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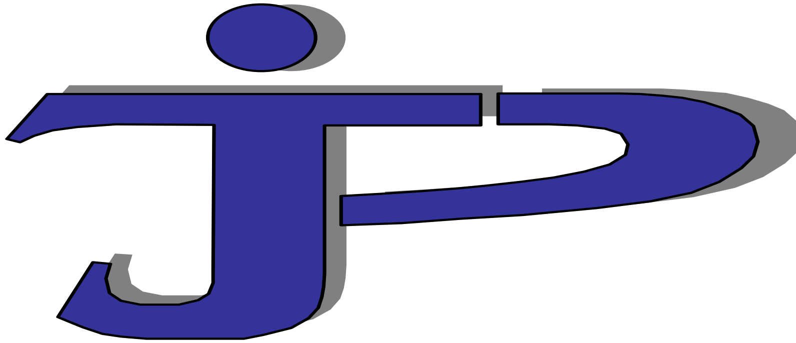

Agustus 2007

JURNAL TEKNOLOGI PERTANIAN

UNIVERSITAS MULAWARMAN

Penelitian

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Isolasi Jamur Penghasil Lipase dari Tanah, Tempe, dan Ragi Tempe (*Isolation of Lipase-Producing Molds from Soil, Tempeh, and Tempeh "Ragi"*) **Yuliani, Chusnul Hidayat, Supriyadi**

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A SIALIDASE FROM HORSE LIVER WAS CO-PURIFIED WITH *β*-GALACTOSIDASE AND CARBOXYPEPTIDASE A

Sialidase Hati Kuda Terdapat sebagai Enzim Kompleks dengan β-Galaktosidase dan Carboxypeptidase A

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ABSTRACT

The solubilized sialidase was purified using anion-exchange chromatography on Fractogel EMD TMAE-650 (M) followed by affinity chromatography on *p*-aminophenyl thio-β-D-galactopyranoside-agarose and chromatofocusing on PBE 94 with a factor and yield of about 18 and 0.2 %, respectively. The enzyme was found to be associated with β-galactosidase and carboxypeptidase A. The purified enzyme liberated sialic acid residues from sialooligosaccaharides (α2,3- was preferred than α2,6-sialyllactose), sialoglycoprotein and ganglioside such as GM3 and GD1a, however, the GM2 and GD1b are not suitable substrates for the sialidase as were also shown for BSM and guinea pig serum. The Neu2en5Ac is a strong competitive inhibitor with K_i of 47.5 µM.

Key words: Horse liver, sialidase, β-galactosidase, carboxypeptidase A, O-acetylated sialic acid.

INTRODUCTION

The interest in studying enzymes concerning sialic acids metabolism from animal-rich *O*-acetylated sialic acid has attracted much attention by the fact that little is known in the *O*-acetylated mechanism of sialic acid (Iwersen *et al*., 1998; Tiralongo *et al*., 2000; Shen dan Schauer, 2000). However, the catabolism of 4-*O*-acetylated neuraminic acid, which blocked most of sialidase activity, was first elucidated as esterase was found in horse liver and demonstrated that it involved in the 4-*O*-acetylated neuraminic acid. The esterase was first modified the acetyl at C-4 allowing the sialidase to hydrolyze the normal sialic acid from sialoglycoconjugate (Schauer *et al*., 1988).

Previously, we have partially purified a sialidase from horse liver (Candra *et al*., 2005). In the present study, we describe purification and characterization of sialidase from horse liver, which is distinct to the first sialidase we have isolated earlier. β-Galactosidase and carboxypeptidase A were co-purified during the purification.

MATERIALS AND METHODS

Materials

Fresh horse liver was obtained from a local slaughterhouse. The liver was excised and stored at –20 °C until used. Fractogel EMD TMAE-650 (M) was purchased from Merck. *p*-Aminophenyl thio-β-D-galactopyranoside agarose and D-galactonic acid γ-lactone (γ-galactonolactone) were obtained from Sigma Chemical Ltd. Polybuffer exchanger 94 and Polybuffer 74 were from Pharmacia LKB. Sialyl-methylumbelliferyl α-glycoside (MU-Neu5Ac) was from Toronto Research Center (Toronto, Canada). α2,3-sialyllactose and α2,6-sialyllactose were obtained from our lab. GD1a and GD1b were purchased from Matreya Inc. (Biotrend Chemikalien GmbH, Köln, Germany). NaCl was obtained from Reidel de Häen (Seelze, Germany). All other reagents used were of analytical grade from Sigma Chemical Ltd. (Deisenhofen, Germany), Serva (Heidelberg, Germany) and Merck (Darmstadt, Germany). Triton X-100 was supplied by Biomol (Hamburg, Germany).

