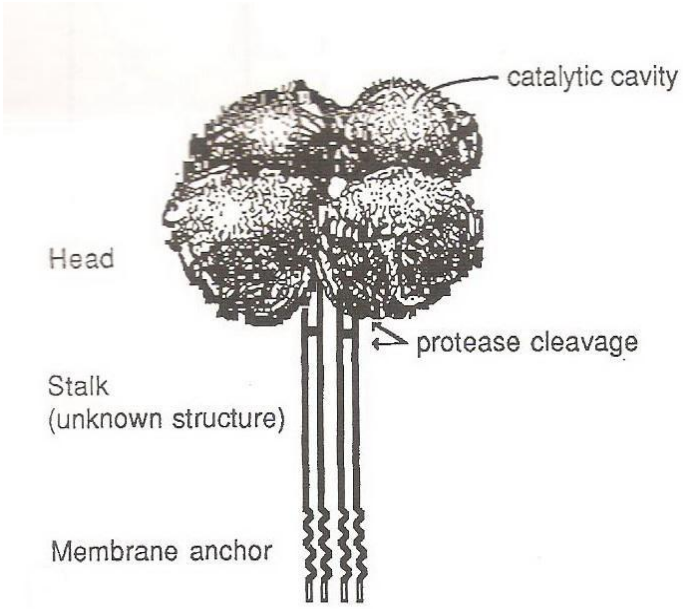

Figure 2-3 The structure of neuraminidase (Air and Laver, 1989)

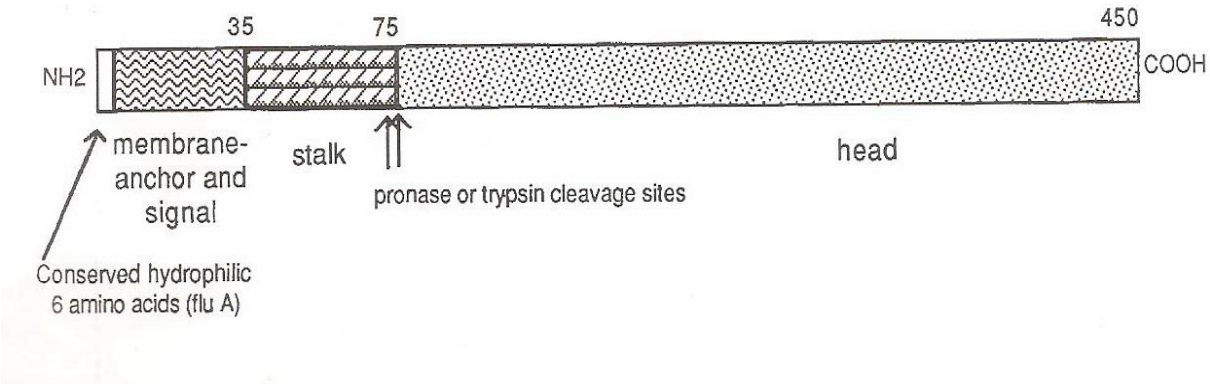

Figure 2-4 A schematic diagram of neuraminidase (Air and Laver, 1989)

2.5 Antiviral drug

The pathogenic avian influenza A (H5N1) virus is highly contagious among birds including poultry in several continents, and poses a serious threat to human health, evidenced by increasing numbers of H5N1 virus-infected patients. Up to date, more than 602 people worldwide have been infected with H5N1 virus (WHO, 2012). Effective control of H5N1 epidemics includes both effective vaccination and antiviral-drug treatment. However, influenza vaccines are not efficient because of antigenic variation. Therefore, during the early infection period, prevention and treatment of patients with H5N1 infection must rely mainly on antiviral drug treatments (Moscona, 2005; Yuthavong, *et al.,* 2009). Four drugs are currently available for the treatment or prophylaxis of influenza infections: the adamantanes (amantadine and rimantadine) and the newer class of neuraminidase inhibitors (zanamivir [Relenza] and oseltamivir [Tamiflu]) (Moscona, 2005). Amantadine and rimantadine target the M2 ion channel of influenza virus to prevent viral uncoating. Also they affect the pH regulation of vesicles in which viral glycoproteins are transported to cytoplasm for assembly. The other classes of antiviral drugs are neuraminidase inhibitors, zanamivir and oseltamivir (Escuret *et al.*, 2011). Influenza A and B viruses bind to the sialic acid glycoconjugates on the host cell by their hemagglutinin molecules. After successfully penetrating and replicating in the cells, neuraminidase cleaves the host cell sialic acid glycoconjugates and the mature viruses bud from the cell surface (Fig. 2-5 (A)) (Bouvier and Palese, 2008). The neuraminidase inhibitors inhibit the cleavage of sialic acid residues, preventing the release of the viruses (Fig. 2-5 (B)) (Moscona, 2005). In contrast to the admantanes, the neuramindase inhibitors are associated with very little toxicity and

are far less likely to promote the development of drug-resistant influenza. As a class, the neuraminidase inhibitors are effective against all neuraminidase subtypes and, therefore, against all strains of influenza, a key point in epidemic and pandemic preparedness and an important advantage over the adamantanes, which are effective only against sensitive strains of influenza A (Gubareva *et al.*, 2000). These new drugs, if used properly, have great potential for diminishing the effects of influenza infection. In the event of an influenza pandemic, it is generally accepted that antiviral drugs will provide the first line of defense against the new virus. Of these, the neuraminidase inhibitors are the drugs of choice (Yuthavong *et al.*, 2009; Kongkamnerd *et al.*, 2012).

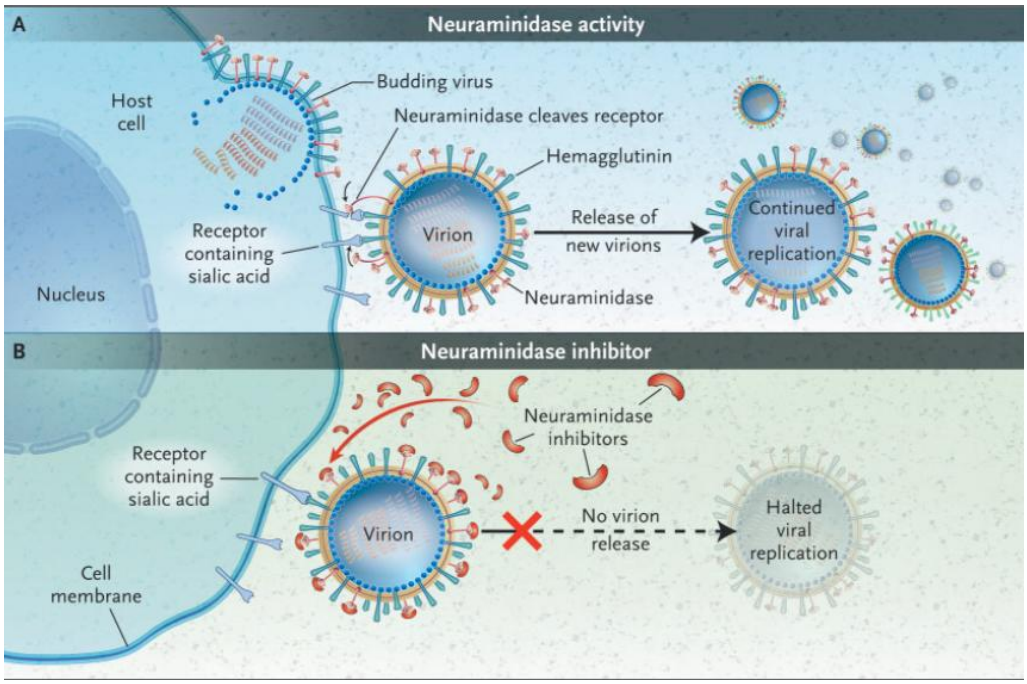

Figure 2-5 Mechanism of Action of Neuraminidase Inhibitor (Moscona, 2005)

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

Autoclave	(Ta chang medical instrument, Taiwan)
Balance	(Satorius, Germany)
Centrifuge tubes	(Oxygen scientific, USA)
Digital balance	(Satorius, Germany)
Gel documentation	(UVP, UK)
Heater	(Chatcharee holding, Thailand)
Incubator	(Gallenkamp, UK)
Laminar flow	(Thermo electron corporation, USA)
Microwave	(Sharp, Thailand)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Magnetic bar	(Lio lab limited partnership)
Microplate reader spectrophotometer	(Biochrom, UK)
Microrefrigerated centrifuge: model 5417	(Eppendrof, UK)
pH meter	(Model 250, Dever instrument)
UV-VIS spectrophotometer	(Thermo scientific, UK)
Spectrofluorometer	(Perkin Elmer,UK)
Vortex	(Scientific industries, USA)
Water bath	(T.S. instrument, Thailand)

3.2 Chemicals

In all experiments, the analytical grade and/or molecular biological grade chemicals and reagents were purchased from various manufacturers; namely, Sigma (USA), Merk (Germany), Fluka (Switzerland), Carlo Erba (Italy), Ajax Finechem (Australia), Difco (USA), Usb (USA), Biorad (USA) and Invitrogen (Canada).

3.3 Antibody and recombinant yeast

Antibody against neuraminidase, recombinant full length and head domain neuraminidase were obtained from Miss Kanidta Niwasabutra.

3.4 Purification column

Phenyl-Sepharose HP column	GE Healthcare Bio-Science AB (Sweden)
Sepharose Q column	GE Healthcare Bio-Science AB (Sweden)
CM sepharose column	GE Healthcare Bio-Science AB (Sweden)

3.5 Thai medicinal plant

Ten types of Thai medicinal plant extracts kindly provided by Miss Chutima Keawpiboon and Miss Kingkaew Piriyakananon were as follows: *Allium sativum*, *Celosia argentea*, *Clerodendrum inerme*, *Colocasia esculenta*, *Phyllanthus amarus*, *Rhizophora apiculata*, *Rhizophora mucronata*, *Sesuvium portulacastrum*, *Suaeda maritime* and *Vernonia cinerea*. The crude samples were extracted with ethanol by the soxhlet and later lyophilized. The dried powder of samples were then dissolved in 1X assay buffer (Appendix A) to make the 10 μg/ ml solution.

3.6 Data analysis program

Graph analysis program (Graph Pad Prism5)

3.7 Research methodology

The research methodology is as follows:

3.7.1 Expression of recombinants in *Pichia pastoris*

3.7.2 Optimization for the recombinant expression in *Pichia pastoris*

3.7.3 Purification of neuraminidase

3.7.4 Characterization of neuraminidase

3.7.1 Expression of recombinants in *Pichia pastoris*

3.7.1.1 Expression of neuraminidase

A single colony of recombinant neuraminidase was inoculated in 2 ml YPD and incubated at 30 °C, 250 rpm for 48 hr. Then, 100 µl of cell culture was transferred into 5 ml BMGY medium and grown until OD_{600} reached 5 to 6. Cells were harvested by centrifugation at 3,000 rpm, 30 °C for 5 min and resuspended in 1 ml BMMY medium and primarily induced with 3% methanol for 7 days. After that, the culture medium was collected by centrifugation at 10,000 rpm, 4 °C for 10 min. The supernatant was analyzed by SDS-PAGE and western blot analysis.

3.7.1.1.1 SDS-PAGE analysis

A protein sample was prepared by mixing with 4X sample buffer to final concentration of 1X sample buffer. The reaction was incubated at 95 °C for 5 min and centrifuged at 13,000 rpm for 5 min. The protein sample was then separated by SDS-PAGE.

3.7.1.1.2 Western blot analysis

After protein separation in SDS-PAGE, the proteins were transferred to PVDF membrane. Briefly, pieces of polyvinylidene difluoride (PVDF) membrane and filter papers were cut to the size of the gel, soaked with methanol and equilibrated in transfer buffer (Appendix B) for about 15 min. Next, the transfer sandwich is prepared starting from the bottom platinum anode with 3 pieces of filter papers, then the membrane, the equilibrated gel and finally another 3 pieces of filter papers. After the sandwich was completely arranged, a glass cylinder was rolled across it to eliminate air bubbles and then secure the stainless steel cathode and the safety cover. The transblotting sandwich was assembled and electroblotted at the constant amperes of 70 mA per gel at 90 min.

After that, the transfer membrane was incubated in 10 ml blocking buffer for 1 hr. at room temperature and then rinsed with 10 ml PBST twice. The membrane was incubated

with primary antibody in blocking buffer for 1 to 2 hr. on rocker platform at room temperature and then washed with 10 ml PBST three times. Next, the membrane was incubated with secondary antibody in blocking buffer for 2 hr. on rocker platform at room temperature and then washed with 10 ml PBST three times. The membrane was incubated with working solution for 3 min at room temperature, wrapped in clean piece of Saran wrap and exposed to a sheet of film for 30 sec. and developed the signal on x-ray film.

3.7.2 Optimization of the recombinant expression in *Pichia pastoris*

The full length and head domain neuraminidase recombinants were inoculated into 2 ml YPD and incubated at 30 °C, 250 rpm until the absorbance at 600 nm reached 5 to 6. Next, cell culture was transferred to 6 ml of BMGY medium before shaking at 250 rpm, 30 °C until the absorbance at 600 nm was 5 to 6 again. The cells were harvested by centrifugation at 4 °C, 5,000 rpm for 10 min before resuspending in 1 ml BMMY.

3.7.2.1 Optimization of the methanol concentration

The cells were resuspended in BMMY and shaken at 30 °C 250 rpm for 7 days. The induction was conducted by the separate addition of 1% to 5% fresh methanol every 24 hr. After that, the culture medium was collected by centrifugation at 10,000 rpm, 4 °C for 10 min. The supernatant was analyzed by SDS-PAGE and western blot analysis.

3.7.2.2 Optimization of the time

To investigate the optimal time of the expression, the cells were incubated for 1 to 7 days at 30 °C, 250 rpm and added every 24 hr. with optimal concentration of methanol obtained from 3.7.2.1. After that, the culture medium was collected by centrifugation at 10,000 rpm, 4 °C for 10 min. The supernatant was analyzed by SDS-PAGE and western blot analysis.

3.7.3 Purification of neuraminidase

The full length and head domain neuraminidase recombinants were grown according to the method described in 3.7.2 using the optimal conditions obtained from 3.7.2.1 and 3.7.2.2. After centrifugation at 10,000 rpm, 4 °C for 30 min, supernatant was concentrated by Centricon concentrators and then purified using phenyl-sepharose HP (5 ml), sepharose Q (5 ml) and carboxylmethyl sepharose (5 ml) columns respectively as described in the followings:

3.7.3.1 Phenyl-sepharose HP column (5ml) was previously equilibrated with 5 column volumes of 35 % $(NH_4)_2SO_4$, 20 mM potassium phosphate, pH 7.0 with 1 ml/min flow rate. Approximately, 70 mg of concentrated solution of sample were loaded to a column. Then, the column was washed with 40 % ammonium sulfate, 20 mM potassium phosphate, pH 7.0 to remove the unbound protein. The 2 ml fractions were later collected and the adsorbed proteins were then eluted with 10 mM NaCl, 10 mM Tris, pH 7.5, pooled and

concentrated. The approximate molecular weight of the purified NA were further analyzed by SDS-PAGE and western blot analysis.

