Studies on the neuraminidase from chick chorioallantoic membranes*

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Abstract The sialoglycoproteins were extracted from the chorioallantoic membranes (CAM) of 17 days-old chick embryo using lithium 3, 5-diiodosalicylate (LIS) and sodium dodecyl sulfate (SDS). The sialoglycoprotein prepared from CAM with LIS was hydrolyzed by bacterial neuraminidase accompanied with the release of sialic acid. However, the sialoglycoprotein extracted by SDS from membranes was not hydrolyzed becuase of the existence of SDS which was a strong inhibitor of the enzyme.

The solubilization of the neuraminidase (N-acetylneuraminylhydrolase, E. C. 3. 2. 1. 18) from the chorioallantoic membranes was carried out with the following solution: 0.1 M sodium phosphate buffer, pH 7.0, 0.1 M KCl, 25% glycerol, 1 mM 2-mercaptoethanol, 0.5% deoxycholic acid sodium salt. Solubilized enzyme was partially purified by the gel filtration (Sephadex G-75) and the ion exchange chromatography (CM-Sephadex C-50). Neuraminidase activities of the intact CAM and solubilized enzyme from CAM were measured using sialoglycoproteins from CAM, chicken ovomucoid, mucin, fetuin, colominic acid, sialyllactose and ganglioside as substrate. The pH optimum was at 4.3 and a marked loss of enzyme activity was observed by heating at 60°C and above for 60 min. Km and Vmax values were determined for colominic acid and fetuin. Though Ca^{2+} did not affect the enzyme activity, Cu^{2+} and Hg^{2+} inhibited the activity extensively.

Introduction

In 1961, Ada and Lind first reported neuraminidase (NAase, sialidase) activity found in the chorioallantois of the chick embryo[1-8]. Chorioallantoic membrane (CAM) contained not only NAase but also sialoglycoproteins, so sialic acid was released from the membrane during incubation in an appropriate condition[4].

The sialic acid are widely distributed as a terminal carbohydrate residue in mammalian tissues, being components of milk oligosaccharides, mucins, gangliosides, and mucopolysac-

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* This report was partially communicated by F. Ibuki at the Annual Meeting of the Agricultural Chemical Society of Japan held at Yokohama (1977).
Received on July 26, 1980

charides and so on. The biological function of sialic acid in glycoproteins has been studied principally by observing the effects of selective removal of this residue by digestion with NAase[5].

Chick embryo incubated for 21 days develops into chick under a suitable condition. During incubation chorioallantoic membrane appears and develops around the embryo and egg white proteins are gradually absorbed through the membrane.

In the preceding paper[4], the nature of membrane bound neuraminidase and correlation of membrane and sialoproteins. This paper deals with the solubilization of the enzyme from the chorioallantoic membrane and several properties of the solubilized enzyme compared with the membrane bound enzyme.

Materials and Methods

1. Materials.

White Leghorn fertile eggs (17 days-old) of both sexes were obtained from Sato Furanjo (Kyoto, Japan). The chorioallantoic membranes from chick embryo were prepared by the same way described in the preceding paper[4]. Chicken ovomucoid was prepared as previously described[4]. Colominic acid, homopolymer of sialic acid was generous gift from Dr. T. Sugimori, Kyoto Research Laboratory, Marukin Shoyu Co. Ltd. (Kyoto, Japan). Fetuin (Type I) from fetal calf serum, bovine brain ganglioside (Type II), N-acetylneuramin-lactose (Type II) from bovine colostrum, and mucin (Type I) from bovine submaxillary glands were purchased from Sigma Chemical Co. Highly purified neuraminidase from *Arthrobacter Ureafaciens* was from Nakarai Chemicals (Kyoto, Japan). All other chemicals were reagent grade.

2. Chemical determination.

Protein was determined by the measurement of absorption at 280 nm spectrophotometrically. The sialic acid content was determined by the thiobarbituric acid reaction[6] after hydrolyzing the glycoprotein in 0.1 N H_2SO_4 at 80°C for 1 hr. The carbohydrate content was determined by the phenol-sulfuric acid procedure of Dubois et al[7].

3. Isolation of crude sialoglycoproteins with sodium dodecyl sulfate[8].

Chorioallantoic membranes (wet weight 3.05 g) were minced well with scissors, and blended with 10 volumes of the cold acetone (−20°C). The suspension was immediately filtered under reduced pressure. These operations were repeated twice. The resulting acetone powder (297 mg) was stored under reduced pressure at 4°C in a desiccator. For the extraction of sialoglycoproteins, acetone powder (297 mg) was mixed with 40 volumes of 0.01 M Tris-HCl buffer, pH 7.8 containing 0.1% sodium dodecyl sulfate (SDS) and the suspension was centrifuged at 12,000 g for 30 min. The supernatant was dialyzed against water at 4°C overnight and dialysate was lyophilized (48.4 mg).