Enzyme assays

Sialidase and β-galactosidase activity were assayed using MU-Neu5Ac and MU-Gal respectively as described in (Candra *et al*., 2000). For natural substrates, the free sialic acid released on sialidase activity assay was determined using thiobarbituric acid method (Warren, 1959). For sialidase assay on *O*-acetylated neuraminic acid using guinea pig serum and bovine submandibular mucin, the free neuraminic acid was detected using Fluorimetric-HPLC (Reuter dan Schauer, 1994). Carboxypeptidase A was assayed in a final volume of 100 µl, 10 µl of enzyme preparation was incubated in 80 mM potassium phosphate buffer pH 5.8 containing 1 mM *N*-CBZ-Phe-Leu 42 °C. After 30 minutes, 200 µL ninhydrin reagen was added to the reaction. The mixture was vortexed and then boiled for 10 minutes. The reaction was cooled on ice for 2 min, and then 700 µL of 50 % ethanol was added. Absorbance of the organic phase at the upper side following centrifugation was read at 570 nm.

Purification of sialidase

All purification processes were carried out at 4 °C. Frozen horse liver (65 g) was homogenized in 240 mL of cold distilled water with ultra turrax 3 times, each 1 min, and centrifuged at 100,000 g for 60 min. The Pellet was then solubilized in about 400 mL of 0.1 M phosphate buffer pH 5.5 containing 0.15 M NaCl, 0.5 % Triton X-100 and 0.25 M sucrose with Potter-Elvehjem apparatus for 10 strokes. After centrifugation at 100,000 *g* for 60 min, the supernatant (solubilisate) was diluted in order to achieved a buffer condition of 0.25 M phosphate buffer pH 5.5 containing 0.05 M NaCl, 0.125 % Triton X-100 and 0.25 M sucrose (buffer A). The diluted solubilisate was then applied to a 50 mL Fractogel EMD TMAE-650 (M) column (3.5x5.2 cm), which has been equilibrated with buffer A, at a flow rate of 1 mL min^{-1}. The column was washed with buffer A and the sialidase was eluted with NaCl gradient in buffer A between 50 and 1000 mM (400 mL). The sialidase fraction from Fractogel was dialysed against buffer A and then applied to a 7 mL of PATG-agarose column (1.4x6.4 cm) equilibrated with buffer A, at a flow rate of 0.5 mL min^{-1}. Bound enzymes were eluted with 1 M γ-galactonolactone in the buffer A. The affinity-purified enzyme was dialyzed against 0.1 M piperazine buffer pH 5.5 containing 0.15 M NaCl, 0.1 % Triton X-100 and 0.25 M sucrose (buffer B). The dialysate was then applied at flow rate of 0.5 mL min^{-1} to 10 mL PBE 94 column (0.48x14 cm), which has been equilibrated with buffer C by first running on 5 mL of eluent (PB 74 diluted 1:10 containing 0.05 M NaCl, 0.05 % Triton X-100 and 0.25 M sucrose, adjusted to pH 4.0) and then switching back to eluent again.

Analytical methods

The protein content was determined using spectrophotometer at 280 nm or by the Bradford method (Bradford, 1976) using Bio-Rad reagen with bovine serum albumin as the standard. SDS-PAGE was performed according to the procedure described in LaemmLi (1970) using 10-16 % gradient gel. Gels were stained for protein with silver nitrate.

