Purification of Neuraminidase from Sub Type H5N1 Influenza Virus

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ABSTRAK

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Neuraminidase pada virus influenza memainkan peranan yang vital dalam keberlangsungan kehidupan virus. Vaksinasi hewan dengan glikoprotein ini menghasilkan respon imun yang dapat melindungi hewan dari infeksi. Suplementasi vaksin konvensional dengan neuraminidase terbukti meningkatkan proteksi dan masa pakai vaksin. Neuraminidase yang dimurnikan dapat juga dipakai untuk pembuatan alat uji serologis untuk membedakan hewan yang positif avian influenza secara serologis akibat vaksinasi atau akibat infeksi. Dalam penelitian ini diuraikan cara purifikasi neuraminidase virus influenza suptipe H5N1. Triton X-100 dan Octyl β-D-glucopyranoside digunakan untuk mengekstraksi dan melarutkan glikoprotein membrane tersebut. Aktivitas enzimatik diukur menggunakan suatu uji yang sederhana, sensitif dan cocok dengan pekerjaan purifikasi tersebut; menggunakan substrat flourokrom 4-methylumbelliferyl-α-D-N-acetyl neuraminic acid. Nuraminidase diabsorbsi secara selektif dengan oxamic-acid agarose. Tingkat kemurnian neuraminidase yang dielusi dari kolom tersebut sebenarnya sudah cukup tinggi. Kemurnian yang lebih tinggi lagi diperoleh dengan separasi dengan gel filtrasi menggunakan Superdex-200. Neuraminidase yang dimurnikan tersebut aktif secara enzimatis dan tidak mengandung hemagglutinin yang terdeteksi baik dengan uji hemagglutinasi maupun dengan antibodi spesifik terhadap hemaglutinin H5N1. Neuraminidase yang dimurnikan tersebut dikenali secara kuat oleh antibodi terhadap suatu daerah pada bagian tengah molekul neuraminidase tetapi hanya dikenali secara lemah oleh antibodi terhadap suatu daerah pada C-terminal neuraminidase virus H5N1. Neuraminidase yang dimurnikan tersebut terdapat dalam bentuk tetramer tetapi terurai menjadi bentuk monomer pada SDS PAGE reduksi, atau sebagian besar bentuk dimer pada SDS PAGE non reduksi.

Kata kunci: Neuraminidase, Influenza, H5N1, *Methylumbelliferyl*, *Oxamic-acid*

ABSTRACT

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Influenza-virus neuraminidase plays vital role in the survival of the organisms. Vaccination of animals with this glycoprotein confers immune responses so that enable it to protect the animals from incoming infection. Supplementation of conventional vaccines with this glycoprotein increases the protection and longevity of the vaccine. Purified neuraminidase can also be used to develop serological tests for differentiation of serologically positive animals due to infection or to vaccination. In this study purification of neuraminidase from influenza virus subtype H5N1 was described. Triton x-100 and Octyl β-D-glucopyranoside were used to extract and diluted the glycoprotein membrane. The enzymatic activity of the neuraminidase was assayed using a fluorochrome substrate, 4-methylumbelliferyl-α-D-N-acetyl neuraminic acid, which was found to be simple, sensitive and suitable for the purification purpose. The neuraminidase was absorbed selectively on an oxamic-acid agarose column. The purity of neuraminidase eluted from this affinity column was high. A higher purity of the neuraminidase was obtained by further separation with gel filtration on Superdex-200. The purified neuraminidase was enzymatically active and did not contain any detectable haemagglutinin, either by haemagglutination assay or by monospecific antibodies raised against H5N1 hemagglutinin. The purified neuraminidase was recognized strongly by antibodies raised against an internal but only weakly by that against C-terminal regions of the neuraminidase protein of H5N1-influenza virus. The purified neuraminidase was in tetrameric forms but dissociated into monomeric form on reducing condition, or mostly dimeric form on non-reducing SDS-PAGE.