4. Isolation of crude sialoglycoproteins with lithium diiodosalicylate[9]

The minced chorioallantoic membranes (9.48 g) were suspended in 0.3 M lithium 3.5-diiodosalicylate (LIS)-0.05 M Tris-HCl buffer, pH 7.5 at a concentration of 25 mg of

membrane protein per ml. Then, 2 volumes of distilled water was added, and stirring was continued for 15 min at 4°C. The mixture was centrifuged at 12,000 g for 30 min at 4°C and the resulting supernatant was mixed with an equal volume of 50% of phenol for 15 min at 4°C. Mixture was centrifuged at 4,000 g for 60 min. The upper aqueous layer was picked up and dialyzed overnight against several changes of distilled water. The clear dialysate was freeze dried (79.4 mg).

5. Solubilization and partial purification of neuraminidase from chorioallantoic membranes[10].

The membranes of chick embryo (17 days-old, 18 g wet weight) were frozen (acetone dry ice bath) and thawed (warm water bath) three times. The tissue was treated with 30 to 35 ml of 0.1 M phosphate buffer, pH 7.0, 0.1 M KCl, 25% glycerol, 1 mM 2-mercapto-ethanol, 0.5% deoxycholic acid sodium salt for 5 to 6 hr at 4°C with gentle stirring. After centrifugation at 12,000g for 30 min at 4°C, the supernatant was dialysed against 0.05 M phosphate buffer, pH 7.0 overnight. Then the dialysate was ultracentrifuged at 50,000 g for 60 min and the supernatant was concentrated by freezing-dry and applied to a column (94.5×2.5 cm) of Sephadex G-75 for the partial purification. The detail condition will be described in the results section.

6. Enzymatic analyses.

The method of neuraminidase activity measurement was reported in the previous paper[4]. Eighty to 400 μg of protein were used in the assay of solubilized NAase from CAM. NAase enzymatic activity was detected by the release of sialic acid from fetuin, colominic acid, mucin, ganglioside and ovomucoid etc. Reaction was terminated by adding HIO_4 reagent and cooled to −20°C. The released sialic acid was calculated with following equation: amount of sialic acid (μg) = 309×2.5×O. D. 549 nm/70.7. The reaction mixture was adjusted pH to 4.3 with acetic acid prior to the assay for enzymatic activities.

Results

1. Chemical analyses of sialoglycoproteins extracted from chorioallantoic membranes.

The analytical values for the components of sialoglycoproteins from CAM were summarized in Table 1. The both sialoglycoproteins prepared with LIS and SDS showed the similar components. Also sialoglycoproteins extracted with LIS and SDS from CAM were found to have high absorption maxima at 260 nm rather than that at 280 nm. This might

Table 1. Chemical composition of the sialoglycoproteins prepared with sodium dodecyl sulfate (SDS) and lithium diiodosalicylate (LIS) from CAM

	sialoglycoportein prepared with SDS	LIS
protein (%)	17	21
sialic acid (%)	0.4	0.3
hexose (%)	3	6

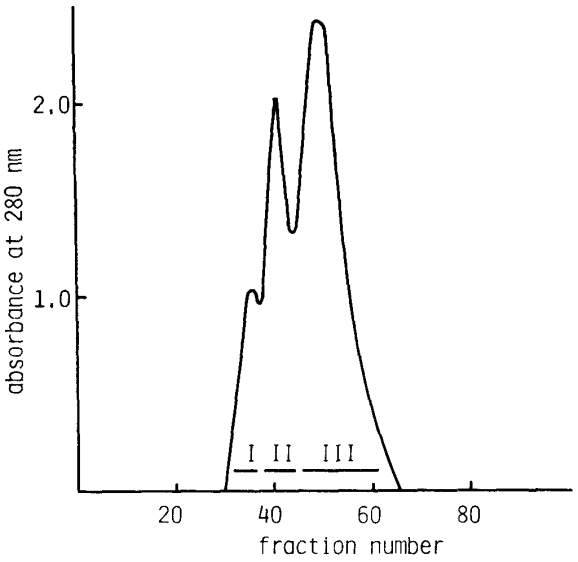

Fig. 1. Gel filtration of sialoglycoproteins solubilized from the chorioallantoic membranes with SDS on Sephadex G-50. The sample in 3 ml of 0.05 M phosphate buffer, pH 7.0 containing 1 % SDS was applied to a column (2.5×93.5 cm). Fractions of 5 ml were collected at a flow rate of 20 ml/hr.