RESULTS

Some different characteristics of sialidase were observed between the sialidase isolated in this present study and the sialidase isolated earlier (Candra *et al*., 2005). The sialidase in the present study needed at least 50 mM NaCl to be maintained in soluble form as well as detergent in the purification steps, as the two conditions was not needed for the sialidase isolated earlier. The sialidase activity could not be activated by incubating at 37 °C in acidic pH at range of 4.8 to 6.0 (data not shown), however, a decrease of activity was observed during the incubation. It also did not bind to the *N*-(*p*-aminophenyl)-oxamic acid agarose as in case of the earlier isolated sialidase.

Purification of sialidase

β-Galactosidase activity, which optimally at pH 4.0, was found to co-elute with sialidase activity following anion-exchange chromatography on Fractogel EMD TMAE-650 (M) (Figure 1).

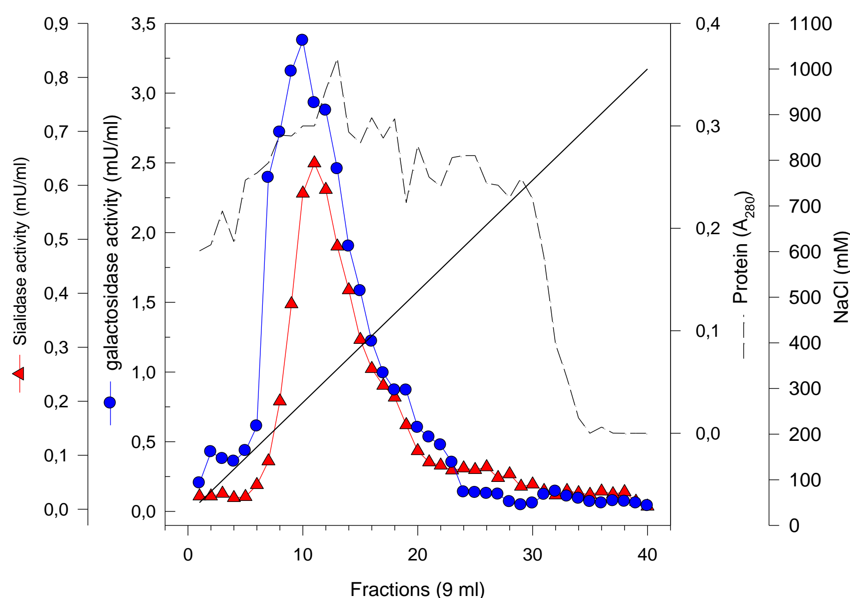

Figure 1. Chromatogram of sialidase and β-galactosidase activities following anion-exchange chromatography on Fractogel EMD TMAE-650 (M). The sample was applied at flow rate of 1.0 mL min^{-1}. Sialidase activity was assayed using MU-Neu5Ac as substrate at pH 4.5; β-Galactosidase activity was assayed using MU-Gal as substrate at pH 4.0.