Key Words: Neuraminidase, Influenza, H5N1, Methylumbelliferyl, Oxamic-acid

INTRODUCTION

Avian influenza caused by influenza virus subtype H5N1 has been one of the most feared infection and has become the most publiciz ed disease since 2003. The poultry industries have suffered enormous economic losses from massive number of chicken deaths and increased expenses for prophylaxis and

biosecurity. The virus causes not only substantial economic losses but also has the potential to cause a global pandemic of human influenza. Signalling the threat is the increasing number of people that have become infected by the virus; in Indonesia alone, from 137 confirmed cases until 20 July 2008, 112 cases were fatal (WHO, 2008).

Influenza viruses have two types of glycoproteins, haemagglutinin (HA) and neuraminidase (NA). The HA binds to a sialoglycan receptor on the target cell surface and mediates fusion of the viral envelope with the endosomal membrane in order to deliver viral genome into the cytoplasm of the target cells. The NA promotes the release of progeny virus from infected cells by destroying receptors on the host cell through hydrolysis of terminal sialic acids of sialoglycans (KLENK and ROTT, 1988; SKEHEL and WILLEY, 2000). Antibodies to the HA are protective and it neutralize viruses which are closely matched antigenically, whereas those to the NA are also protective but infection-permissive (DEROO *et al.*, 1996; JOHANSSON *et al.*, 1989). When animals are immunised with the same amount of purified HA and NA, the titre of conferred antibody against NA is comparable to that against HA, indicating both glycoproteins are equally immunogenic (JOHANSSON *et al.*, 1989). However, immunisation of animals with conventional vaccines confers antibodies most of which are directed toward HA. The antibody levels towards NA is much lower, or undetected, especially following primary immunisation. This is because HA exist in much higher molar ratio in the virion (JOHANSSON and KILBOURNE, 1990). Since in conventional vaccine protection is attributed to the HA, changes in the HA due to antigenic drift may result in vaccination failure. Neuraminidase also undergoes antigenic drift but its rate is much slower. For that reason, inclusion of NA in a vaccine against influenza reduces the occurrence of vaccination failure (JOHANSSON and BRETT, 2007; JOHANSSON *et al.*, 1998).

One of the disadvantages of vaccination policy to control avian influenza is the difficulty in differentiating chicken serologically positive due to infection or vaccination, and this greatly impedes the surveillance and control programmes. One strategy that has been demonstrated to be effective for the differentiating infected from vaccinated animals (DIVA) is the 'heterologous NA strategy'(CAPUA *et al.*, 2002). This strategy requires that chicken are vaccinated with vaccines containing the same HA subtype as the challenge virus but a different NA subtype. Infection is monitored by a serological test that is used to detect antibody to the NA subtype of the challenge or field virus. This strategy has been planned to be used in Indonesia. Heterologous H5N2 vaccines have been introduced and intended to replace the H5N1 vaccines. If this strategy was implemented successfully, the prevalence of ongoing H5N1 infection could then be easily monitored by the presence of antibodies to N1-neuraminidase. For this approach the availability of a serological test for detection of the antibodies to N1 neuraminidase is essential. Development of such test however requires purified NA from the H5N1 virus, and this was attempted in this study.

A number of methods for purification of influenza-virus NA have been available. The influenza virus glycoproteins can be separated from the virion by various detergents and separated either by anion exchange chromatography (JOHANSSON *et al.*, 1989), oxamic-acid affinity chromatography (DEROO *et al.*, 1996) or immuno-affinity chromatography (GERENTES *et al.*, 1996). As an alternative to the detergents, some worker demonstrated the use of proteases (pronase, bromelain or trypsin) to selectively cleavage the NA heads (AITKEN and HANNOUN, 1980; BURMEISTER *et al.*, 1991; MCKIMM-BRESCHKIN *et al.*, 1991). The NA is separated from the protease by a gel filtration chromatography.

The NA of H5N1 virus, as far as we were aware, has not been purified to date. This subtype of influenza virus is much more virulence than those whose NA have been purified previously. This high virulence of H5N1 virus causes lower amount of virus to be produced during the passage of virus inoculated chicken embryos. In addition, the amount and type of proteins in the harvested alantoic fluid may also be different compared to those with the less virulent viruses. For that reason some modification of the published methods may be necessary. The present study demonstrates the effectiveness of the approach taken in which Octyl β-D-glucopyranoside and Triton X-100 were used for extraction and solubilisation, umbelliferyl assay for detection, and oxamic-acid-affinity and Superdex-200 gel filtration chromatographies for separation of the H5N1-virus NA.