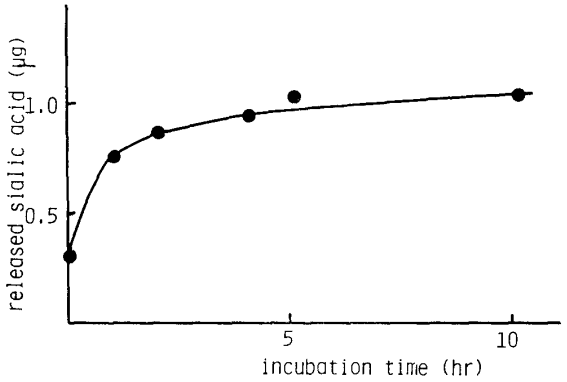

Fig. 2. The rate of sialic acid release from a sialoglycoprotein prepared from chorioallantoic membranes with LIS by bacterial neuraminidase. Assay mixture contained 2.6 mg sialoglycoprotein, 2.0 ml of 0.1 M acetate buffer, pH 5.0 and 0.02 unit of the enzyme. An aliquot of 0.2 ml was taken out for the assay.

be caused by the presence of the nucleic acid material.

The sialoglycoprotein obtained from the CAM with SDS was chromatographed on a Sephadex G-50 (fine) column as shown in Fig. 1. The three peaks were observed and each fraction indicated the bar was collected, dialysed and analysed. The first peak (I) contained 13% of protein, 4% of sialic acid and 0.4 % of hexose, the second peak (II), 8 %, 1% and 0.2% and the third peak (III) 7 %, 1 % and 0.1 %, respectively. The first peak fraction contained much more sialic acid among the those fractions, however, it was so difficult to remove SDS completely by dialysis which inhibited the neuraminidase activity in large extent. Consequently, the sialoglycoproteins prepared with SDS from CAM and that fractionated with a Sephadex gel chromatography using the medium contained SDS were impossible to use as the substrate of neuraminidase. In the following experiments, the sialoglycoprotein obtained with LIS was used as neuraminidase substrate without further fractionation.

As shown in Fig. 2, about 50% of total sialic acid was released by purified bacterial neuraminidase after 5 to 10 hr incubation. This result shows sialoglycoproteins from CAM can be expected being a suitable substrate for intact membrane neuraminidase.

2. Partial purification of chorioallantoic membrane neuraminidase.

The frozen solubilized neuraminidase from CAM was clarified by centrifugation and the supernatant was applied to a column (2.5×95 cm) packed with Sephadex G-75 equilibrated before with 0.05 M phosphate buffer, pH 7.0. Elution was carried out with the same buffer at a flow rate of 25 ml/hr. Five ml of fractions were collected. An aliquot of the

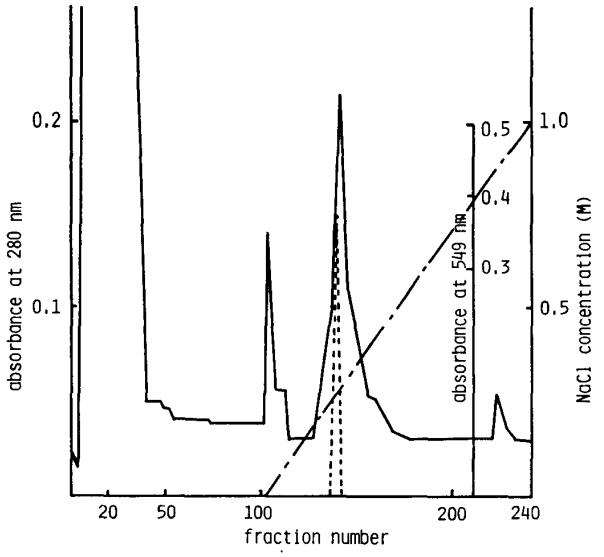

Fig. 3. The ion exchange chromatography of solubilized membrane neuraminidase obtained by Sephadex G-75 gel filtraration on a column of CM-Sephadex C-50. The clear supernatant in 0.05 M Tris-HCl buffer, pH 7.0 after the ultracentrifugation of solubilized neuraminidase treated with Sephadex G-75, was applied to a column (2.0×20 cm) of CM-Sephadex C-50 equilibrated with Tris-HCl buffer (0.05 M, pH 7.0). Elution was carried out with the same buffer at a flow rate of 22 ml/hr. The fraction volume was 4 ml. After the sufficient washing of the column, the linear NaCl gradient elution was started. Colominic acid was used as substrate in the assay. ———, absorbance at 280 nm (protein) ; ·····, absorbance at 549 nm (activity) ; -•-•-•-, NaCl concentration.