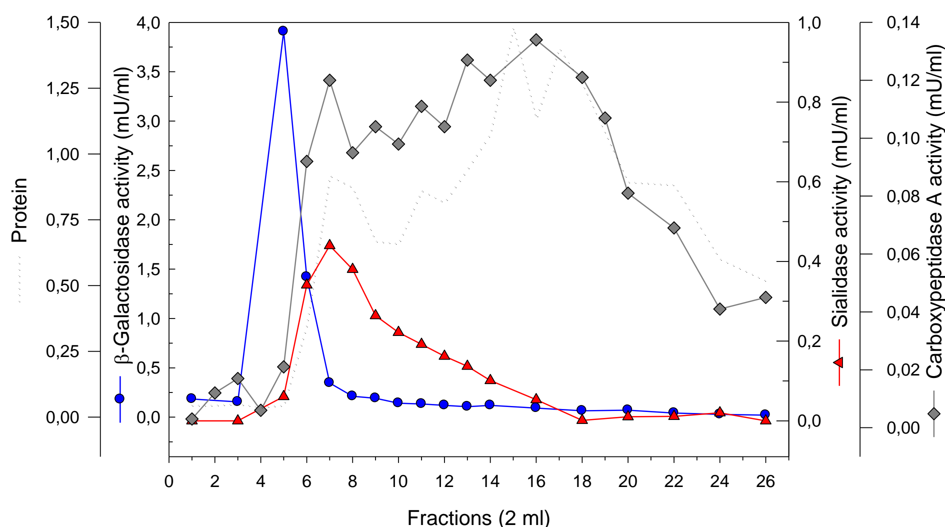

Figure 2. Chromatogram of sialidase, β-galactosidase and carboxypeptidase A activities following affinity chromatography on PATG-agarose. The sample was applied to PATG-agarose column (1.4x6.4 cm) at flow rate of 0.5 mL min^{-1}, which was equilibrated with 25 mM phosphate buffer containing 50 mM NaCl, 0.25 M sucrose and 0.125 % Titon X-100. The column was eluted with 1 M γ-galactonolactone in the equilibration buffer, and 2 mL fractions were collected. Sialidase and β-galactosidase activity were assayed as described in Figure 1, while carboxypeptidase was assayed at pH 5.8, 42°C for 30 minutes.

With the expectation that the sialidase isolated was occurred as enzyme-complex with β-galactosidase and carboxypeptidase A, a specific affinity medium for β-galactosidase, PATG-agarose, was used in the purification following anion-exchange on Fractogel EMD TMAE-650 (M). Following this affinity chromatography (Figure 2), the sialidase was purified about 9-fold with a yield of 0.6 %.

The sialidase was bound very tightly to the PATG-agarose and could only be eluted with high concentrated γ-galactonolactone. It started to elute when 0.5 M of γ-galactonolactone was applied and an optimum elution was when 1.0 M of the γ-galactonolactone used.

After this step, the expectation whether the sialidase isolated occurred as an enzyme-complex with β-galactosidase and carboxypeptidase A, as usually found for lysosomal sialidase was enhanced because the peak of carboxypeptidase A was also found in the same fraction where sialidase-activity peak was found. This explanation can also be seen in further purification step with chromatofocusing on PBE 94 (Figure 3). Following this step sialidase activity was purified by about 18-fold with a yield of 0.2 %. The β-galactosidase and carboxypeptidase were co-purified by factor of about 291-fold with yield of 6.5 % and 32-fold with yield of 0.4 % respectively (Table 1).

SDS-PAGE

The final preparation of sialidase was subjected to SDS-PAGE to investigate the sub-unit compositions of the enzymes. The SDS-PAGE under reducing condition of the enzyme preparation exhibits four bands (72, 78, 85 and 105 kD (data not shown). The protein band of 105 kD is predicted to be correspond to β-galactosidase activity, this 105 kD and 85 kD protein bands were disappeared in the SDS-PAGE under reducing condition giving a protein band with molecular weight over 200 kD.