MATERIALS AND METHODS

Virus isolation

The influenza virus subtype H5N1 used in this study was isolated from a hen dying during an avian influenza outbreak in Gunung Sindur Bogor, West Java, Indonesia in 2003 (A/Ck/West Java/1067/2003). This isolate is genetically similar to the Indonesian reference strain A/Ck/Bl/2003 (Dr. Indi NPL Dharmayanti, personal communication).

Extraction and purification of neuraminidase

The virus was grown by innoculating 11-day-specific-pathogen-free-chicken embryos with 10^3 lethal egg dose (LED)/egg. After 30 hour incubation at 37°C,

allantoic fluid was collected and clarified at 5 000 x *g* for 30 minutes. The virus particles contained in the fluid were pelleted at 35 000 x *g* for 30 minutes, suspended in PBS at about 1/300 original volume and kept at -70°C until used. Extraction of NA from the viral particles was performed according to a previous method (HOCART *et al.*, 1995). Briefly, an equal volume of 6% Triton X-100 (Sigma Aldrich chemicals) solution in 0.3 *M* sodium acetate (pH 5.5) containing 2 m*M* phenylmethanesulfonyl fluoride (PMSF) (Sigma Aldrich Chemicals) was added to the freshly thawed viral suspension and stirred for 1 hour at 25°C. Insoluble viral materials were pelletted at 35 000 x *g* for 30 minutes, the HA/ NA rich material contained in the supernatant was removed. The NA was purified from the HA/ NA rich supernatant by an affinity chromatography. A 5-ml N-(*p*-aminophenyl) oxamic acid agarose (Sigma Aldrich chemicals) was packed into a PD-10 column (Bio Rad Laboratories), activated by washing with 6 column volumes (CV) of 0.1 *M* sodium bicarbonate buffer (pH 9.1) containing 0.1% Octyl *β*-D-glucopyranoside (Sigma Aldrich chemicals) followed by 5 CV of 0.05 *M* sodium acetate buffer pH 5.5 containing 0.1% Octyl β-D-glucopyranoside. The HA/NA-rich supernatant was loaded into the column; unbound materials were removed by washing the column with 10 CV of 0.15 *M* sodium acetate buffer containing 0.1% Octyl β-D-glucopyranoside. Bound NA was eluted from the column with 5 CV of 0.1 *M* sodium bicarbonate buffer containing 0.1% Octyl *β*-D-glucopyranoside and 2 m*M* CaCl2. The NA-rich solution was concentrated to about 10% original volume using a 10-kDa-molecular-cut-off-spin concentrator.

Further purification of the NA was performed on gel filtration chromatography using a 65 x 1.5 cm, superdex-200 column (Amersham Pharmacia Biotech). A 2-ml- volume of the concentrated oxamic-acid absorbed protein solution was loaded into the column, proteins were eluted from the column with a solution containing 40 m*M* sodium acetate, 150 m*M* NaCl, and 2 m*M* $CaCl_2$ pH 7.0 at a flow rate of 0.7 ml^{-1}. Fractions (2.5 ml) were collected and NA content in each fraction was determined. Fractions containing NA were pooled and concentrated.

Neuraminidase activity

Neuraminidase activity was detected using a fluorochrome 4-methylumbelliferyl-α-D-N-acetyl neuraminic acid (Sigma Aldrich chemicals) as a substrate of the enzyme. The substrate was first diluted in double distil deionised H_2O at 5 m*M* concentration, aliquoted then keep at -20°C until use. A 5 µl volume of freshly thawed substrate was added to an equal volume of serially diluted sample, mixed and incubated for 10 minutes at 37°C. A 5-µl volume of this mixture was placed on the surface of a dark-coloured ceramic plate and examined under a 10-watt UV light. This UV light was similar to that shop keepers use to examine counterfeit money. The intensity of fluorescence emitted by the mixture corresponded to the NA activity of the sample.