fraction was adjusted pH to 4.3 with acetic acid prior to the assay. Expected results were not gained. Namely, the activity peak coincided in the main protein peak and the ratio of activity to protein did not increase before and after the gel filtration. The collected active fraction in gel chromatography was treated on Sephadex G-100. However, further purification did not proceed.

One trial for the purification with CM-Sephadex C-50 was carried out. A typical result is shown in Fig. 3. An enormous non-active fraction was eluted as non-absorbed fraction and a sharp peak were obtained as active fractions with a linear NaCl gradient elution. These fractions were collected. However this active enzyme fraction were found to be very unstable and almost the activity was lost during the steps for the dialysis and concentration. Therefore, the experiments hereafter were carried out using the enzyme treated with Sephadex G-75 gel filtration.

3. The neuraminidase activities of intact chorioallontoic membrane and solubilized enzyme from membranes against various substrates.

A comparative studies of the rates of hydrolysis of various substrates by the intact and solubilized enzyme of CAM are shown in Fig. 4 to 7. Chicken ovomucoid, colominic acid, sialyllactose and ganglioside were hydrolyzed more efficiently than mucin by intact CAM. Fetuin was not attacked by intact CAM. Ovomucoid, colominic acid, sialyllactose were not hydrolyzed than fetuin and mucin by solubilized neuraminidase. Ganglioside was scarecely

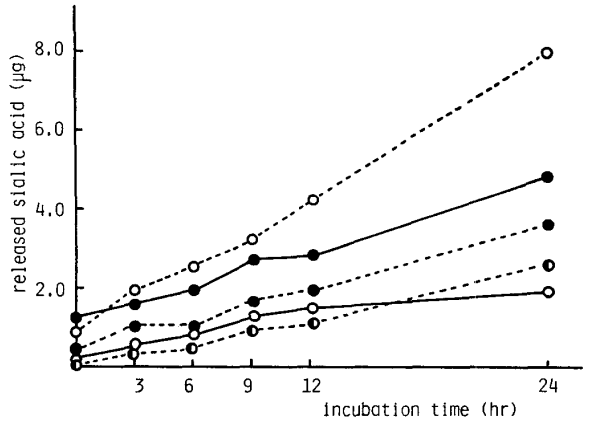

Fig. 4. The hydrolysis of various substrates containing sialic acid by intact chorioallantoic membranes. Assay mixture contained 100 mg of CAM, 2.0 ml of 0.01 M acetate buffer, pH 4.3 and various substrates. ○·····○, colominic acid 1 mg, ●——●, ovomucoid 30 mg; ●·····●, bovine submaxillary gland mucin 2 mg; ○——○, fetuin 5 mg; ◐·····◐, 100 mg of CAM alone.

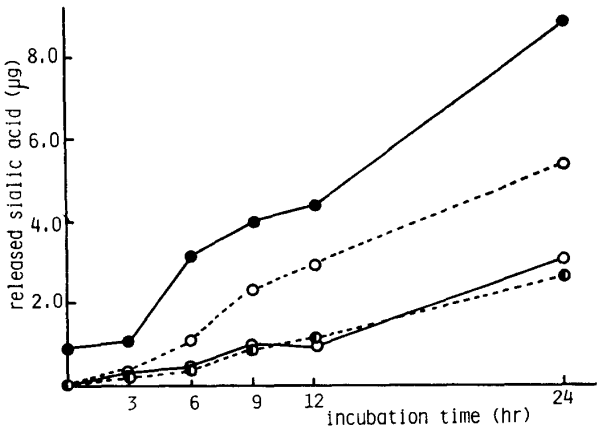

Fig. 5. The hydrolysis of various substrates containing sialic acid by intact chorioallantoic membranes. The assay condition was the same as described in Fig. 4. ●——●, sialyllactose 2 mg; ○·····○, ganglioside 3.8 mg; ○——○, sialoglycoprotein from CAM with LIS 2 mg; ◐·····◐, 100 mg of CAM alone.