3.7.3.2 The Sepharose Q column (5ml) was preequilibrated with 5 column volumes of 20 mM diethanolamine pH 8.5 at 1 ml/min flow rate. Approximately, 70 mg of concentrated solution of sample were loaded to a column. Then, the column was washed with 20 mM diethanolamine pH 8.5 to remove the unbound protein. The 2 ml fractions were collected and the adsorbed protein was eluted with linear NaCl gradient from 0 to 400 mM containing 20 mM diethanolamine buffer, pH 8.5. The eluted fractions were pooled and concentrated. The approximate molecular weight of the purified NA were further analyzed by SDS-PAGE and western blot analysis.

3.7.3.3 CM sepharose column (5ml) was preequilibrated with 5 column volumes of 20 mM diethanolamine pH 8.5 with 1 ml/min flow rate. Approximately, 70 mg of concentrated solution of sample were loaded to a column. Then, the column was washed with 20 mM diethanolamine pH 8.5 to remove the unbound protein. The 2 ml fractions were collected and the adsorbed protein was eluted with linear NaCl gradient from 0 to 400 mM containing 20 mM diethanolamine buffer, pH 8.5. The eluted fractions were pooled and concentrated. The approximate molecular weight of the purified NA were further analyzed by SDS-PAGE and western blot analysis.

3.7.3.4 The assay for neuraminidase activity

The neuraminidase activity can be quantitated by measuring the fluorescence of 4-methylumbelliferone after cleaving the 2’-(4-methylumbelliferyl)-α- *D*- N-acetylneuraminic acid (MUNANA). Briefly, the

reaction mixture was composed of 10 μl sample, 10 μl of 1X assay buffer and 30 μl of 500 μM MUNANA. The sample was then incubated at 37 ^{o}C for 60 min and 150 μl of stop solution (Appendix B) was later added. The released product, 4-methylumbeliferone was fluorospectrophotometrically determined by Perkin Elmer 1420 multilabel counter. The excitation and the emission wavelengths were 355 nm and 460 nm, respectively. Figure 3-1 showed the reaction catalyzed by NA for the assay.

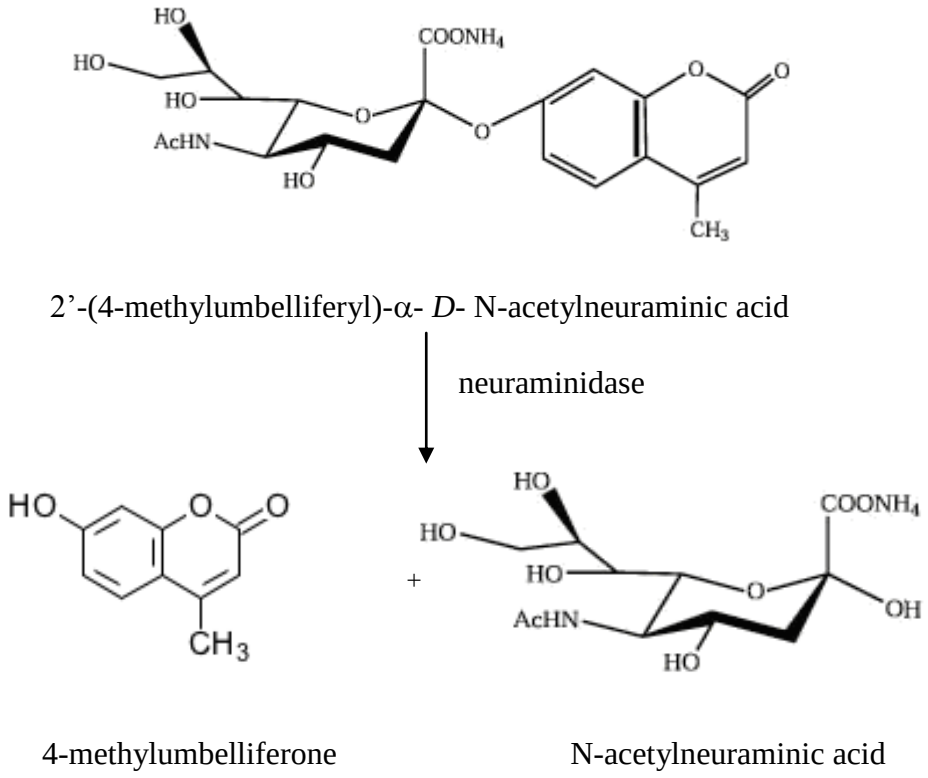

Figure 3-1 Cleavage of neuraminidase.

The substrate, 2'-(4-Methylumbelliferyl)-α- *D*- N-acetylneuraminic acid was cleaved by neuraminidase enzyme to release the products, 4-methylumbelliferone and N-acetylneuraminic acid.

3.7.3.5 Protein determination

The protein concentration was determined by Bradford's protein assay method. 5 µl of sample and 300 µl of Bradford reagent were mixed in 96 well plates before incubating at room temperature for 5 min and later measured for the absorbance at 595 nm. The protein concentrations were determined from the standard curve of bovine serum albumin (BSA) shown in the Appendix D.

3.7.4 Characterization of neuraminidase

3.7.4.1 Kinetics analysis

3.7.4.1.1 Determination of the Michaelis-Menten constant (K_m)

For the determination of Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), 10 µl of the purified neuraminidases and 10 µl of 1X assay buffer were incubated at 37 ^{o}C for 1 hour with 30 µl of MUNANA spanning the concentration range of 0-2000 µM. After that, 150 µl of stop solution was added. The released 4-methylumbeliferone was measured by using Perkin Elmer 1420 multilabel counter. The excitation wavelength was 355 nm and emission wavelength was 460 nm. Apparent kinetic constants, K_m and V_{max} were determined by fitting the collected data to a Michaelis-Menten equation by non-linear regression analysis using GraphPad Prism 5 (GraphPad software).

3.7.4.1.2 Determination of the inhibition constant (K_i)

For the determination of inhibition constant (K_i), the concentrations of both the enzyme and the inhibitors were fixed and the substrate concentrations were varied from 1000–0 μM by and 2x dilutions for 10 times. Then, the enzyme was added with the various concentrations of inhibitors from 0-20 μM, 10 μl of the purified neuraminidases, 30 μl of MUNANA was incubated at 37 ^{o}C for 1 hour with 10 μl of oseltamivir carboxylate. After that, 150 μl of stop solution (Appendix B) was added. The released 4-methylumbeliferone was measured by using Parkin Elmer 1420 multilabel counter. The excitation wavelength was 355 nm and emission wavelength was 460 nm. The velocities of the reactions were plotted against the concentration of inhibitor and the data were analysed according to secondary plot (Weerakalat, 2000)

3.7.4.2 Screening of inhibitors from Thai medicinal plant extracts

To study the effect of the extract on the activities of the purified neuraminidase, 10 μl of the purified neuraminidases, 30 μl of 1000 μM MUNANA were incubated with 10 μl of 10 μg/ ml of each Thai medicinal plant extract at 37 ^{o}C for 1 hr. After that, 150 μl of stop solution (Appendix B) was added. The released 4-methylumbeliferone obtained was then fluorospectrophotometrically determined using Parkin Elmer 1420 multilabel counter at the excitation and emission

wavelengths of 355 nm and 460 nm respectively. The specific activities and % inhibition were later determined.

CHAPTER IV

RESULTS

4.1 Expression of recombinants in *Pichia pastoris*

The recombinant full length (pPICZαA-NA) and head domain (pPICZαA-SA) of neuraminidase were inoculated in YPD at 30 °C. Then, the cell cultures were transferred into BMGY medium and grown until OD_{600} reached 5 to 6. Cells were harvested and resuspended in BMMY medium and primarily induced with 3% methanol for 7 days. The results of SDS-PAGE (A) and western blotting (B) in Figure 4-1 illustrated the protein bands of expressed recombinant pPICZαA-NA and pPICZ αA-SA with the sizes of approximately 47 kDa which confirmed that the full length (pPICZαA-NA) and head domain (pPICZαA-SA) of neuraminidase could be secreted from the recombinant pPICZαA-NA and pPICZ αA-SA.

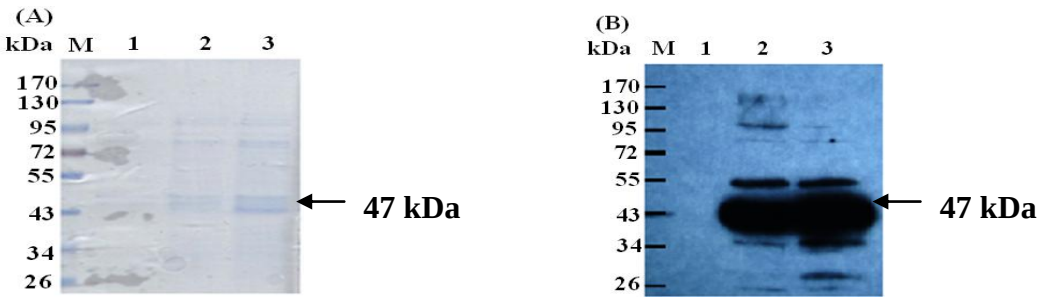

Figure 4-1 SDS-PAGE (A) and western blot (B) analysis of culture media from recombinant full length and head domain neuraminidase in the presence of 3% methanol for 7 days.

Lane M : protein markers

Lane 1 : culture medium from recombinant pPICZαA

Lane 2 : culture medium from recombinant pPICZαA-NA

Lane 3 : culture medium from recombinant pPICZαA-SA

4.2 Optimization of the recombinant expression in *Pichia pastoris*

4.2.1 Optimization of the methanol concentration

The cell medium was shaken at 30 °C for 7 days. The optimal concentration of methanol was determined by the addition of 1% to 5% of fresh methanol to maintain induction every 24 hr. From the results in Figure 4-2, 4-3 and 4-4, the culture media from recombinant pPICZαA-NA and recombinant pPICZαA-SA were analyzed by SDS-PAGE (A) and western blot (B). The protein bands of approximately 47 kDa could be detected in the culture media after the induction with 1 to 5% of fresh methanol compared with culture medium from recombinant pPICZαA. The intensity of the 47 kDa protein band was increased according to the increase of methanol concentration to 4 %. Therefore, 4% methanol was subsequently selected to study the optimization of the time.

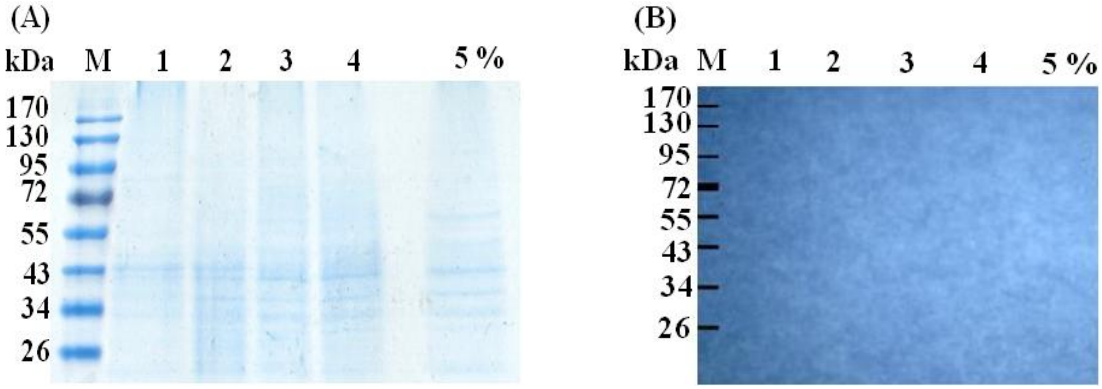

Figure 4-2 SDS-PAGE (A) and western blot (B) analysis of culture media from recombinant pPICZαA induced with 1-5% methanol for 7 days

Lane M : protein markers

Lane 1-5 : culture media from recombinant pPICZαA induced with 1-5% methanol

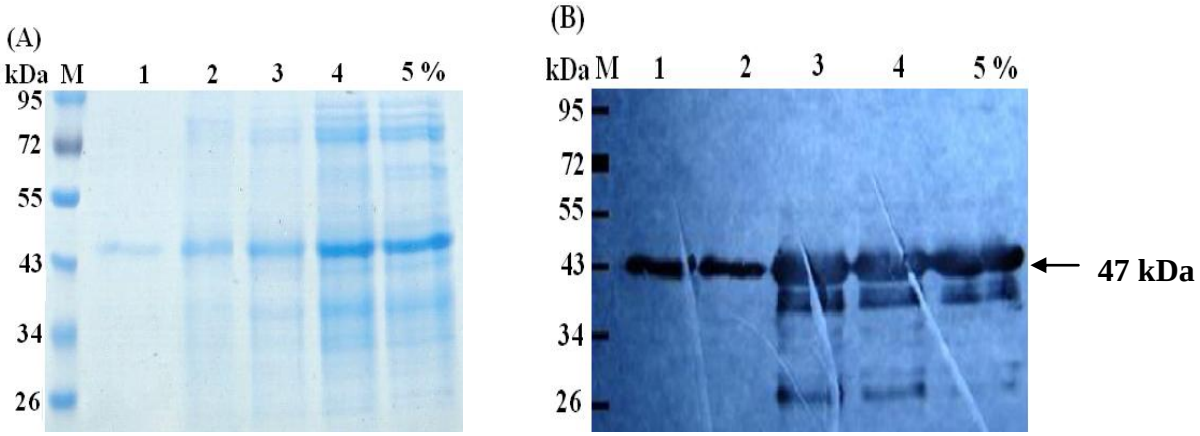

Figure 4-3 SDS-PAGE (A) and western blot (B) analysis of culture media from recombinant full length neuraminidase induced with 1-5% methanol for 7 days.