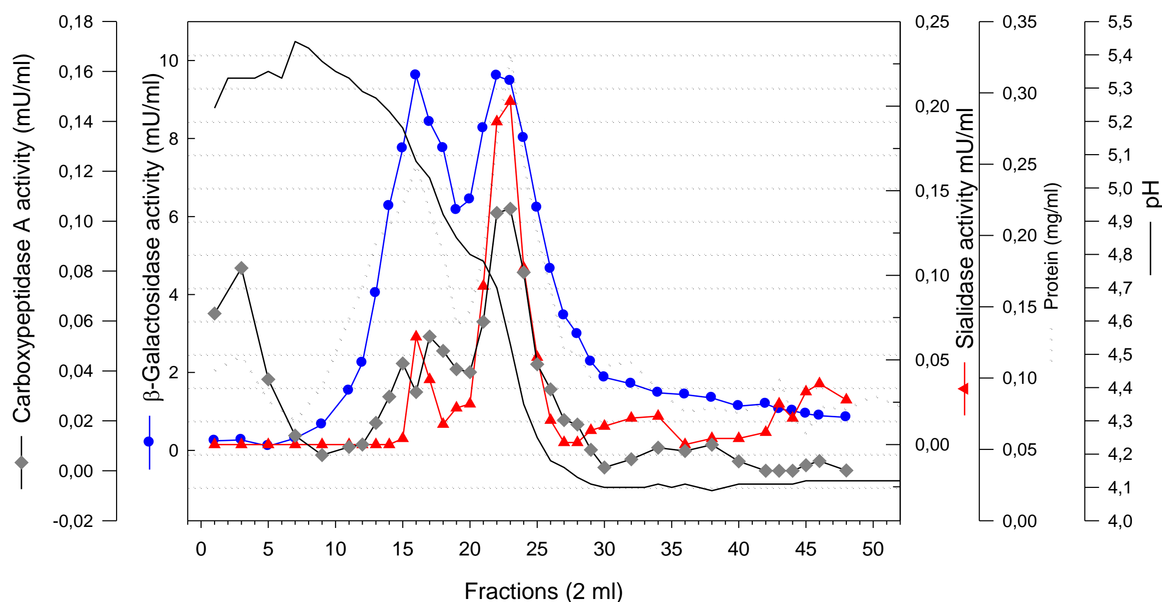

Figure 3. Chromatogram of sialidase, β-galactosidase and carboxypeptidase A activity following chromatofocusing with PBE 94. The sample was applied at flow rate of 0.5 mL min^{-1}, and 2 mL fractions were collected.

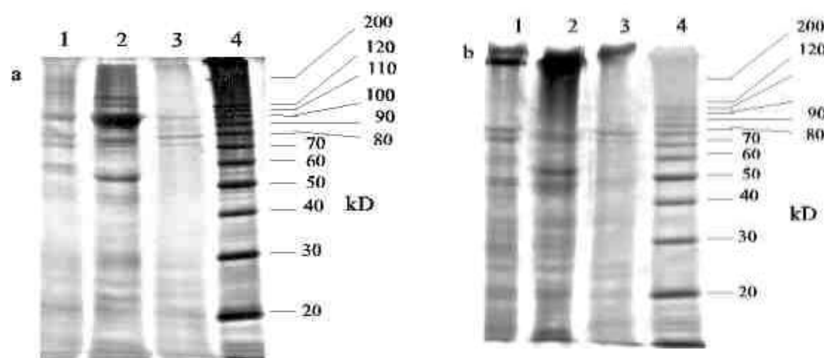

Figure 4. SDS-PAGE on a gradient gel between 10 and 16 % at reducing (a) and non-reducing (b) condition of sialidase poll at each step of purification. Lane 1, Fractogel EMD TMAE-650 (M) pool; Lane 2, PATG-agarose pool; Lane 3, PBE 94 pool; Lane 4, Molecular mass standard.

Table 1. Purification of sialidase from horse liver.

Fractions	Protein (mg)	Specific activity (μU mg^{-1})			Yield (%)			Purification factor		
		Sial	β-Gal	CarbA	Sial	β-Gal	CarbA	Sial	β-Gal	CarbA
Homogenate	13,822.8	82	231	26	100.0	100.0	100.0	1.0	1.0	1.0
Supernatant	8,663.8	77	219	33	58.9	59.3	79.0	0.9	1.0	1.3
Fractogel	975.3	84	1,431	38	7.2	43.7	10.3	1.0	6.2	1.5
PATG-agarose	9.3	752	25,802	743	0.6	7.5	1.9	9.2	111.7	28.6
Chromatofocusing	1.4	1,500	67,423	851	0.2	6.5	0.4	18.3	291.9	32.7

Note: Sial = sialidase, β-Gal = β-galactosidase, CarbA = carboxypeptidase A

Properties of the purified sialidase

Temperature- and pH optimum

The effects of temperature and pH on the activities of the sialidase were determined in acetate or phosphate buffer using MU-Neu5Ac. As shown in Figure 5, the temperature and pH optimum for the sialidase are 42-46 °C and 4.5, respectively.

pH stability

The stability of the sialidase at various pHs was determined by keeping the enzymes in 0.1 M acetate or phosphate buffers ranging in pH from 3.5 to 7.0 at 37 °C prior to standard assaying at pH 4.5 (Figure 6a). The sialidase was stable at pH 4.5, 5.0 and 6.0, however rapidly lost its activity at pH 4.0 or 7.0. At 4 °C, the sialidase was quite stable in all of the pH-range tested except at pH 4.0 with a rapid lost of activity (data not shown).

Freeze-thaw effects

The sialidase was also stable to the effects of freeze-thawing (Figure 6b). Following 4 freeze-thaw cycles, sialidase in 25 mM piperazine buffer pH 5.5 containing 0.25 M sucrose, 0.05 M NaCl and 0.01 % Triton, only lost approximately 14 % of its activity.