Enzyme linked immunosorbent assay (ELISA)

In addition to the 'methylumbelliferyl assay', the presence of N1 NA is also confirmed with an ELISA using a panel of antibodies: (1) anti-Neuraminidase CT (specific antibodies raised against synthetic peptide with sequence similar to a 15 conserved amino acid at carboxy terminus of H5N1 NA protein (Genbank accession no. CAC95053), (2) anti-neuraminidase IN (specific antibodies raised against synthetic peptide with sequence similar to a 16 conserved amino in the middle of H5N1 NA protein (Genbank accession no. CAC95053), (3) anti-hemagglutinin NT (specific antibodies raised against synthetic peptide with sequence similar to a 15 conserved amino acid at amino terminus of H5N1 hemagglutinin protein (Genbank accession no. AAT76166), and (4) anti-hemagglutinin IN (specific antibodies raised against synthetic peptide with sequence similar to 14 conserved amino acid in the middle of H5N1 hemagglutinin protein (Genbank accession no. AAT76166). All of those antibodies were obtained from Proscience Inc. Poway CA (USA). ELISA was performed according to the procedure provided by the antibodies' producer.

SDS PAGE and Immunoblotting

The SDS-PAGE was performed in a Mini-Protean-3 cell (Bio Rad Laboratories). Stacking and separating gels contained 4% and 10% acrylamide, respectively. A 20-µl volume of samples containing about 100 µg protein and 1x reduced or non reduced sample buffer were loaded into the gels and electrophoresis was run until the leading dye reached about 0.5 cm from the bottom of the gel. Detergent contained in the samples were removed before loading by methanol and chloroform precipitation (ROSENBERG, 1996). Separated proteins in one set of the gel were stained with Coomasie blue, whereas those in the other set were transferred onto a nitrocellulose membrane using a Mini Transblot (Bio Rad laboratories) and a transfer buffer consisting of 25 m*M* Tris, 192 m*M* glycine and 20% methanol. Proteins in the blot were incubated with serum from chicken previously immunised with an inactivated H5N1 vaccine then, probed with horseradish-peroxidise-labelled anti-chicken IgY (Sigma Aldrich chemicals).

RESULTS

The N-(*p*-aminophenyl) oxamic acid agarose absorbed NA selectively from the Triton extract of the H5N1 virus. From 510 inoculated embryonated eggs, about 60 ml eluate from the oxamic-acid column was obtained. After adjusting the pH of the eluate to about 7 and concentrating to 2.3 ml, the concentrated eluate contained 0.63 mg/ml of total protein. Its NA enzymatic activity was detected in 10^{-6} dilution by the 'methylumbelliferyl assay'. No hemagglutination activity was detected in this eluate either before or after the concentration. When this concentrated sample was fractionated further with a gel-filtration chromatography using a high resolution media, Superdex-200, it contained almost exclusively a single protein or at least a group of proteins of similar size (Figure 1). Based on our previous experience with similar Superdex-200 column, proteins eluted at that elution volume had a molecular weight of about 200 kDa. This major elute contained NA but not hemagglutinating activity. Only a small proportion of protein(s) (<10%), peaked at 105 ml elution volume, accompanied the major peak (Figure 1). This protein, or group of proteins, were predicted to have a molecular weight of about 50 kDa. Neuraminidase and hemagglutinating activities were not detected in this minor eluate.

The umbelliferyl and hemagglutination assays which showed that the aminophenyl-oxamic-absorbed H5N1 proteins contained exclusively NA, without contaminating haemagglutinin agreed with ELISA results. In ELISA, the protein recognized strongly by anti neuraminidase-IN antibody, and weakly by anti neuraminidase-CT antibody (Figure 2). The oxamic-acid absorbed protein did not recognized by specific antibodies to H5-hemagglutinin, neither by antibody to the N-terminal region nor by antibody to a middle region of H5N1 hemagglutinin (Figure 2). Similar results were obtained from ELISA experiment on the gel-filtration-purified oxamic-acid-absorbed protein (Figure 3).