Lane M : protein markers

Lane 1-5 : culture media from recombinant pPICZαA-NA induced with 1-5% methanol

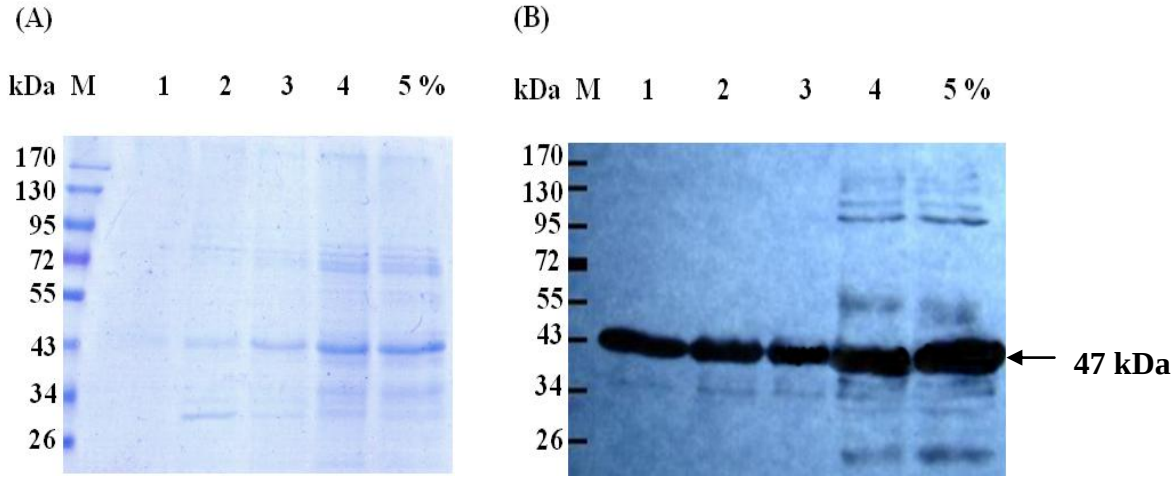

Figure 4-4 SDS-PAGE (A) and western blot (B) analysis of culture media from recombinant head domain neuraminidase induced with 1-5% methanol for 7 days.

Lane M : protein markers

Lane 1-5 : culture media from recombinant pPICZαA-SA induced with 1-5% methanol

4.2.2 Optimization of the time

Once the optimal concentration of methanol was obtained at 4 %, the optimal time was investigated by adding 4 % of fresh methanol to maintain the induction every 24 hr. The cells were incubated for 1 to 7 days at 30 °C, 250 rpm. In Figure 4-5, 4-6 and 4-7, the culture media from recombinant pPICZαA-NA and recombinant pPICZαA-SA were analyzed by SDS-PAGE (A) and western blot (B). From the results, the protein bands of approximately 47 kDa could be observed in the culture media after induced with 4% of fresh methanol for 1 to 7 days compared with culture medium from recombinant pPICZαA. The band intensity of 47 kDa protein was increased after 5 days of induction. Hence, the optimal time for induction selected to express full length (pPICZαA-NA) and head domain (pPICZαA-SA) of neuraminidase was 5 days.

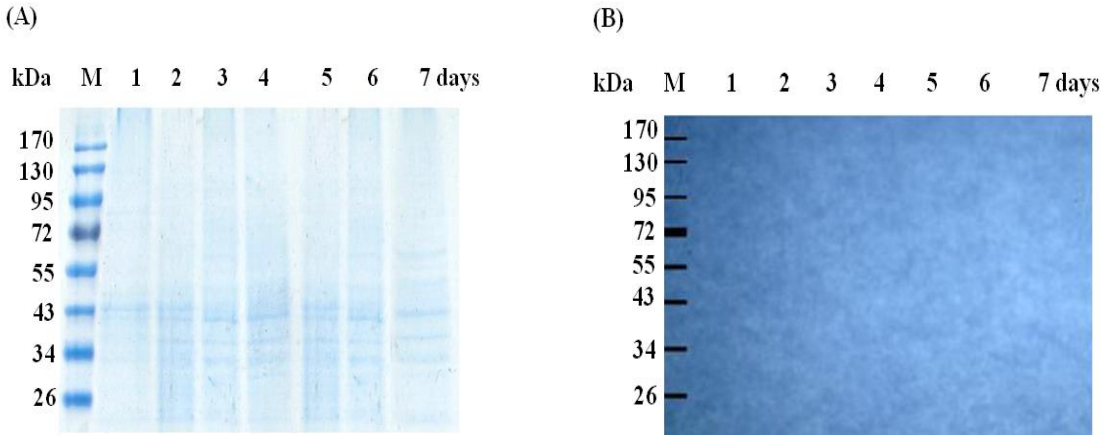

Figure 4-5 SDS-PAGE (A) and western blot (B) analysis of culture media from recombinant pPICZαA induced with 4% methanol for 1-7 days.

Lane M : protein markers

Lane 1-7 : culture media from recombinant pPICZαA induced with 4% methanol for 1-7 days

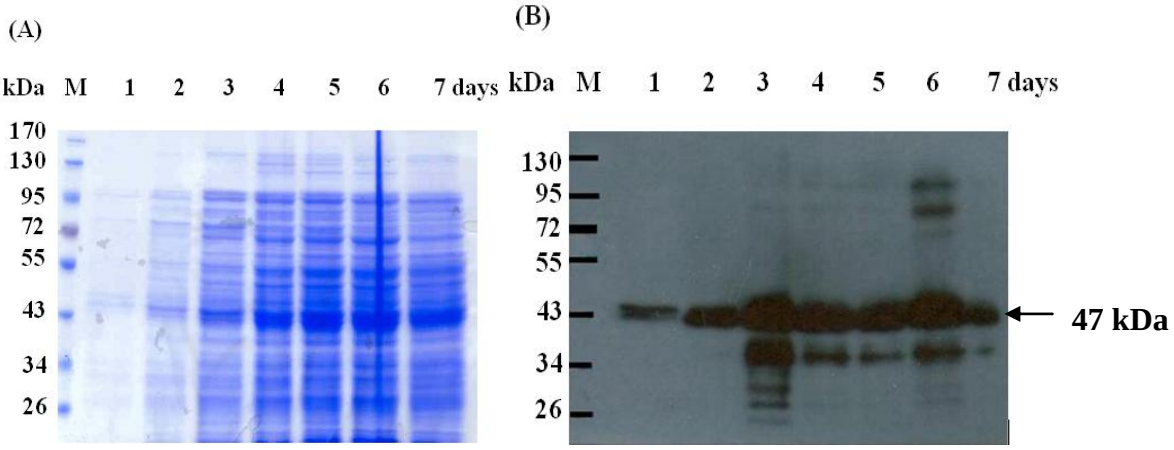

Figure 4-6 SDS-PAGE (A) and western blot (B) analysis of culture media from recombinant full length neuraminidase induced with 4% methanol for 1-7 days.

Lane M : protein markers

Lane 1-7 : culture media from recombinant pPICZαA-NA induced with 4% methanol for 1-7 days

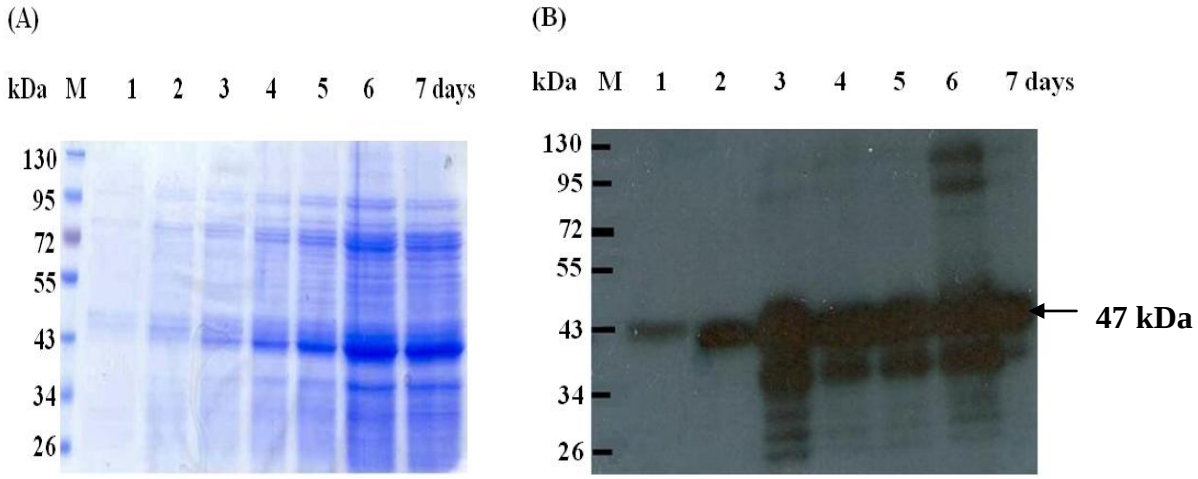

Figure 4-7 SDS-PAGE (A) and western blot (B) analysis culture media from recombinant head domain neuraminidase induced with 4% methanol for 1-7 days.

Lane M : protein markers

Lane 1-7 : culture media from recombinant pPICZαA-SA induced with 4% methanol for 1-7 days

In addition, the expressed recombinant proteins from the full length and the head domains were later compared to the inactivated virus in order to verify that the expressed recombinant proteins were the viral NA. The results from SDS-PAGE and western blot in Figure 4-8 showed that the protein bands of recombinant pPICZαA-NA and pPICZαA-SA approximately at 47 kDa were apparent in the culture media after being induced with 4% of fresh methanol for 4 and 5 days. Hence, the obtained recombinants were H5N1 neuraminidase.

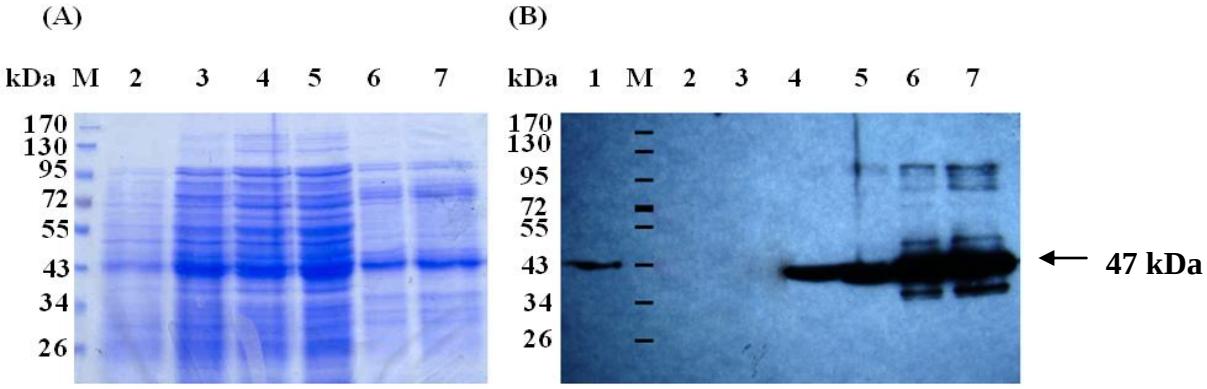

Figure 4-8 SDS-PAGE (A) and western blotting (B) analysis of culture media from recombinants full length and head domain neuraminidase induced with 4% methanol for 4 and 5 days.

Lane M : protein markers

Lane 1 : inactivated H5N1 virus,

Lane 2, 3 : culture media from recombinant pPICZαA induced with 4% methanol for 4, 5 days

Lane 4, 5 : culture media from recombinant pPICZαA-NA induced with 4% methanol for 4, 5 days

Lane 6, 7 : culture media from recombinant pPICZαA-SA induced with 4% methanol for 4, 5 days

4.3 Purification of neuraminidase

The recombinant pPICZαA-NA and pPICZαA-SA were grown at 30 °C, 250 rpm for 5 days in the presence of 4 % of fresh methanol to maintain the induction every 24 hr. After centrifugation at 10,000 rpm at 4 °C for 30 min., the supernatant was concentrated by Centricon concentrators. Then, the activities of neuraminidase were assayed and the protein contents were determined. The results obtained were tabulated in Table 4-1. From the table, it can be seen that the total proteins of the crude extract from recombinant full length neuraminidase was 7.58 mg, the total activities were equal to 487.66 µmol/min and the specific activities were 64.37 µmol/min/mg protein. For the crude extract from recombinant head domain neuraminidase, it was shown that the total proteins were 7.03 mg, total activities was 481.99 µmol/min and specific activities were 68.53 µmol/min/mg protein.

Table 4-1 The activities of full length and head domain neuraminidase

Fraction	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg protein)
Crude extract from recombinant full length neuraminidase	7.58	487.66	64.37
Crude extract from recombinant head domain neuraminidase	7.03	481.99	68.53

4.3.1 Purification of recombinant full length (pPICZαA-NA) neuraminidase

4.3.1.1 Phenyl-sepharose HP column

The crude extract from recombinant full length neuraminidase was purified by phenyl-sepharose HP column. The flow through proteins were initially obtained by 40 % ammonium sulfate, 20 mM potassium phosphate, pH 7.0 and adsorbed fractions were later eluted by 10 mM NaCl, 10 mM Tris, pH 7. The results were illustrated in Figure 4-9 with 2 peaks of proteins in the first flow through peak and the second eluted peak. Both peaks were, the flow through fractions from 10-50 and the eluted fractions from 68-80. Then, fraction 15 and 71 were analyzed by SDS-PAGE and western blot. It was found that the proteins were detected in the flow through but no band of protein was visible in the eluted. (Figure 4-10).

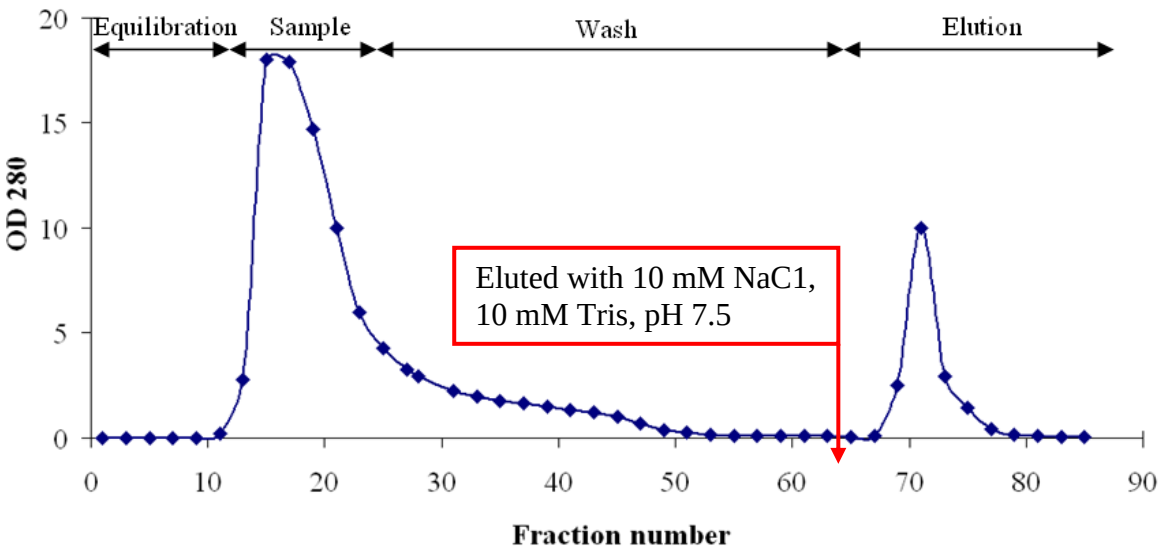

Figure 4-9 Chromatographic purification profile of recombinant full length neuraminidase. Approximately 70 mg of sample was loaded on phenyl-sepharose HP column (5x1 ml) previously equilibrated with 35 % $(NH_4)_2SO_4$, 20 mM potassium

phosphate, pH 7.0 flow rate: 1 ml/min eluted with 10 mM NaC1, 10 mM Tris, pH 7.5 ,2 ml fractions were collected.