Influence of oligovalent cations and inhibitors to the sialidase activity

The Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} in chloride form between 0.25 and 10 mM had an inhibitory about 5 to 40 % of sialidase activity (data not shown). On the other hand, sialidase activity could be increased by about 20 % with the addition of 0.5 mM aluminium chloride. However, at higher concentration $AlCl_3$ was inhibitory (Figure 7a). The activation of sialidase activity by aluminum chloride disappeared when 1 mM EDTA was added (Figure 7b).

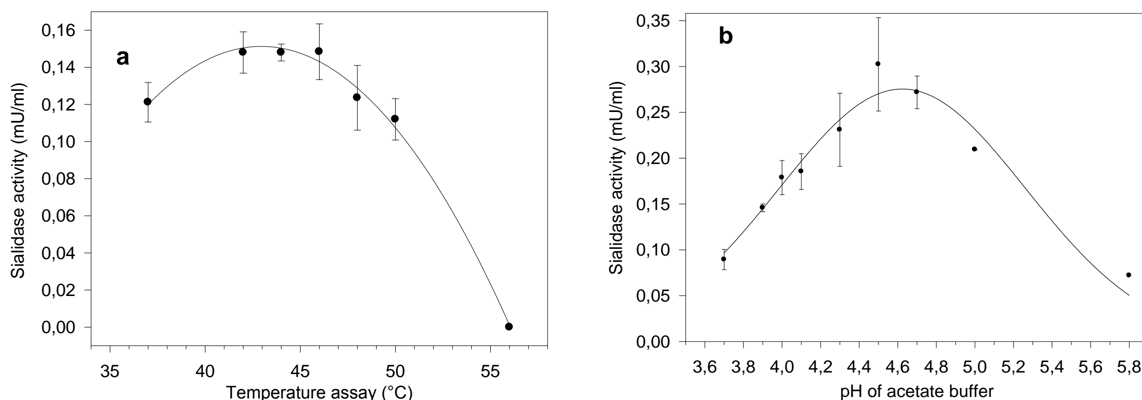

Figure 5. Influence of pH and temperature to the sialidase activity. (a) Temperature experiment was performed in 70 mM acetate buffer pH 4.5 containing 0.1 mM MU-Neu5Ac, **(b)** pH Experiment was performed in 70 mM acetate and phosphate buffer for pH 3.7-5.0 and 6.0 containing 0.1 mM MU-Neu5Ac respectively at 46 °C.

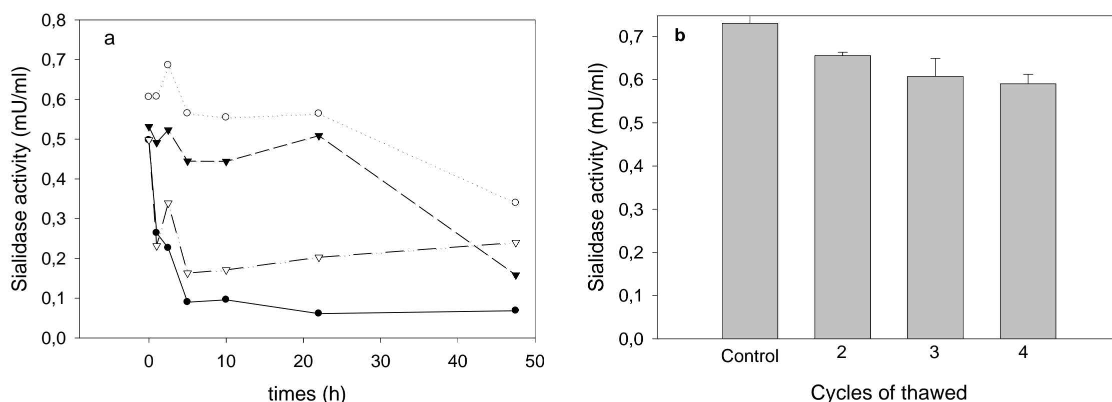

Figure 6. Effect of pH on the stability of sialidase activity. (a) The enzyme was placed in 0.1 M acetate buffer or phosphate buffer ranging in pH between 4.0 and 7.0 at 37 °C, and then the sialidase activity was measured with MU-Neu5Ac substrate at pH 4.5. ●H 4.0; , ○ pH 5.0; ▲, pH 6.0; △pH 7.0. **(b)** The enzyme was thawed from –80 C in piperazine buffer pH 5.5 containing 0.05 M NaCl, 0.25 M sucrose and 0.1 % Triton X-100.