Results of SDS-PAGE and immunoblot analyses from different stage of NA purification are presented in Figure 4. The banding patterns of proteins in non-reduced samples were different from those of reduced counterpart, indicating the presence of intensive disulphide bonding. Some of the major proteins that were present in the whole viral proteins were absent in the Triton X-100 extract indicating selective extraction by the detergent (Figure 4 line 1 and 2). The non reduced oxamic-acid absorbed H5N1-protein consisted of one conspicuous bands at ~ 85 kDa and the other less prominent bands at ~28 kDa, ~55 kDa and >98 kDa (Figure 4a, line 3). When this oxamic-acid absorbed H5N1-protein was purified further using gel filtration chromatography, the ~ 85 kDa band became more dominant, whereas other bands were unapparent (Figure 4A, line 4). Under reducing condition, the ~ 85 kDa and >98 kDa bands were absent. Two most conspicuous bands, ~ 60 and ~ 28 kDa, were seen (Figure 4C, line 4). On immunoblot analysis, the non-reduced ~ 85 KDa and the > 98 kDa band were recognised strongly by the H5N1 antiserum (Figure 4B). The ~ 60 kDa reduced band was also strongly recognised but not the ~28 kDa.

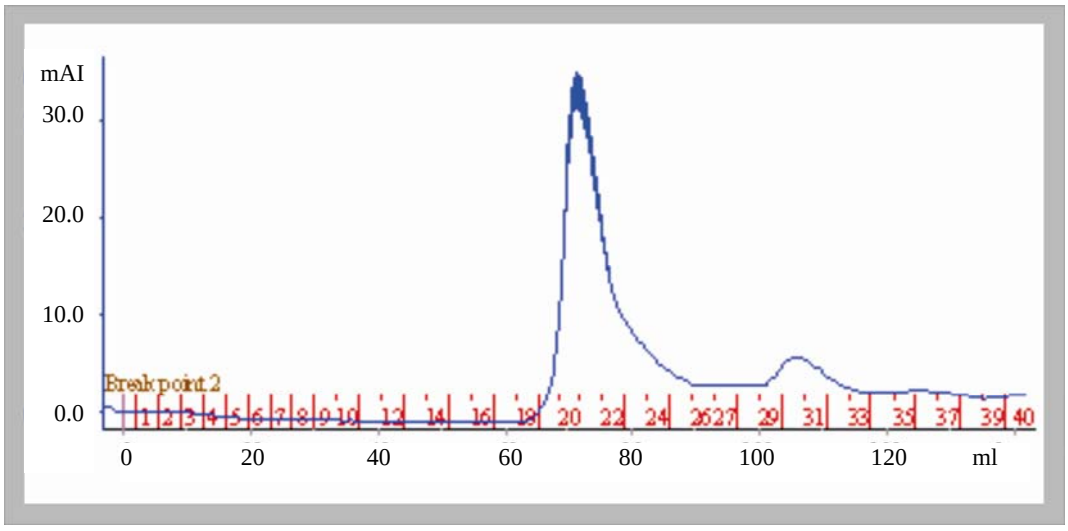

Figure 1. Chromatogram of oxamic-acid absorbed H5N1 protein on Superdex-200 column. The dominant peak contained enzymatically active NA.