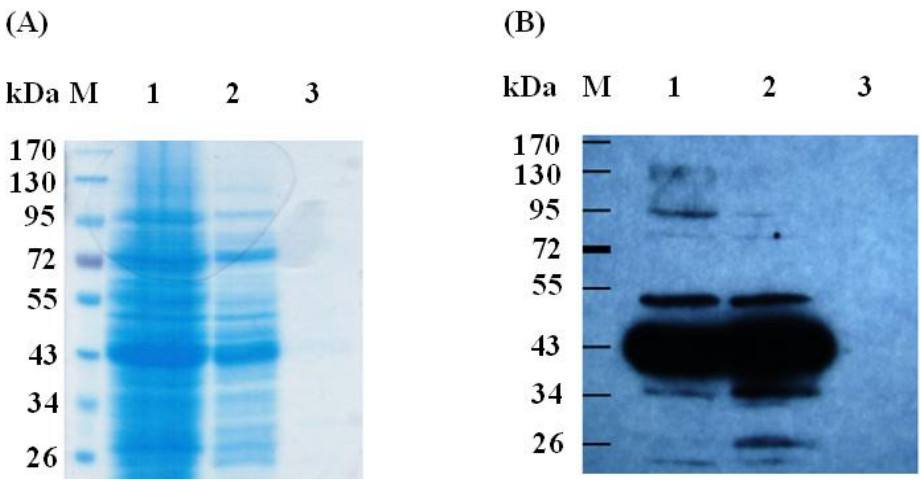

Figure 4-10 SDS-PAGE (A) and western blot analysis (B) of purified full length neuraminidase from phenyl-sepharose HP column

Lane M : protein markers

Lane 1 : crude extract from recombinant full length neuraminidase

Lane 2 : fraction number 15 (flow through)

Lane 3 : fraction number 71 (elution)

4.3.1.2 Sepharose Q column

Since the first phenyl-sepharose column could not separate the crude extract from recombinant full length neuraminidase, the sample was then again separated by sepharose Q column and the obtained chromatogram was shown in Figure 4-11. The flow through proteins were initially obtained by 20 mM diethanolamine pH 8.5 and adsorbed fractions were expected eluted by linear NaCl gradient from 0 to 400 mM containing 20 mM diethanolamine buffer, pH 8.5. Unfortunately,

only one peak of protein was obtained indicating that the proteins still could not be separated by this column.

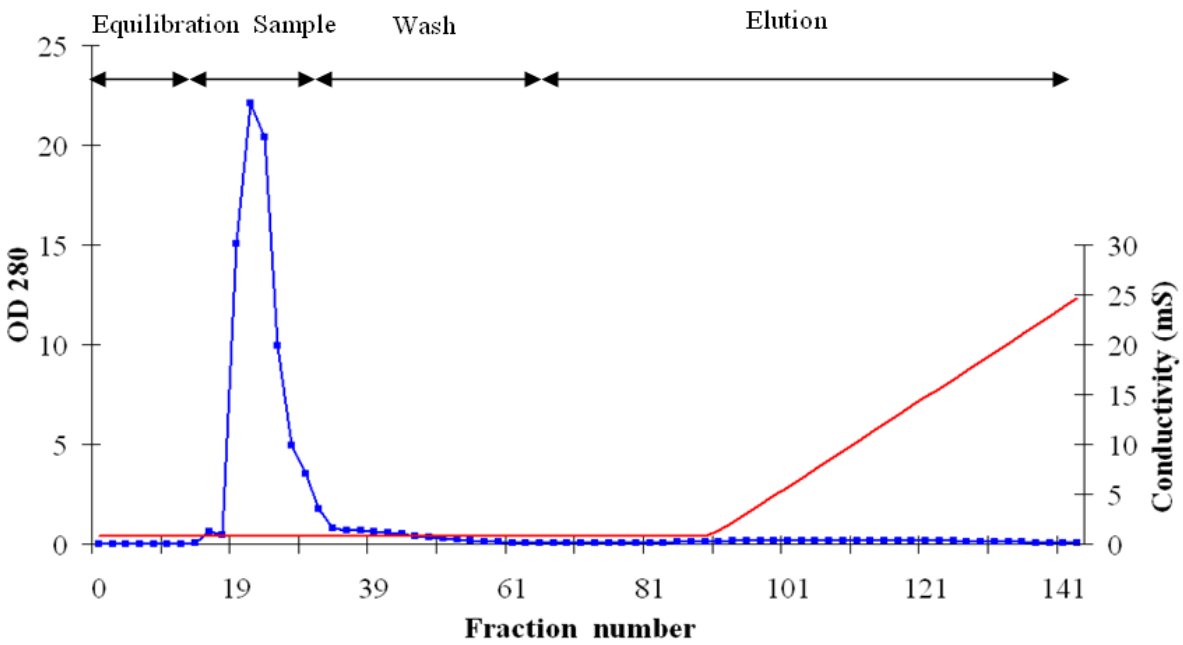

Figure 4-11 Chromatographic purification profile of recombinant full length neuraminidase. Approximately 70 mg of sample was loaded on Sepharose Q column (5x1 ml) equilibrated with 20 mM diethanolamine pH 8.5; flow rate 1 ml/min and eluted with linear NaCl gradient from 0 mM to 400 mM in 20 mM diethanolamine buffer, pH 8.5, 2 ml fractions were collected.

4.3.1.3 CM sepharose column

From the results of the first and second purification, the sample of proteins could not be separated. Therefore, the crude extract from recombinant full length neuraminidase was then purified by CM sepharose column and the chromatogram was shown in Figure 4-12. The flow through proteins were initially obtained by 20 mM diethanolamine pH 8.5 and adsorbed fractions were later eluted by linear NaCl gradient from 0 to

400 mM containing 20 mM diethanolamine buffer, pH 8.5. Similar results as the phenyl-sepharose HP and sepharose Q column were obtained that no peak was detected in the eluted fractions but all proteins were present in the flow through.

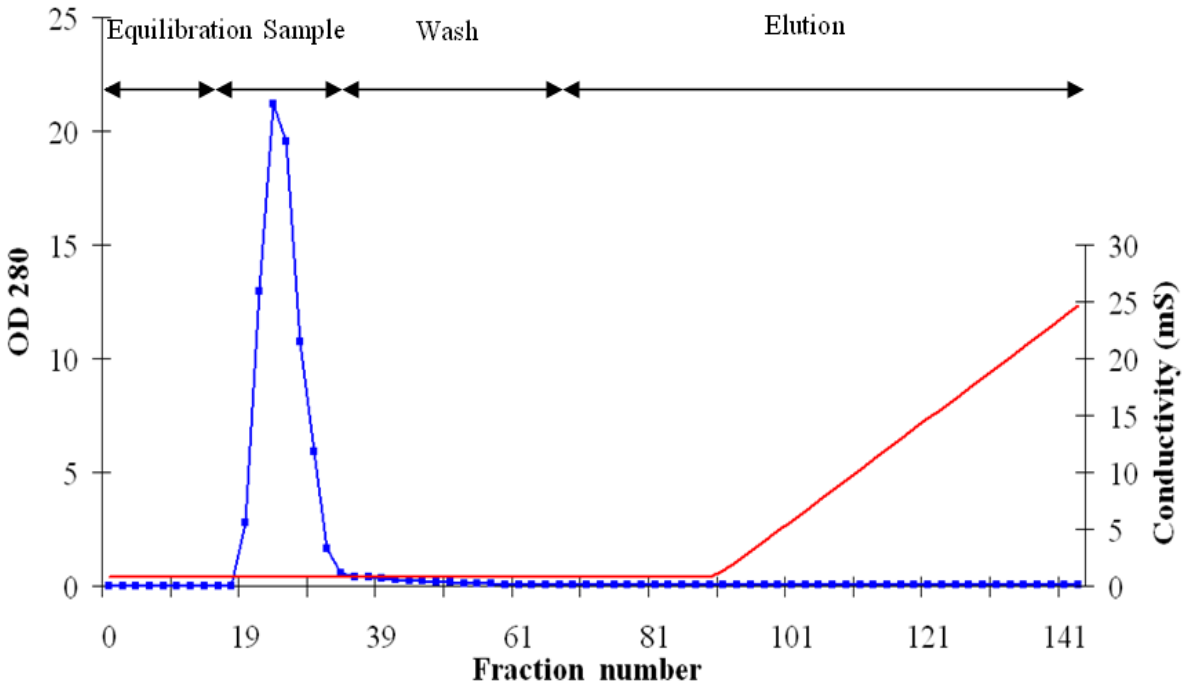

Figure 4-12 Chromatographic purification profile of recombinant full length neuraminidase. Approximately 70 mg of sample was loaded on CM Sepharose column (5x1 ml) equilibrate with 20 mM diethanolamine pH 8.5; flow rate 1 ml/min and eluted with linear NaCl gradient from 0 mM to 400 mM in 20 mM diethanolamine buffer, pH 8.5, 2 ml fractions were collected.

From overall results of the recombinant full length (pPICZαA-NA) neuraminidase, it was shown that the expressed protein could not be purified.

4.3.2 Purification of recombinant head domain (pPICZαA-SA) neuraminidase

4.3.2.1 Phenyl-sepharose HP column

The crude extract from recombinant head domain neuraminidase was purified by phenyl-sepharose HP column. The flow through proteins were initially obtained by 40 % ammonium sulfate, 20 mM potassium phosphate, pH 7.0 and adsorbed fractions were later eluted by 10 mM NaCl, 10 mM Tris, pH 7. The results were illustrated in Figure 4-13 that no peak was detected in the eluted fractions but all proteins were present in the flow through fractions. The peak appeared to show very small shoulder at fractions 30-40.

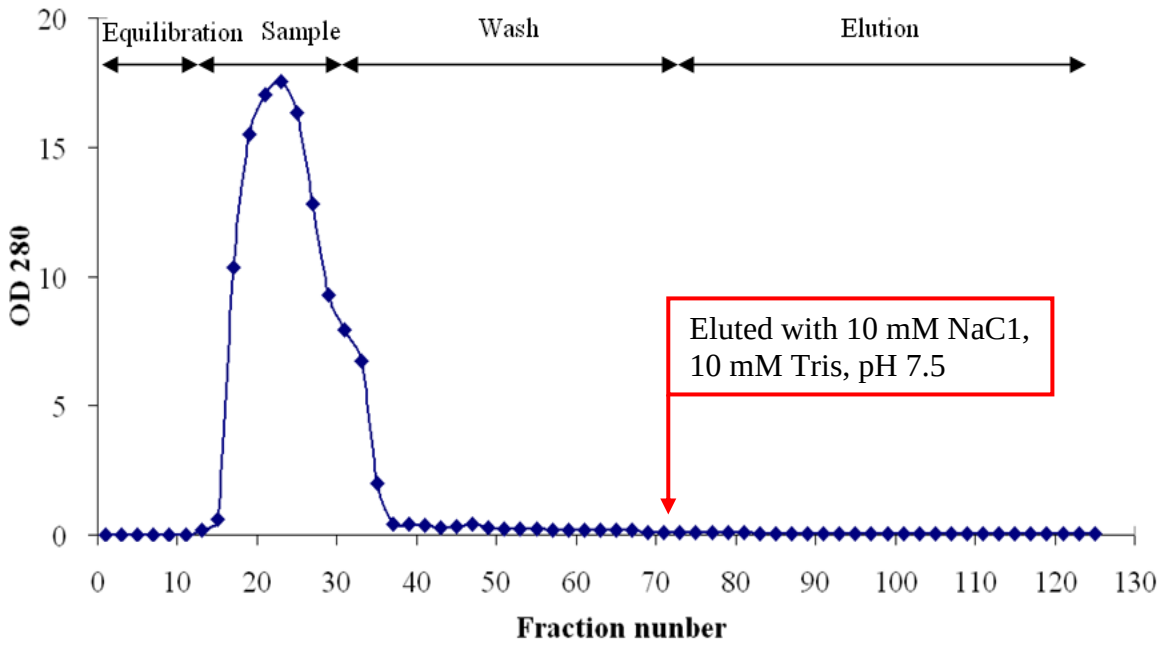

Figure 4-13 Chromatographic purification profile of recombinant head domain neuraminidase. Approximately 70 mg of sample was loaded on phenyl-sepharose HP column (5x1 ml) previously equilibrated with 35 % $(NH_4)_2SO_4$, 20 mM potassium

phosphate, pH 7.0 flow rate: 1 ml/min eluted with 10 mM NaC1, 10 mM Tris, pH 7.5, 2 ml fractions were collected.

4.3.2.2 Sepharose Q column

Since the first phenyl- sepharose column could not separate the crude extract from recombinant head domain neuraminidase, the sample was then again separated by sepharose Q column and the obtained chromatogram was shown in Figure 4-14. The flow through proteins were initially obtained by 20 mM diethanolamine pH 8.5 and adsorbed fractions were later eluted by linear NaCl gradient from 0 to 400 mM containing 20 mM diethanolamine buffer, pH 8.5. When the proteins from those 2 peaks were analyzed by SDS-PAGE and western blot, the proteins in 3 fractions of 21, 23 and 25 in the flow through and, the eluted proteins in fractions 101, 103 and 105 were detectable with the approximate size of 47 kDa as illustrated in Figure 4-15. From the analysis by the western blot, it was found that the protein was partially purified. Since the results detected from the western blot revealed the presence of protein bands in both the flow through and the eluted fractions, the fractions 17-45, and 91-137, were then pooled and assayed for the activities of neuraminidases. It was found that the activities were detected only in the eluted pool, 91-137, the total proteins were 4.11 mg and the total activities were 361.52 µmol/min. The specific activities were equal to 87.96 µmol/min/mg protein with the purification folds of 1.28. Finally, the yield of the

purification was 75.01 %. The results of the activities from the purified head domain NA were tabulated in Table 4-2.