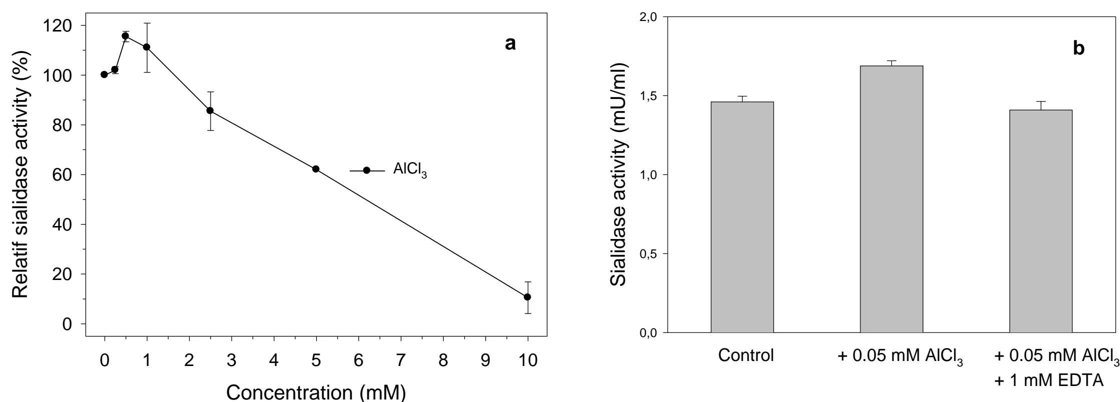

Figure 7. Influence of Al^{3+} to sialidase activity. (a) Influence of different concentrations of Al^{3+} on sialidase activity, **(b)** Influence of Al^{3+} at concentration of 0.5 mM with and without EDTA on sialidase activity.

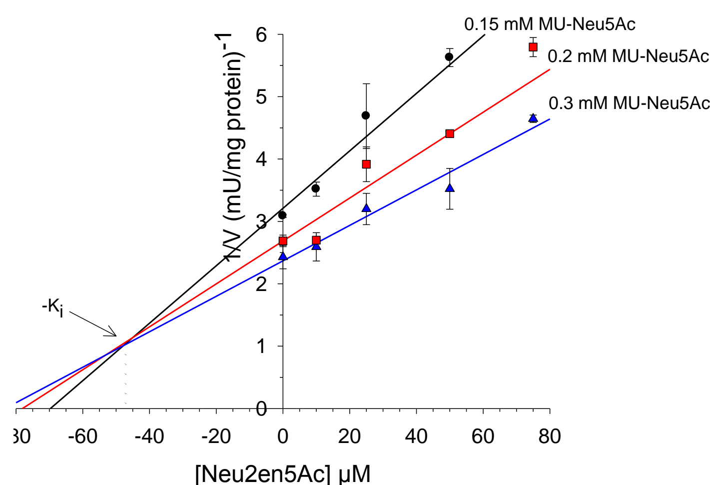

Figure 8. Dixon plot for the inhibition of sialidase activity by Neu2en5Ac. The sialidase activity was assayed in 60 mM acetate buffer pH 4.5 containing Neu5,2en between 0 and 70 µM for each MU-Neu5Ac concentration used at 46°C for 60 minutes. . ● , 0.15 mM MU-Neu5Ac; ■ , 0.20 mM MU-Neu5Ac; ▲ , 0.3 mM MU-Neu5Ac.

Inhibition of the sialidase

Neu5Ac and *N*-(*p*-aminophenyl)-oxamic acid up to concentration of 1 mM showed no inhibitory effects on sialidase activity. However, a light inhibitory effect of about 20 % was showed at concentration of 5 mM. On the other hand, Neu2en5Ac was a strong competitive inhibitor for the sialidase with K_i of 47.5 µM (Figure 8).