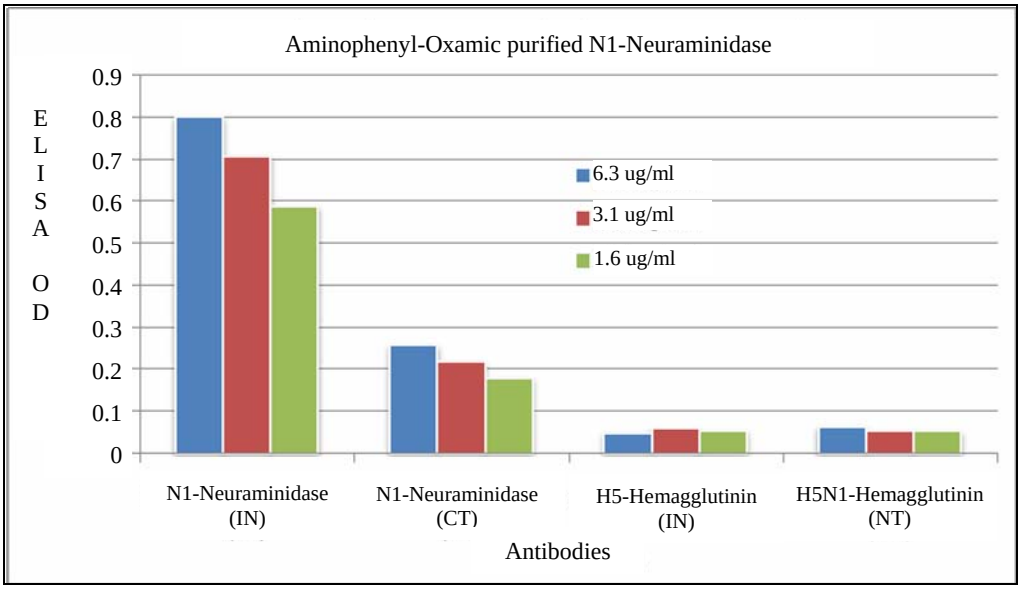

Figure 2. Recognition in ELISA of the oxamic-acid purified NA at different concentration by antibodies against amino-acid-sequence in the middle of H5N1 neuraminidase ((N1-Neuraminidase (IN)), carboxyl-terminal H5N1 neuraminidase (N1-Neuraminidase (CT)), amino-acid-sequence in the middle of H5N1-hemagglutinin ((H5-Hemagglutinin (IN)), and amino-terminal H5N1 hemagglutinin (N1-Hemagglutinin (NT))

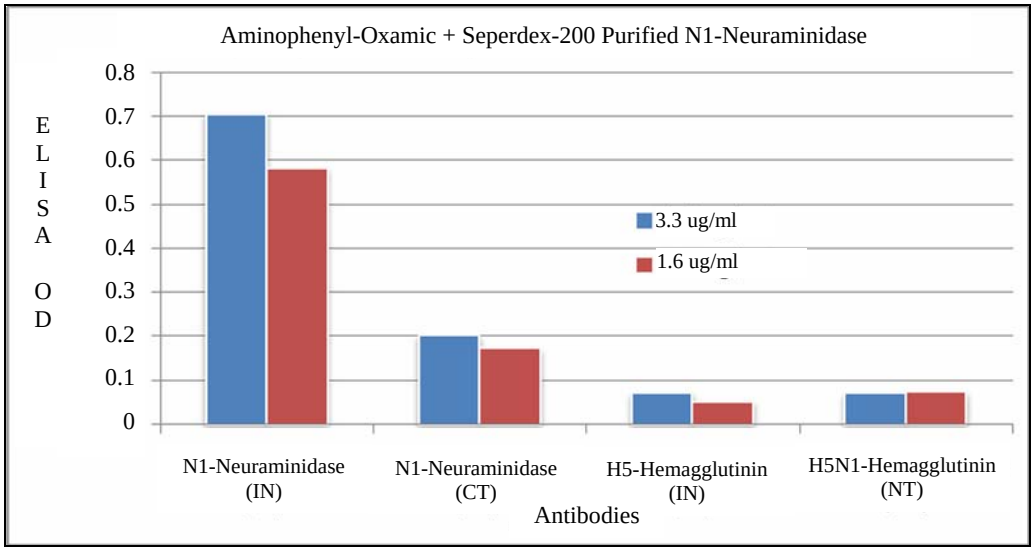

Figure 3. Recognition in ELISA of the gel-filtration-purified NA at different concentration by antibodies against amino-acid-sequence in the middle of H5N1 neuraminidase ((N1-Neuraminidase (IN)), carboxyl-terminal H5N1 neuraminidase (N1-Neuraminidase (CT)), amino-acid-sequence in the middle of H5N1-hemagglutinin ((H5-Hemagglutinin (IN)), and amino-terminal H5N1 hemagglutinin (N1-Hemagglutinin (NT))