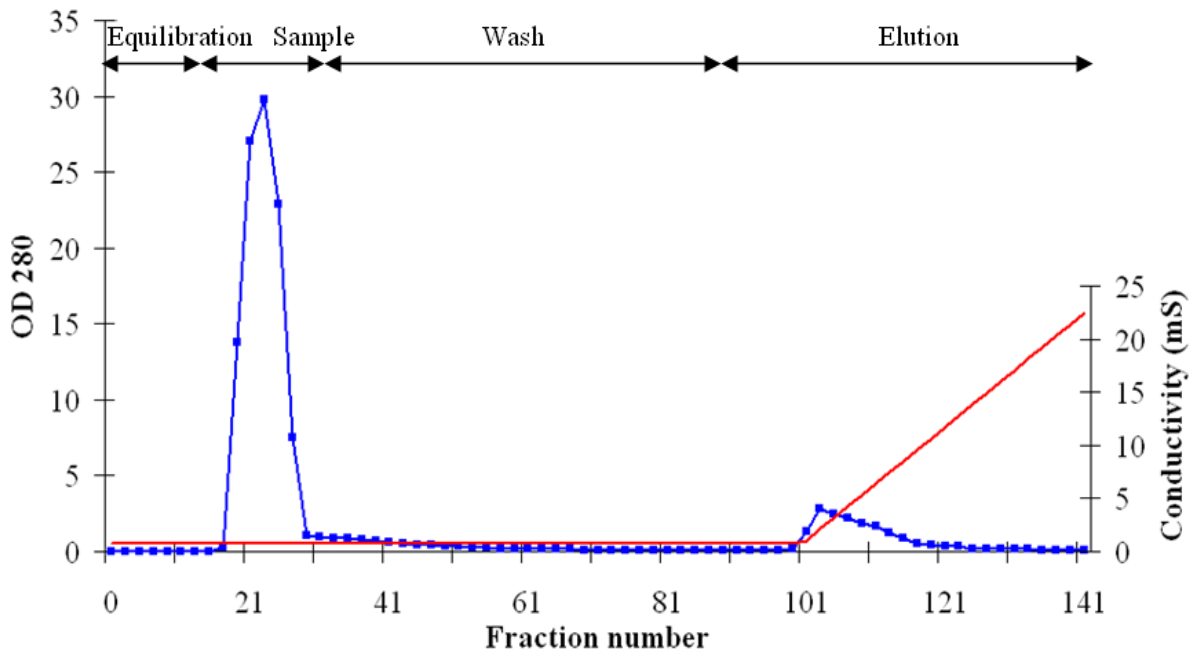

Figure 4-14 Chromatographic purification profile of recombinant head domain neuraminidase Approximately 70 mg of sample was loaded on sepharose Q column (5x1 ml) equilibrated with 20 mM diethanolamine pH 8.5; flow rate 1 ml/min and eluted with linear NaCl gradient from 0 mM to 400 mM in 20 mM diethanolamine buffer, pH 8.5, 2 ml fraction.

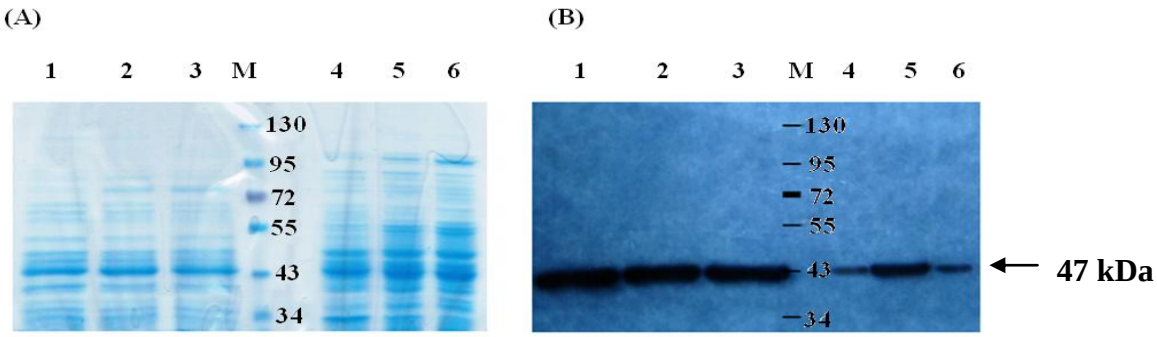

Figure 4-15 SDS-PAGE (A) and Western blot analysis (B) of purified head domain neuraminidase by sepharose Q column

Lane M : protein markers

Lane 1-3 : fraction 21, 23 and 25 (flow through)

Lane 4-6 : fraction 101, 103 and 105 (elution)

Table 4-2 Purification of head domain neuraminidase

Fraction	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/ mg protein)	Purification (folds)	Activity Yield (%)
Crude extract from recombinant head domain neuraminidase	7.03	481.99	68.53	1	100
Flow through Fractions 17 -45 from Sepharose Q	2.26	0	0	0	0
Elution Fractions 91 - 137 from Sepharose Q	4.11	361.52	87.96	1.28	75.01

4.4 Characterization of head domain neuraminidase

Since the full length neuraminidase could not be purified, only the recombinant of the head domain was characterized as follows:

4.4.1 Kinetics analysis

The head domain neuraminidase was characterized by the determination of Michaelis-Menten constant (K_m) and maximum velocity (V_{max}). The enzyme preparations were incubated with the increasing final concentrations of substrates, MUNANA, spanning the range from 0-300 µM. The velocities of the head domain neuraminidase as then plotted against the substrate concentrations illustrated in Figure 4-16. The result showed that the enzyme apparently followed the Michaelis-Menten equation. At low concentrations of MUNANA from 0-100 µM, the velocities of the head domain neuraminidase increased dramatically. When the concentrations of substrate increased further from 100 to 300 µM, the velocity started to level off at the steady state and possibly became saturated at 300 µM. The apparent kinetic constants, K_m and V_{max} were then determined by fitting the collected data to a Michaelis-Menten equation by non-linear regression analysis using GraphPad Prism 5 (GraphPad software) as tabulated in Table 4-3.

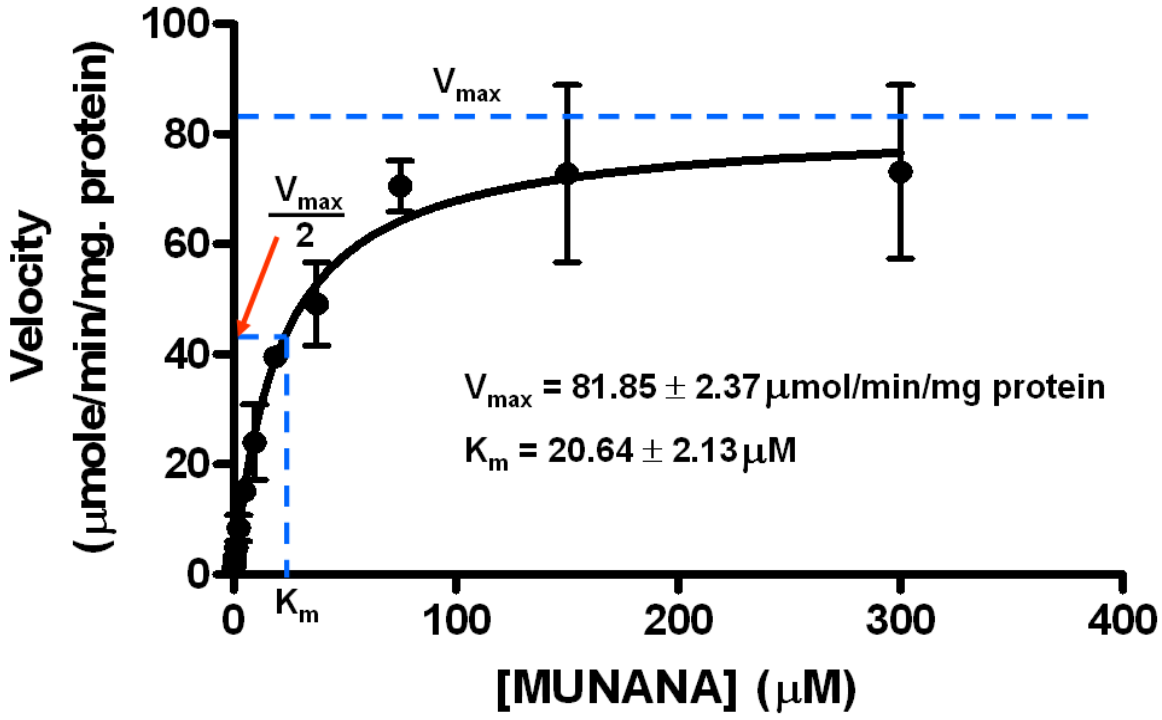

Figure 4-16 The effect of MUNANA concentrations on the activities of purified head domain neuraminidase. The kinetic parameters K_m and V_{max} were obtained by nonlinear regression analysis of experimental steady-state data using the GraphPad Prism 5 computer program (GraphPad software)

Table 4-3 Kinetic analysis of the recombinant head domain neuraminidase

	K_m ± SD (μM)	**V_{max} ± SD (μmol/min/ mg protein)**
Head domain neuraminidase	20.64 ± 2.13	81.85 ± 2.37
Virus NA (Remeix-Welt *et al*., 2006)	21 ± 1	-
Virus NA (Yuthavong *et al*., 2009)	15 ± 3	-
Virus NA (Schmidt *et al*., 2011)	24.16 ± 1.1	-

4.4.2 Inhibition of oseltamivir carboxylate

In this work, the effect of the drug, oseltamivir carboxylate, concentrations on the activities of head domain NA was investigated. The activities of the head domain neuraminidase were determined in the presence of 0-20 μM oseltamivir carboxylate and the results were illustrated in Figure 4-17. It can be seen that the slopes of the inverse plot were increased with the elevated drug concentrations. It was clearly shown from the plot that the same Y intercept was obtained from each concentration of the drug whereas the K_m was increased with the increase of the inhibitor concentrations from 5 -20 μM. This indicates that the inhibition was competitive.

Then, the secondary plot was later obtained from the relationship between the slope of Lineweaver-Burk plot of head domain neuraminidase (from Figure 4-17) against the concentrations of substrate, MUNANA, in the presence of elevated concentrations of Tamiflu from 5-20 μM and the results were illustrated in Figure 4-18. The straight line was consequently obtained and the inhibition constant (K_i) was determined as 84.15 nM. Subsequently, the IC_{50} of oseltamivir carboxylate was determined from plotting between the % inhibition and the logarithm concentration of oseltamivir carboxylate, the values of IC_{50} was found to be equaled to 30.03 nM (Appendix H). The characterization of the recombinant head domain neuraminidase kinetics was tabulated in Table 4-4.

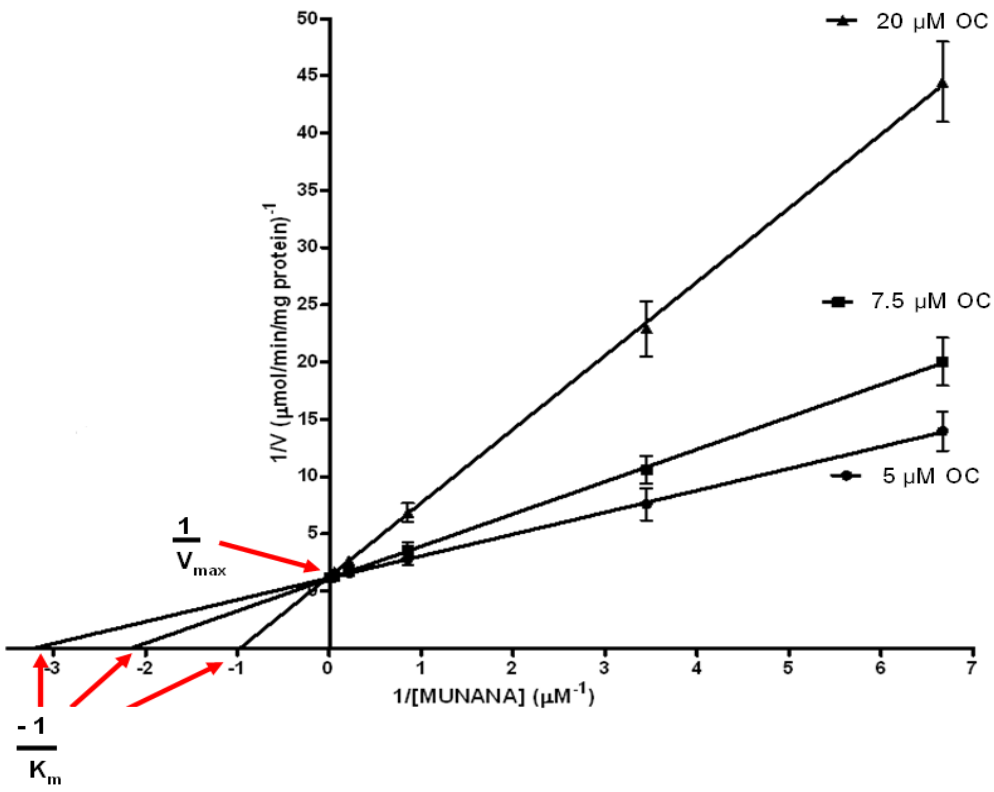

Figure 4-17 Lineweaver-Burk plot of head domain neuraminidase in the presence of ($[I]_1$), 7.5 ($[I]_2$) and 20 ($[I]_3$) μM of OC (oseltamivir carboxylate)

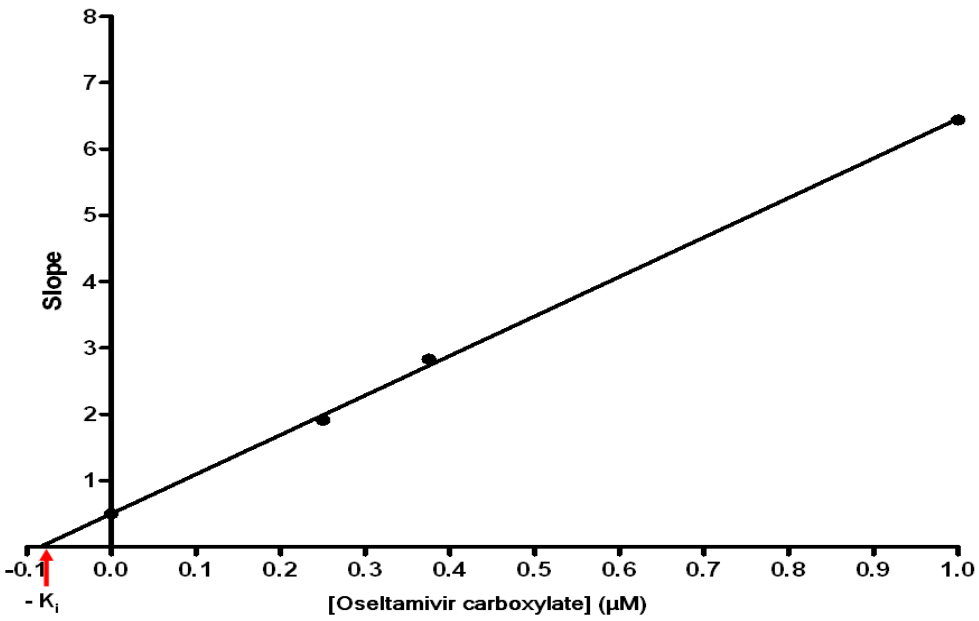

Figure 4-18 Secondary plot for the inhibition of oseltamivir carboxylate

Table 4-4 Kinetic analysis of the neuraminidase head domain in the presence of inhibitor

	K_i (nM)	**IC_{50} (nM)**
Head domain neuraminidase	84.15	30.03
Virus NA (Remeix-Welt *et al*., 2006)	127 ± 31	2.3 ± 0.3
Virus NA (Yuthavong *et al*., 2009)	0.0013 ± 0.0004	0.0121
Virus NA (Schmidt *et al*., 2011)	3.78 ± 0.2	3.72 ± 0.45

4.4.3 Screening of inhibitors from Thai medicinal plant

The extracts from ten types of natural Thai medicinal plants were studied for the inhibitory effect on the activities of neuraminidase as follows; *Allium sativum* (garlic), *Celosia argentea* (red cockscomb), *Clerodendrum inerme* (sammanga), *Colocasia esculenta* (born), *Phyllanthus amarus* (loog tai bai) (born), *Rhizophora apiculata* (gong garng bai lek), *Rhizophora mucronata* (gong garng bai yai), *Sesuvium portulacastrum* (pak bia talay), *Suaeda maritime* (cha krarm) and *Vernonia cinerea* (ya dorg kao). From Table 4-5, *Vernonia cinerea* (ya dorg kao) resulted in the smallest specific activity of the NA at 0.62 µmol/min/mg protein whereas the highest was obtained from *Rhizophora mucronata* (gong garng bai yai) at 326.21 µmol/min/mg protein. The antiviral drug, oseltamivir carboxylate, gave the specific activity at 8.05 µmol/min/mg protein. The other 8 types of medicinal plants showed the results of approximately 240 to 290 µmol/min/mg protein

Table 4-5 The inhibitory activities of Thai medicinal plant extracts compared to the antiviral drug, the oseltamivir carboxylate and purified head domain neuraminidase.