Substrate specificity of sialidase

Sialyllactose, fetuin and the gangliosides, GM3 and GD1a, were found to act as substrates for the purified enzyme, how ever GD1b and GM2 were not (Table 2). The enzyme showed very high activity toward α2,3-sialyllactose compared to α2,6-sialyllactose or ganglioside mixture from bovine brain. The apparent Michaelis constants for some substrate show that the enzyme has high affinity towards α2,3-sialyllactose (Table 3).

Table 2. Substrate specificity of sialidase from horse liver

Substrates	Concentration (µM)	Sialidase activity (µU/mg protein)
GM3	3.9	98.5
GD1a	6.8	522.3
Fetuin	9.2	42.6
GM2	4.6	0.0
GD1b	4.5	0.0
BSM		0.0
Guinea pig serum		0.0

Table 3. The K_m and V_{max} values for various sialidase substrates

Substrates	K_m (µM)	V_{max} (µU/mg protein)
MU-Neu5Ac	117.3	520.5
2,3-sialyllactose	338.9	680.0
2,6-sialyllactose	132.1	207.8
Gangliosides mixture from bovine brain	50.0	228.0

DISCUSSION

Following a previous report, we have purified horse liver sialidase with another purification method with the application of a specific affinity media for β-galactosidase, PATG-agarose. A large amount of β-galactosidase was obtained during the purification on PATG-agarose. However, most of the β-galactosidase activity was removed by applying chromatofocusing on PBE 94. The purification of sialidase using PATG-agarose were successfully applied in the case that the sialidase was occurred as enzyme-complex with other tow enzymes, β-galactosidase and carboxypeptidase A (Verheijen *et al*., 1982; Verheijen *et al*., 1987; Hiraiwa *et al*., 1996; Hiraiwa *et al*., 1997). The expectation that the sialidase isolated is an enzyme-complex with the two other enzymes was raised because the carboxypeptidase A activity was detected in the enzyme preparation following purification on PATG-agarose, and the activities of the three enzymes were again detected in the same fractions. This evidence shows that the sialidase isolated in this present study was distinct to the sialidase reported at previous study because b-galactosidase could be completely removed from the last sialidase following affinity chromatography on *N*-(*p*-aminophenyl)-oxamic acid-agarose (Candra *et al*., 2005). Activation of sialidase activity by incubating the enzyme preparation (solubilisate) in acidic pH-condition at 37°C for 90 min was also different. The sialidase activity from the present study decreased following the incubation, as not in case of the sialidase from the previous study. This result is, unfortunately, very difficult to explain. One possible explanation is that the livers were obtained from different breeds of horses, however the exact breed of the horses is unknown.

Taking the consideration that eucaryotic sialidases have molecular weight around 50 kD or more (Vinogradova *et al*., 1998; van der Spoel *et al*., 1998; Potier *et al*., 1990; Hata *et al*., 1998; Fronda *et al*., 1999; Miyagi dan Tsuiki, 1995), it appears probably that the protein band at approximately 72°kD could be the sialidase, since this band was only observed in the sialidase-positive fractions and not in other fractions. Experiments to resolve this puzzle using Blue native-PAGE (BN-PAGE) (Schägger dan von Jagow (1991) were unfortunately unsuccessful, possibly because the sialidase was unstable or the enzyme-complex conformation was disturbed, which gave negative effects to the sialidase activity. Hiraiwa *et al*. (1996) demonstrated that sialidase, which occurred as enzyme-complex with β-galactosidase and carboxypeptidase A, could be dissociated by incubation in pH 7.5 and its activity was irreversibly inactivated even following reconstitution of the complex.

As our interest in the catabolism of *O*-acetylated sialic acid, particularly 4-*O*-acetylated sialic acid, experiments using blood serum of guinea pig, which contained about 32 % of 4-*O*-acetylated sialic acid, was performed using thiobarbituric acid test or fluorimetric-HPLC. The purified enzyme could not release sialic acids from the guinea pig blood serum, as well as from BSM. Some sialidases were also reported that BSM was not suitable for their substrate (Hata *et al*., 1998; Hiraiwa *et al*., 1988)

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