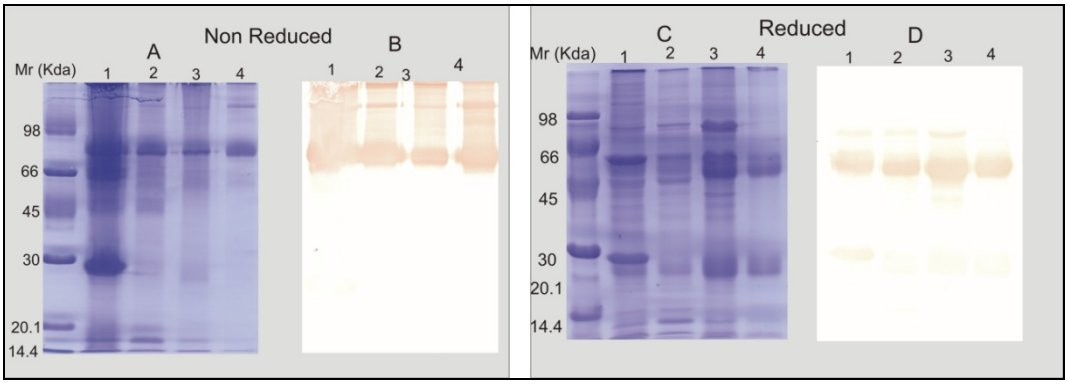

Figure 4. SDS-PAGE and immunoblot analysis on samples taken from different stages of H5N1-neuraminidase purification. Samples in A and B were devoid of reducing agent (non reduced), whereas samples in C and D reduced by β-mercaptoethanol

Collumn 1= H5N1 virus
Collumn 2= Triton-100-extracted H5N1 virus
Collumn 3= aminophenyl-oxamic-absorbed H5N1- protein
Collumn 4= gel-filtration-purified-aminophenyl-oxamic absorbed protein
A and C= SDS-PAGE gels stained with Coomasie blue
B and D= blots probed with serum from chicken immunised with commercial H5N1 vaccine

DISCUSSION

This study shows that H5N1 neuraminidase is effectively purified using oxamic-acid-agarose affinity column chromatography, followed by gel-filtration chromatography. The purified NA was enzymatically active and recognised by specific antibodies raised against H5N1 neuraminidase. Triton X-100, used in this study, extracted mainly the surface glycoproteins, HA and the NA, from the core viral proteins (HOCART *et al.*, 1995; JOHANSSON *et al.*, 1988). Since the concentration of HA is much higher than that of NA, the most likely contaminating proteins in the NA purification would be the HA (JOHANSSON *et al.*, 1989). In our purified NA, however, the presence of HA could not be detected either by hemagglutination assay (HA) or ELISA using specific antibodies against the glycoprotein. This is desirable especially if the purified NA is to be used for the development of a diagnostic test, especially the heterologous NA DIVA test which requires a complete removal of HA.

Being insoluble in water, the NA required detergents in its extraction and purification. Glycoside detergents have been used for extraction and purification of influenza-virus NA (JOHANSSON *et al.*, 1989; POWERS *et al.*, 1996). These detergents have some important advantages because they are mild, can be removed by dialysis and and do not absorb 280 nm light, at which wave length proteins are usually detected. However, this detergent is prohibitively expensive. Polyoxyethylene detergents such as Triton x-100 and Triton x-114 are also effective for extraction of surface glycoproteins of influenza virus (BRETT and JOHANSSON, 2005; GERENTES *et al.*, 1998; GERENTES *et al.*, 1996). Tritons are cheap and widely available but they can not be removed by dialysis and absorb 280 nm wavelenght. For extraction of surface glycoproteins in this study, we used Triton x-100 since this stage did not require the dialysis and measurement at 280 nm. In the later steps of purification, Octyl β-D-glucopyranoside was used because the steps required measurement at 280 nm and concentration by ultrafiltration. We found that the combination of those detergents was satisfactory.

An alternative to using detergents in the NA purification is to using proteases such pronase, trypsin or bromelain (AITKEN and HANNOUN, 1980; BURMEISTER *et al.*, 1991; MCKIMM-BRESCHKIN *et al.*, 1991; WARD *et al.*, 1982). These proteases are believed to selectively cleave the NA head from the rest of the virion. The NA head having high molecular weight can be separated from the proteases which have much lower molecular weight by a gel filtration chromatography. In addition, the NA head is relatively easier to manipulate because it is soluble in water. In our experience with the trypsin or pronase, however, the yield of purified NA was very low and enzymatically inactive (Data not presented). The reason for the low yield was unknown, but we speculate that the NA was digested by the protease into small fragments.