	Specific activity (µmol/min/mg protein)	**% Inhibition**
NA activity	436.98	0
Oseltamivir carboxylate	8.05	98.16 ± 0.01
Vernonia cinerea (ya dorg kao)	0.62	99.86 ± 0.01
Suaeda maritime (cha krarm)	244.23	44.32 ± 0.42
Sesuvium portulacastrum (pak bia talay)	245.68	43.15 ± 1.48
Rhizophora apiculata (gong garng bai lek)	251.76	42.61 ± 0.16
Phyllanthus amarus (loog tai bai)	253.34	41.90 ± 2.79
Colocasia esculenta (born)	255.46	41.50 ± 0.75
Celosia argentea (red cockscomb)	258.46	41.09 ± 1.89
Allium sativum (garlic)	278.76	36.44 ± 0.66
Clerodendrum inerme (sammanga)	291.74	33.10 ± 1.89
Rhizophora mucronata (gong garng bai yai)	326.21	25.62 ± 2.21

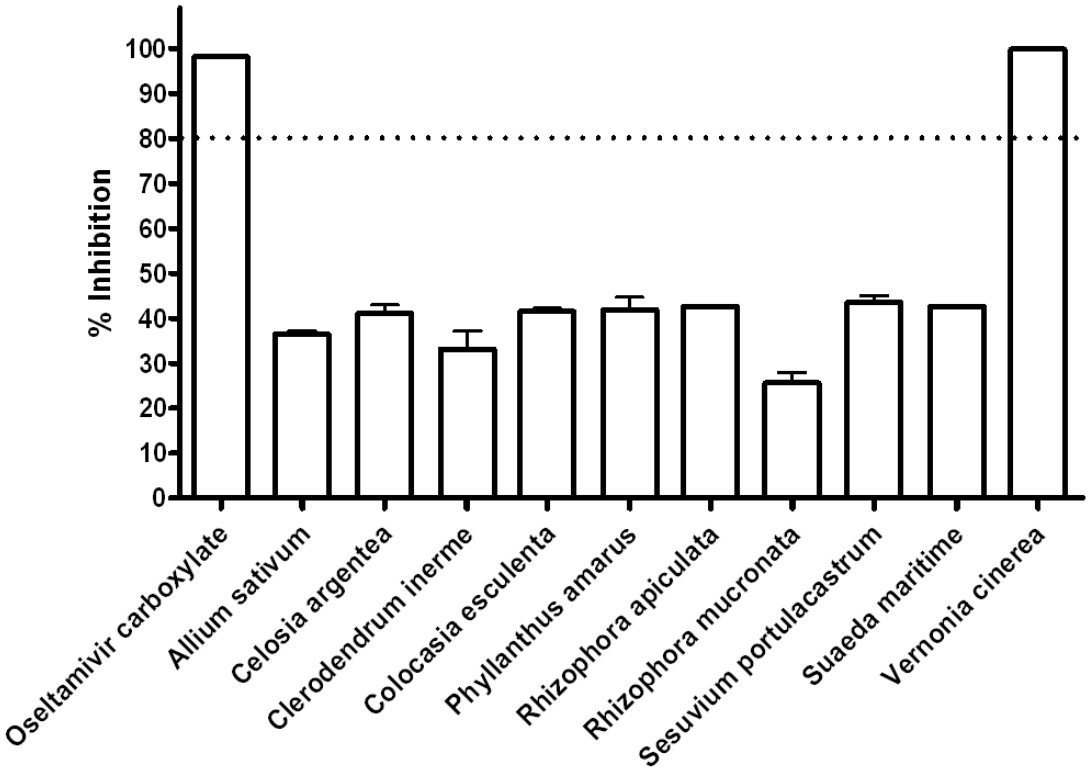

Figure 4-19 Inhibitory activities of Thai herbal extracts and oseltamivir carboxylate

Figure 4-19 showed the comparison of the inhibitions obtained from all the ten types of medicinal plant extracts and the antiviral drug, oseltamivir carboxylate. It can be concluded that *Vernonia cinerea* (ya dorg kao) extract showed the highest 99.86 ± 0.01 % inhibition of the activity of neuraminidase closed of the drug effect at 98.16 ± 0.01 %. On the contrary, *Rhizophora mucronata* (gong garng bai yai) extract gave only 25.62 ± 2.21 % inhibition and not that much different from the other eight types of extracts.

CHAPTER V

DISCUSSION

The pathogenic avian influenza A (H5N1) virus is highly contagious among birds including poultry in several continents and poses a serious threat to human health owing to its virulence with high lethality, endemic presence, increasingly large host reservoir, and significant ongoing mutation (Yuthavong *et al*., 2009). Previous studies revealed that neuraminidase is a major surface glycoprotein containing the enzymatic activity essential for influenza virus replication and infection (Gong *et al*., 2007). In the full length (pPICZαA-NA) neuraminidase, there are three distinct domains parts namely; globular head domain containing the enzyme active site, a highly variable stalk region, and *N*- terminal signal anchor domain whereas the head domain (pPICZαA-SA) neuraminidase only consists of globular head domain functioning as antigen together with the enzyme which catalyzes the release of virus from the host cells (Air and Laver, 1989; Ohuchi *et al*., 2006). Therefore, this research was focussed on the expression, purification and characterization of the full length and head domain of neuraminidase from avian flu virus (H5N1) in *Pichia pastoris*.

5.1 Expression of recombinants in *Pichia pastoris*

The expression of recombinant full length (pPICZαA-NA) and head domain (pPICZαA-SA) neuraminidase were initially tested in the presence of 3% methanol for the period of 7 days. The proteins obtained were analyzed by SDS-PAGE and later confirmed by western blot by the application of antibody specific to the protein

neuraminidase. From the results, it was found that the sizes of the expressed recombinant full length (pPICZαA-NA) and head domain (pPICZαA-SA) were approximately 47 kDa (Figure 4-1). Since the expected size of recombinant head domain neuraminidase (pPICZαA-SA) was estimated at only about 40 kDa (Niwasabutra, 2009), the size increase of the expressed neuraminidase might have been from the post-translation modification in *Pichia pastoris* such as N-glycosylation (Cereghino *et al*., 2002). The result obtained was consistent with previous studies. In yeast expression system of *Pichia pastoris*, both N-linked and O- linked glycans can be observed in heterologous proteins (Bretthauer and Castellino, 1999). The expression of recombinant head domain neuraminidase from avian influenza virus A/Viet Nam/DT-036/2005 (H5N1) in *Pichia pastoris* was carried out by Yuthavong *et al* (2009). Their results similarly revealed that the protein size was approximately 72 kDa higher than the expected 45 kDa from the N-linked glycosylation. In addition, Martinet *et al* (1997) reported that the N-linked glycosylation was detected with the additional 30-40 residues of mannoses. This resulted in the size increase of the expressed neuraminidase from 43 kDa to 65 kDa.

5.2 Optimization of the recombinant expression in *Pichia pastoris*

The optimal methanol concentration and incubation time are the important factors for the expression. Too high the concentration of methanol may lead to low expression of protein or even death. On the other hand, too low the concentration of methanol added in the reaction may not be sufficient. Similarly, the shorter time may not be suitable for expression of yeast cell whereas the longer time may attribute to

cell damage. In this research, the expressed protein was secreted into the culture medium and the expression level of protein was induced in the presence of different methanol concentrations and time of induction. The optimal conditions for the expression of both recombinant neuraminidases were found to be 4 % of methanol for the period of 5 days. The obtained results were different from Martinet *et al* (1997) and Yuthavong *et al* (2009). Martinet *et al* (1997) induced the expression by 0.5% methanol for the period of 2 days whereas Yuthavong *et al* (2009) was 2-3% of methanol for 4 days. The differences of the optimal conditions may stem from the differences of the strains or the host cells applied in the studies. Influenza virus A/Victoria/3/75 (H3N2) was used in Martinet *et al* (1997) whereas recombinant from influenza virus A/Vietnam/DT-036/2005 (H5N1) was used in Yuthavong *et al* (2009) and this work was the recombinant from influenza virus A/chicken/Thailand/Nontaburi/CK162/2005.

5.3 Purification of neuraminidase

5.3.1 Purification of recombinant full length (pPICZαA-NA) neuraminidase

When the crude extract from recombinant full length neuraminidase was separated by hydrophobic phenyl-sepharose HP and again with sepharose Q, it could not be purified (Figure 4-9 to 4-11). This may be explained from the same net positive charges present in the molecules of full length neuraminidase (Burgess, 2008) which is similar to the anion exchange column. Therefore, the proteins were then subsequently purified by cation exchange chromatography using CM sepharose column (Figure 4-12). However, the recombinant full length neuraminidase from avian influenza virus (H5N1)

still could not be purified. This is consistent with the fact that the purification of this recombinant has not yet been previously reported. However, Dalakouras *et al* (2006) studied the purification of recombinant full length neuraminidase in influenza virus A/Beijing/262/95 (H1N1) in *expres*SF+ insect cell by affinity chromatography using the immobilized ligand, bearing phenylalanine and isoleucine linked on the chlorotriazine ring (Phe-Trz-Ile) which displayed high affinity for NA. Nevertheless, the protein could only be partially purified and that some unclarified crude extract was still detectable.

5.3.2 Purification of recombinant head domain (pPICZαA-SA) neuraminidase

The crude extract from recombinant head domain neuraminidase was separated by hydrophobic phenyl-sepharose HP, anion exchange sepharose Q. The recombinants could not be purified by hydrophobic phenyl-sepharose HP column. The purification was nonetheless obtained from the anion exchange sepharose Q columns (Figure 4-14). The results showed that 1.28 folds of purification was obtained and the activity yield was approximately 75% (Table 4-2). The recombinant protein homogeneity was supported by the evidence from the western blot analysis. The homogeneity was also detected by SDS-PAGE. The MW was estimated at 47 kDa. (Figure 4-15). From the literature, this recombinant head domain neuraminidase from avian influenza virus (H5N1) has not been previously purified. This work therefore was the first report on the head domain neuraminidase purification.

5.4 Characterization of head domain neuraminidase

5.4.1 Kinetics analysis

The enzymatic properties of the head domain neuraminidase were studied. From the results in Figure 4-16, the velocities of the head domain neuraminidase were plotted against the substrate, MUNANA, concentrations. The head domain NA apparently followed the Michaelis-Menten equation. At the low concentration of MUNANA around 0-100 µM, the velocity was increased dramatically indicating the first order reaction with respect to the MUNANA. When the concentration of substrate was increased to 100 µM, the velocity started to be levelled and saturation was reached at 300 µM indicating that the reaction has reached the zero order with respect to substrate. From the results, the obtained value of K_m approximately 20.64 ± 2.13 µM, was closed to that reported by Remeix-Welt *et al*. (2006), Yuthavong *et al*. (2009) and Schmidt *et al*. (2011) who had studied the enzymatic properties of native virus. Remeix-Welt *et al*. (2006) studied the enzymatic properties of virus NA (Influenza virus strain A/HongKong/156/97 (H5N1)) and obtained the value of K_m equaled to 21 ± 1 µM. Yuthavong *et al*. (2009) determined the K_m from Influenza virus strain A/Vietnam/DT-036/2005 (H5N1) and 15 ± 3 µM was obtained. Schmidt *et al*. (2011) studied enzymatic properties of the original Hokkaido virus NA (Influenza virus strain A/Hokkaido/15/02 (H1N1)) and the K_m was equal to 24.16 ± 1.1 µM.

5.4.2 Inhibition of oseltamivir carboxylate

Oseltamivir carboxylate**,** commercially called Tamiflu is the antiviral drug used to cure and protect the patients infected by influenza A and B viruses. Normally, the drug is orally administered and shows inhibition upon the activities of NA by acting as the transition state analogue inhibitor towards sialic acid, found on the surface proteins of normal host cells.

The effect of oseltamivir carboxylate on the activities of NA was investigated. From Figure 4-17**,** it was clearly seen that oseltamivir carboxylate could inhibit the activities of the enzyme indicating that it was the inhibitor. The kinetic plots also indicated that the inhibition was competitive. Moreover, when the Lineweaver-Burke plot was obtained, the values of K_i and IC_{50} were determined from the secondary plots of Lineweaver-Burk plot of head domain neuraminidase (Figure 4-17) against the concentrations of oseltamivir carboxylate in Figure 4-18 and the results were tabulated in Table 4-4. From the table, it can be seen that the values of K_i and IC_{50} of oseltamivir carboxylate at 84.15 and 30.03 nM were not much different from Remeix-Welt *et al*. (2006), Yuthavong *et al*. (2009) and Schmidt *et al*. (2011).

Remeix-Welt *et al*. (2006) reported the values of K_i and IC_{50} of oseltamivir carboxylate in Influenza virus strain A/HongKong/156/97 (H5N1) at 127 ± 31 and 2.3 ± 0.3 nM, respectively. On the contrary, Yuthavong *et al*. (2009) reported much lower values of K_i at 0.0013 ± 0.0004 and IC_{50} at 0.0121 nM from the neuraminidase in influenza virus strain A/Vietnam/DT-036/2005 (H5N1). On the other hand, the values of K_i and IC_{50} of oseltamivir carboxylate obtained from Schmidt *et al*. (2011) in the original Hokkaido virus NA

(Influenza virus strain A/Hokkaido/15/02 (H1N1)) were 3.78 ± 0.2 and 3.72 ± 0.45 nM respectively. The discrepancies might have been the results from the differences in the strains of avian influenza virus (Figure 5-1), sources of oseltamivir carboxylate or variation in the assay conditions (Yuthavong *et al*., 2009).

K_i is difined as the inhibitory power depending upon the affinity of the enzyme for inhibitor. It is the dissociation constant of the enzyme-inhibitor complex, or the reciprocal of the binding affinity of the inhibitor to the enzyme (Cheng and Prusoff, 1973). The lower K_i reflects the increased affinity for inhibitor (Pruett, 2002). Hence, the lower K_i of oseltamivir carboxylate obtained in this study indicated that the drug can effectively inhibit the activity of neuraminidase. However, the K_i was higher than those of the other studies, it could be suggested that this strain of H5N1 might have possibly shown resistance to the oseltamivir carboxylate.

IC_{50} is the value which expresses the concentration of inhibitor required to produce 50 percent inhibition of an enzymatic reaction at a specific substrate concentration (Cheng and Prusoff, 1973). The higher concentration of oseltamivir carboxylate was required to inhibit the enzyme activity (Table 4-4) indicating the increase of the resistance to antiviral activity of this strain. From the results of K_i and IC_{50}, it can be revealed that this strain could resist to antiviral drug oseltamivir carboxylate. Therefore, such strain can be applied for the search of a new antiviral drug with the higher efficiency of NA than the normally used standard oseltamivir carboxylate.

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Chicken/Thailand/Nontaburi        --------------------------------------------------
Neuraminidase_Hongkong            QKQEIKMNPNQKIITIGSICMVVGIISLMLQIGNIISVWVSHIIQTWHP-
Neuraminidase_Vietnam             ------MNPNQKIITIGSICMVTGIVSLMLQVGNMISIWVSHSINTGNQ-
Neuraminidase_Japan/Hokkaido      ------MNPNQKIITIGSISMAIGIISLILQIGNIISIWASHSIQTGSQN

Chicken/Thailand/Nontaburi        --------------------------------------------------
Neuraminidase_Hongkong            ------------------NQPEPCNQSINFYTEQAAASVTLAGNSSLCPI
Neuraminidase_Vietnam             ------------------HQAEPISN-ANFLTEKAVASVKLAGNSSLCPI
Neuraminidase_Japan/Hokkaido      HTGICNQRIITYENSTWVNQTYVNINNTNVVAGKDKTSVTLAGNSSLCPI

Chicken/Thailand/Nontaburi        -------KDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGSLLNDKH
Neuraminidase_Hongkong            SGWAIYSKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKH
Neuraminidase_Vietnam             NGWAVYSKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKH
Neuraminidase_Japan/Hokkaido      RGWAIYTKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKH
                                         ************************************:******

Chicken/Thailand/Nontaburi        SNGTVKDRSPHRTLMSCPVGEAPSPYNSRFESVAWSASACHDGTSWLTIG
Neuraminidase_Hongkong            SNGTVKDRSPYRTLMSCPVGEAPSPYNSRFESVAWSASACHDGISWLTIG
Neuraminidase_Vietnam             SNGTVKDRSPHRTLMSCPVGEAPSPYNSRFESVAWSASACHDGTSWLTIG
Neuraminidase_Japan/Hokkaido      SNGTVKDRSPYRALMSCPLGEAPSPYNSRFESVAWSASACHDGMGWLTIG
                                  **********:*:*****:************************ .*****

Chicken/Thailand/Nontaburi        ISGPDSGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTD
Neuraminidase_Hongkong            ISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTD
Neuraminidase_Vietnam             ISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTD
Neuraminidase_Japan/Hokkaido      ISGPDNGAVAVLKYNGIITETIKSWKKRILRTQESECVCVNGSCFTIMTD
                                  *****.*************:*****::.*********.********:***

Chicken/Thailand/Nontaburi        GPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYPDAGEITCVCRD
Neuraminidase_Hongkong            GPSNEQASYKIFKIEKGRVVKSVELNAPNYHYEECSCYPDAGEITCVCRD
Neuraminidase_Vietnam             GPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYPDAGEITCVCRD
Neuraminidase_Japan/Hokkaido      GPSNGAASYRIFKIEKGKVTKSIELDAPNYHYEECSCYPDTGTVMCVCRD
                                  ****  **::***:***:*.**:**:***************:* : *****

Chicken/Thailand/Nontaburi        NWHGSNRPWVSFNQNLEYQIGYICSGVFGDTPRPNDGTGSCGPVSSNGAY
Neuraminidase_Hongkong            NWHGSNRPWVSFNQNLEYQIGYICSGVFGDSPRPNDGTGSCGPVSLNGAY
Neuraminidase_Vietnam             NWHGSNRPWVSFNQNLEYQIGYICSGVFGDTPRPNDGTGSCGPVSSNGAY
Neuraminidase_Japan/Hokkaido      NWHGSNRPSVSFNQNLDYQIGYICSGVFGDNPRPKDGEGSCNPVTVDGAD
                                  ******** *******:*************.***:** ***.**: :**

Chicken/Thailand/Nontaburi        GVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFSVKQDIV
Neuraminidase_Hongkong            GVKGFSFKYGNGVWIGRTKSTSSRSGFEMIWDPNGWTETDSSFSLKQDII
Neuraminidase_Vietnam             GVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFSVKQDIV
Neuraminidase_Japan/Hokkaido      GVKGFSYRYGNGVWIGRTKSNRLRKGFEMIWDPNGWTDTDSDFSVKQDVV
                                  ******::************.  *.************:***.**:***::

Chicken/Thailand/Nontaburi        AITDWSGYSGSFVQHPELTGLNCIRPCFWVELIRGRPKE-STIWTSGSSI
Neuraminidase_Hongkong            AITDWSGYSGSFIQHPELTGLNCMRPCFWVELIRGRPKE-KTIWTSGSSI
Neuraminidase_Vietnam             AITDWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKE-STIWTSGSSI
Neuraminidase_Japan/Hokkaido      AMTDWSGYSGSFVQHPELTGLDCMRPCFWVELIRGRPREKTTIWTSGSSI
                                  *:**********:********:*:*************:* .*********

Chicken/Thailand/Nontaburi        SFCGV---------------------
Neuraminidase_Hongkong            SFCGVNSDTVGWSWPDDAELPFTIDK
Neuraminidase_Vietnam             SFCGVNSDTVGWSWPDGAELPFTIDK
Neuraminidase_Japan/Hokkaido      SFCGVNSDTANWSWPDGAELPFTIDK
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Figure 5-1 Amino acid sequence alignment of the recombinant head domain neuraminidase(influenza strain A/chicken/Thailand/Nontaburi /CK162/2005(H5N1)) compared with virus neuraminidase (influenza virus strain A/HongKong/156/97 (H5N1), influenza virus strain A/Vietnam/DT-036/2005 (H5N1) and influenza virus strain A/Hokkaido/15/02 (H1N1))

5.4.3 Screening of inhibitors from Thai medicinal plant extracts

Previous studies have shown that the alterations of amino acids on the NA gene result in the adaptation and the evolution of the virus causing the contagion in living organisms (Suzuki, 2005). On the other hand, the alterations of the amino acids in the active sites result in the structure of the enzyme. The virus will then show resistance to the antiviral drug, oseltamivir, which is used for the treatment of the disease. The amino acid alterations are at the positions of 119 (Glu 119 Val), 292 (Arg 292 Lys), 294 (Asn 294 Ser) and 275 (His 275 Tyr) (Kiso *et al*., 2004). From the resistance to antiviral drugs resulted from the development of the genes in virus, the new drug is therefore required.

The drug, oseltamivir, is chemically synthesized substance which can be naturally derived from shikimic acid, the precursor of the groups of alkaloids, flavonoids and tannins, extractable from *Illicium anisatum* L. which generally contains polyphenolic and flavonoid compounds (Cook and Howard, 1966; Kim *et al.*, 2009) There are also many other types of medicinal plants which can exhibit inhibitory effect on the virulence of the virus. For example, geranium can be extracted with alcohols to obtain polyphenols, flavonoid and phenolic acids (Mukhtar *et al*., 2008). The polyphenolic component, catachins and theaflavins can be obtained from the extracted aqueous of green tea. Polyphenols can be obtained from the extracts of *Cistus incanus* and from pomgranate (Hudson, 2009). Hence, the medicinal plants were interesting for the attempt to search for the new antiviral drug.

In this work, the inhibitory effects on the head domain NA from 10 types of Thai medicinal plant extracts were investigated. From the results, the extract from *Vernonia cinerea* (ya dorg kao) showed the highest inhibition at 99.86 ± 0.01 % which was quite similar to the drug, oseltamiver at 98.16 ± 0.01 %. On the other hand, the extract from *Rhizophora mucronata* (gong garng bai yai) gave only 25.62 ± 2.21 % inhibition and not that much different from the other eight types of extracts.

Vernonia cinerea has been known to show antimicrobial, antibacterial and antioxidant activities and contains the phytochemical compounds such as sterols, flavonoids, sesquiterpene lactones and terpenoid (Rajendran and Abirami, 2012). It is most likely that shikimic acid should be present as the precursor of those compounds. If the extract from this plant can be further studied for the chemical composition, *Vernonia cinerea* may represent the new Thai antiviral drug for avian flu H5N1 so that no import should be required in the future.

CONCLUSION

In this research, the full length and head domain neuraminidase from avian influenza A virus H5N1 were successfully expressed in *Pichia pastoris* .The optimal conditions were the induction with 4% methanol for the period of 5 days and the sizes of expressed products were both approximately 47 kDa. The purifications were carried out for both products but only the head domain could be partially purified. The kinetics of the enzyme and the inhibition were determined. The nearly complete 99% inhibition was obtained from the antiviral drug, oseltamivir. The extract from the Thai medicinal plant, *Vernonia cinerea* also yielded similar percentages of inhibition.

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APPENDICES

APPENDIX A

MEDIUM PREPARATION

1. Glycerol-complex Medium (BMGY) 1 L

Peptone	20	g
Yeast Extract	10	g

All components were dissolved in 700 ml distilled water and then sterilized at 121 °C, 151b/in^2 for 15 min. Next, the mixture was cooled down at room temperature and added with following chemicals:

1 M potassium phosphate buffer, pH 6.0	100	ml
10X yeast nitrogen base	100	ml
500X biotin (4x10^{-5} % biotin)	2	ml
10X glycerol	100	ml

Stored at 4 ° C

2. Methanol-complex Medium (BMMY) 1 L

peptone	20	g
yeast Extract	10	g

All components were dissolved in 700 ml distilled water and then sterilized at 121 °C, 151b/in^2 for 15 min. Next, the mixture was chilled at room temperature and added with the following chemicals:

1 M potassium phosphate buffer, pH 6.0	100	ml
10X yeast Nitrogen Base	100	ml
500X biotin (4x10^{-5} % biotin)	2	ml

10X M (0.5 % methanol)	100	ml

Stored at 4 ° C

3. Yeast Extract Peptone Dextrose Medium (YPD) 1L

peptone	20	g
yeast Extract	10	g

All components were dissolved in 900 ml distilled water and then autoclaved at 121 °C, 15 1b/in^2 for 15 min. Next, the mixture was chilled at room temperature and then added with 100 ml of 20 % dextrose.

4. Yeast Extract Peptone Dextrose Medium plate (YPD plate) 1L

peptone	20	g
yeast Extract	10	g
agar	15	g

All components were dissolved in 900 ml distilled water and then autoclaved at 121 °C, 15 1b/in^2 for 15 min. Next, the mixture was chilled at room temperature and then added with 100 ml of 20 % dextrose.

APPENDIX B

REAGENT PREPARATION

1. Polyacrylamide gel electrophoresis

1.1. Stock reagent

1.1.1. 30 % acrylamide, 0.8 % bis-acrylamide 100 ml

acrylamide	29.2	mg
N, N–methylene-bis-acrylamide	0.8	g

1.1.2. 1.5 M Tris-HCl, pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

The pH of solution was adjusted to 8.8 by HCl and the volume was brought up to 100 ml with distilled water.

1.1.3. 1 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

The pH of solution was adjusted to 6.8 by HCl and the volume was brought up to 100 ml distilled water.

1.1.4. 10 % ammonium persulfate

ammonium persulfate	0.1	mg
distilled water	1	ml

1.1.5. 10 % SDS

SDS	0.1	mg
distilled water	1	ml

1.2. 5X Sample buffer for SDS-PAGE

1 M Tris-HCl, pH 6.8	0.6	ml
glycerol	2.5	ml
10 % SDS	2	ml
2-mercaptoethanol	0.5	ml
1 % bromophenol blue	1	ml
distilled water	3.4	ml

One part of 5X sample buffer was added to four parts of sample. The mixture was heated at 95 °C for 5 min and centrifuged at 12,000 rpm for 5 min before loading to the gel.

1.3. SDS-PAGE

4.3.1. 12 % separating gel

distilled water	3.3	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml
30 % acrylamide solution	4	ml
10 % SDS	0.1	ml
10 % ammonium persulfate	0.1	ml
TEMED	0.004	ml

4.3.2. 5.0 % stacking gel

distilled water	1.4	ml
1 M Tris-HCl, pH 6.8	0.25	ml
30 % acrylamide solution	0.33	ml
10 % SDS	0.02	ml
10 % ammonium persulfate	0.02	ml
TEMED	0.002	ml

4.4. 10X Electrophoresis buffer for SDS-PAGE 1 L

Tris (hydroxymethyl)-aminomethane	30.3	g
glycine	144	g
SDS	10	g

The distilled water was used for adjusting volume to 1 liter.

4.5. Staining solution

coomassie brilliant blue R-250	0.5	g
ethanol	250	ml
glacial acetic acid	50	ml

The distilled water was used for adjusting volume to 500 ml and mixed well.

4.6. Destaining solution

methanol	100	ml
glacial acetic acid	100	ml

Added distilled water to 1000 ml and mix.