The most common method for assay of enzymatic activity of NA are the thiobarbiturate assay (TARIGAN *et al.*, 2007; WARREN, 1959) and Umbellyferyl assay (BUXTON *et al.*, 2000; LAMBRE *et al.*, 1989; POTIER *et*

al., 1979; WETHERALL *et al.*, 2003). The later is much more sensitive and accurate but it requires a fluorometer to measure the intensity of fluorescence. Most laboratories are not equipped with a fluorometer and this restricts the use of the assay. In this study, we use a small fluorescence light similar to that used by shopkeepers to detect counterfeit money to substitute the fluorometer. Although this modified assay was unsuitable for quantitative purpose due to difficulty in determining the endpoint dilution, the assay was very simple, rapid and sensitive. More importantly, the assay was unaffected by the presence of detergent used in this study. The thiobarbiturate assay was quantitative and did not require specialised equipment however the assay did not work in the presence of detergents used in this study.

The binding selectivity of oxamic-acid agarose for the H5N1 neuraminidase was satisfactory. Nevertheless, a single cycle of loading sample into the column and eluting the bound NA was not sufficient to recover all NA contained in the samples. Three or four cycles of sample loading were required to completely absorb the NA in the loading sample, and this inevitably resulted in voluminous eluate. The use of octyl glucopyranoside in the elution buffer was of advantage because the elute needed to be concentrated. The purity of NA after the oxamic-acid agarose chromatography was quite high and did not contain any detectable HA. Neuraminidase preparation with such extent of purity is likely to be adequate for a number of applications. When higher degree of purity was required a gel filtration chromatography as additional purification step, as shown in this study, was necessary.

The NA of influenza-A virus is a mushroom-shaped glycoprotein composed of four identical, single-chain polypeptide (FIELDS *et al.*, 1981). When the NA is analysed on SDS PAGE under reducing condition, the tetrameric glycoprotein dissociates completely into monomer (DEROO *et al.*, 1996; MCKIMM-BRESCHKIN *et al.*, 1991). Once detached from the virion, the NA easily dissociates. Previous studies have demonstrated that a NA derived from expression of H3N2-NA gene in insect cells existed in a mixture of three forms ~ 220 kDa tetrameric, ~130 kDa dimeric and ~54 kDa monomeric forms, even in the absence of any reducing agents. When the preparation was analysed by SDS PAGE under non-reducing condition the tetrameric and dimeric forms migrated as dimeric chains of 110 kDa (DEROO *et al.*, 1996). In the present study, the ~28 kDa band which was dominant in the reducing but not in non-reducing SDS PAGE is considered to be the fragment of monomeric NA. The reason why this fragment was not recognised by the H5N1 antiserum is unknown but the fragment might represent the non immunogenic part of the NA. The >98 kDa, the ~85 kDa and the ~55 kDa bands that appeared on the non-reducing SDS PAGE were considered to be the tetarmeric, dimeric and monomeric forms, respectively. In line with DEROO *et al.*, (1996), the tetrameric and dimeric forms were dissociated into monomeric form under reducing condition. The ~85 kDa- dimeric NA obtained in this study might be the conjugation between a complete monomer with an incomplete, -non-immunogenic part of monomer. Under reducing condition this conjunction dissociated into ~60 kDa complete and ~28 kDa incomplete monomers.

The fact that the purified NA was recognised strongly by the antibodies against the middle but only weakly against the C-terminal regions of the NA protein of a H5N1 virus may indicates that the NA purified in this study has high similarity in the middle but only low similarity in the C terminus with the H5N1 neuraminidase against which the antibodies were raised. The ~28-kDa incomplete, non immunogenic monomeric form is probably derived from the N or C terminus of the NA.

In conclusion, our results demonstrate the advantages of Triton X-100 and Octyl glucopyranoside detergents in solubilising the NA from the virion. High purity of NA was obtained by the use of an oxamic-acid-agarose-affinity chromatography, and gel filtration chromatography on Superdex-200 column further improved the purity. The Umbelliferyl assay offered a rapid and sensitive assay to monitor the enzymatic activity of the neuraminidase.

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