2. Western blot analysis buffer solutions

2.1. Transfer buffer

Tris base ($C_4H_{11}NO_3$)	5.08	g
glycine	2.9	g
SDS	0.37	g
methanol	200	ml

Added distilled water to 1000 ml and mix

2.2. Blocking buffer

PBST	100	ml
skim milk	5	g

2.3. 1X PBS

NaCl	8	g
KCl	8	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g

The pH of solution was adjusted to 7.4 by HCl and the volume was brought up to 1,000 ml distilled water and then autoclaved at 121 ° C, 15 1b/in^2 for 15 min.

2.4. PBST

1X PBS	500	ml

Tween 20	0.25	ml

2.5. Working solution

2.5.1. Solution A

100 mM Tris-HCl pH 8.5	2.5	ml
90 mM coumaric acid	11	µl
250 mM luminol	25	µl

2.5.2. Solution B

100 mM Tris-HCl pH 8.5	2.5	ml
30% H_2O_2	1.5	µl

3. Enzyme assay buffer solutions

3.1. 325 mM MES

MES	6.9	g

The pH of solution was adjusted to 6.5 by NaOH and the volume was brought up to 100 ml with distilled water.

3.2. 0.1 M MES

MES	2.1	g

The pH of solution was adjusted to 6.5 by NaOH and the volume was brought up to 100 ml with distilled water.

3.3. 10 mM $CaCl_2$

CaCl2	0.15	g

Added distilled water to 100 ml and mixed

3.4. 0.1 M $CaCl_2$

$CaCl_2$	1.47	g

Added distilled water to 100 ml and mixed

3.5. Stop solution

absolute Ethanol	100	ml
distilled water	250	ml
glycine	3.04	g

The pH of solution was adjusted to 10.7 by NaOH and the volume was brought up to 400 ml with distilled water.

3.6. 4 mM 4-MU (standard stock)

4-MU	7.9	mg

Added distilled water to 10 ml and mixed

3.7. 5 mM MUNANA (stock solution)

MUNANA	5	mg
Sterile distilled water	2,240	µl

Mixed well, aliquot and stored at -20°C.

3.8. 1X assay buffer:

0.1 M MES (pH-6.5)	32.5	ml
0.1 M $CaCl_2$	4	ml
distilled water	63.5	ml

Mixed and stored at 4ºC

3.9. 40 μM 4-MU (working stock):

4 mM 4-MU	10	μl
1X assay buffer	990	μl

APPENDIX C

PREPARATION AND STANDARD CURVE FOR NEURAMINIDASE ACTIVITY

1. Standard curve of 4-MU

The standard curve of 4-MU was constructed using enzymatic assay method for neuraminidase activity. The method was as follows;

1. The serial dilution of 4-MU was diluted to 2X by using 1X of assay buffer;. The initial concentration of 4-MU was 12.5 μM.

2. 100 μl diluted 4-MU was added into 96 wells microplate.

3. 100 μl stop solution was added into wells containing diluted 4-MU and mixed well.

4. The fluorescent intensity of 4-MU was detected at 355 nm (excitation wavelength) and at 460 nm (emission wavelength).

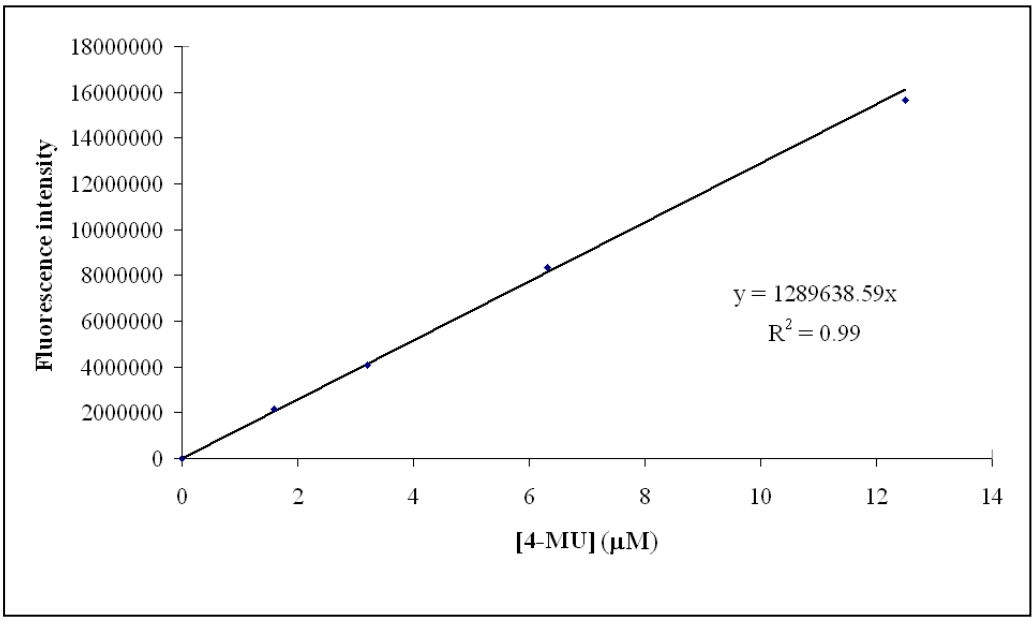

Figure C-1 Standard curve of 4-MU

APPENDIX D

ENZYMATIC REACTION OF NEURAMINIDASE

There are several methods for determination of neuraminidase activity. One of the most commonly used methods is fluorometric assay which has many advantages over the others. This fluorometric assay is simple, highly sensitivev and less time consuming.

Principle:

2'-(4-methylumbelliferyl)-α- *D*- N-acetylneuraminic acid (MUNANA) is used as a fluorogenic substrate. 4-methylumbelliferone (4-MU) is introduced after neuraminidase cleavage from MUNANA. Therefore, neuraminidase activity can be measured by quantification of their fluorogenic products, 4-MU. The fluorescence intensity is recorded at excitation wavelength of 355 nm and emission wavelength of 460 nm (Figure 3-1).

APPENDIX E

PREPARATION AND STANDARD CURVE FOR PROTEIN DETERMINATION

1. Standard curve of BSA

The standard curve of BSA was constructed using Bradford protein assay method for protein determination. The method was as follows;

1. 1 μg/μl BSA was diluted with distilled water from 0.1-0.6 μg (Table E-1).

2. 5 μl BSA from stock solution was added into 96 well microplate.

Table E-1 Reagent volume for preparation of standard curve

BSA (μg)	**Reagent volume (μl)**	
	1 μg / μl of BSA	**dH_2O**
0	-	1000
0.1	100	900
0.2	200	800
0.3	300	700
0.4	400	600
0.5	500	500
0.6	600	400

3. 300 μl of Bradford's reagent was added and incubated for 5 min.

4. The product was measured by an increase in the absorbance at 595 nm.

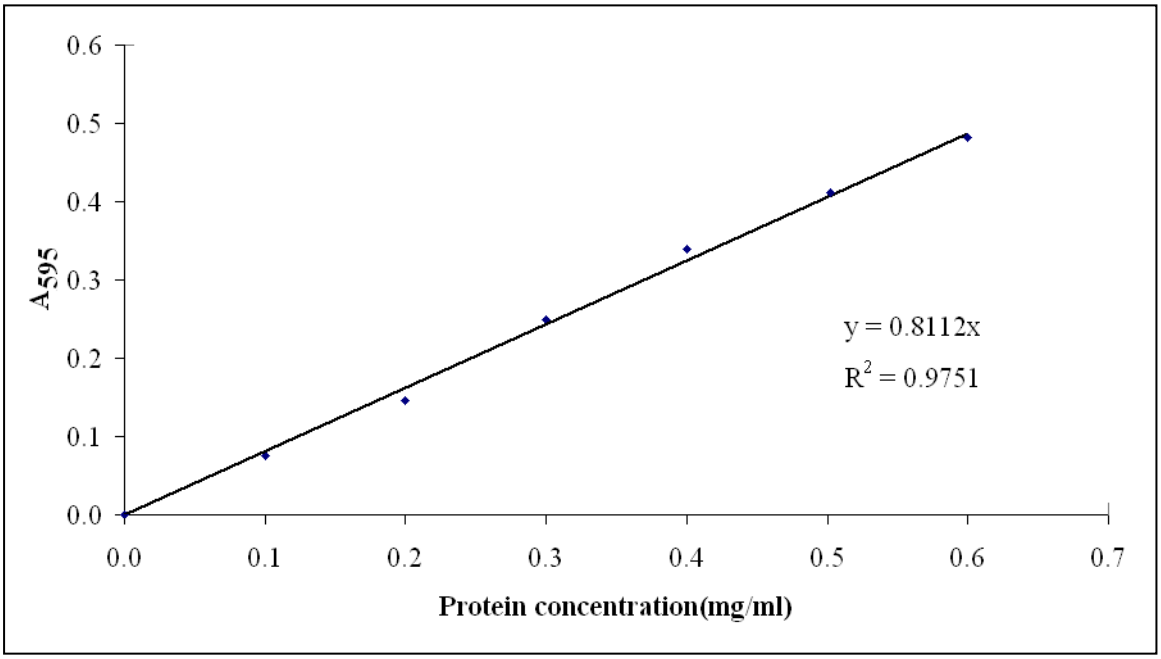

Figure E-1 Standard curve of BSA

APPENDIX F

THAI MEDICINAL PLANTS

Table F-1 Ethanobotanical data of investigated Thai medicinal plants

Plant species	Family	Part tested	Traditional uses
Allium sativum	Alliaceae	Bulbs	Wounds, ulcers, skin infections, flu, athlet's foot, some virus, respiratory aliments, high blood pressure, cold, colic
Celosia argentea	Amaranthaceae	Flower	Antioxidant, anti-inflammatory, antiviral, antibacterial activity
Clerodendrum inerme	Verbenacceae	Leaves	Antiseptic, arrests bleeding, asthma, hepatitis, stomach pains
Colocasia esculenta	Araceae	Roots	Colorectal, arrests bleeding, asthma, hepatitis, stomach pains, uterine stimulant
Phyllanthus amarus	Euphorbiaceae	Leaves	Astringent, cooling, diuretic, stomachic, febrifuge,antiseptic, hepatitis
Rhizophora apiculata	Rhizophoraceae	Leaves	hepatitis
Rhizophora mucronata	Rhizophoraceae	Leaves	hepatitis
Sesuvium portulacastrum	Aizoaceae	Leaves	hepatitis
Suaeda maritime	Chenopodiaceae	Leaves	hepatitis
Vernonia cinerea	Asteraceae	All	Cold and fever, cough dysentery, hepatitis, snake bite, reduce the desire for food, smoking, hypertension

From Bandaranayake, W.M. (1998)

APPENDIX G

CALCULATION METHOD

1. Neuraminidase activity

$$\text{activity} = \frac{\text{amount of 4-MU (µmole)}}{\text{time (min)}}$$

One unit (1 U) was defined as 1 µmole of 4-MU produced per minute at 37 °C

2. Total protein

The absorbance value at 595 nm was calculated by:

$$Y = aX + b$$

Where

Value of X axis = standard protein concentration (mg/ml)

Value of Y axis = absorbance at 595 nm

3. Specific activity of neuraminidase

$$\text{specific activity} = \frac{\text{activity}}{\text{concentration of protein}}$$

4. % inhibition

$$\textbf{\% inhibition} = \left[\frac{SN - SI}{SN}\right] \times 100$$

SN: specific activity of neuraminidase

SI: specific activity of neuraminidase after adding the inhibitor

APPENDIX H

CALCULATION OF HALF MAXIMAL INHIBITORY CONCENTRATION

The half maximal inhibitory concentration (IC_{50}) is defined as the concentration of inhibitor that inhibits 50% of the activity of neuraminidase and can be determined by Microsoft office exel.

1. The linear relationship equation was plotted between the logarithm of inhibitor concentration (X-axis) and the percentage of inhibition (Y-axis).
2. By presenting Y as 50 in the linear equation, IC_{50} could be calculated from the obtained values of X and the anti-log x.

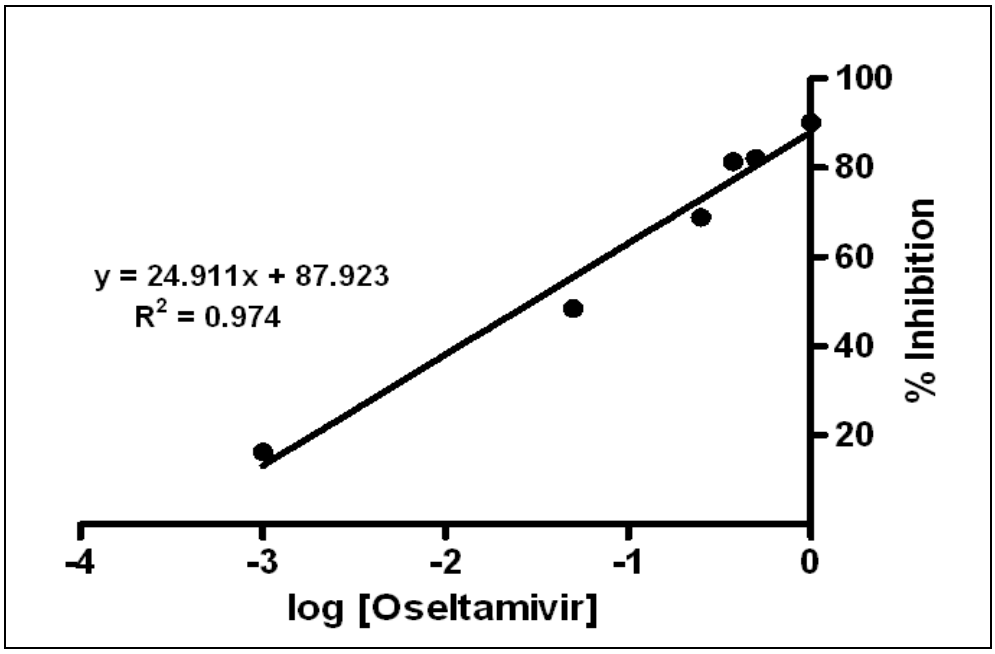

Figure H-1 Inhibition of neuraminidase activity by oseltamivir carboxylate

BIOGRAPHY

Miss Puchita Rerksongkrou was born on July 13, 1985 in Bangkok, Thailand. She graduated with the Bachelor Degree of Science from the Department of Botany, program on genetic, Faculty of Science, Chulalongkorn University in 2008. In that year, she furthered her Master's Degree of Science in Biotechnology from Program in Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University, at which she finished in April of 2012. During her studies, part of her work was presented as poster presentation at The 22nd Annual Meeting of the Thai Society for Biotechnology "International Conference on Biotechnology for Healthy Living" on the topic of "Cloning and expression of neuraminidase from avian flu virus (H5N1) in *Pichia pastoris*" at Prince of Songkla University, Trang Campus, Thailand on 20-22 October, 2010.