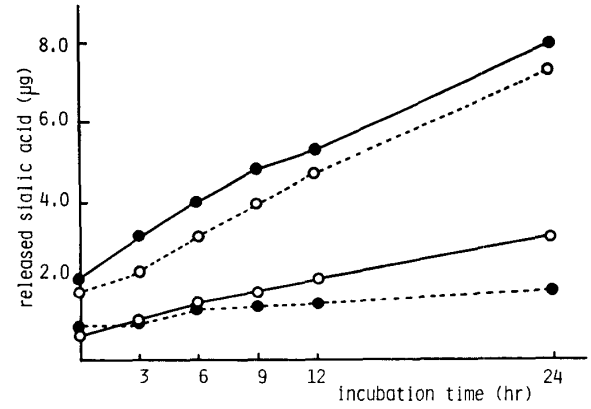

Fig 6. The hydrolysis of various substrates containing sialic acid by solubilized neuraminidase from CAM. The assay mixture contained 0.5 ml of enzyme solution, 2.0 ml of 0.01 M acetate buffer, pH 4.3 and substrate. ○·····○, colominic acid 0.5 mg; ●——●, ovomucoid 30 mg; ○——○, fetuin 5 mg; ●·····●, bovine submaxillary gland mucin 2 mg.

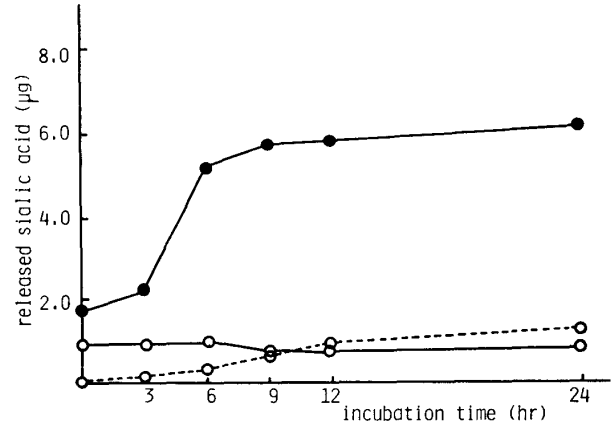

Fig. 7. The hydrolysis of various substrates containing sialic acid by solubilized neuraminidase from CAM. The assay procedure was the same as described in Fig. 6. ●——●, sialyllactose 2 mg; ○·····○, ganglioside 3.8 mg; ○——○, sialoglicoprotein from CAM with LIS 10 mg.

hydrolyzed. In the case of sialoglycoprotein prepared from CAM with LIS, the content of sialic acid might be considered to be too little to detect.

4. Several properties of solubilized neuraminidase from chorioallantoic membranes.

The enzyme had the optimum pH at 4.3 showing a fairly sharp peak with use of colominic acid as substrate as shown in Fig. 8. On the other hand, the pH-activity curve

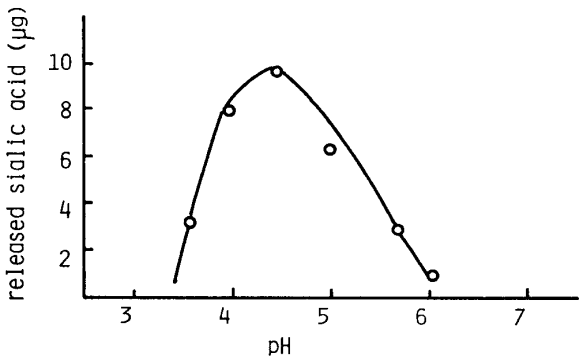

Fig. 8. Effect of pH on the enzyme reaction. Solubilized enzyme was assayed with colominic acid as substrate.

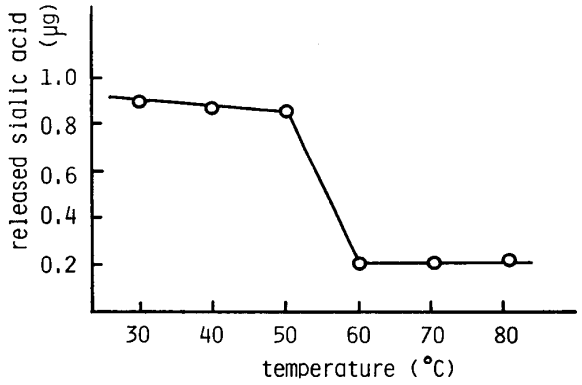

Fig. 9. Heat stability of solubilized enzyme from CAM. The enzyme was incubated at 30°C to 80°C for 60 min. After the incubation, the enzyme activity was measured with colominic acid.

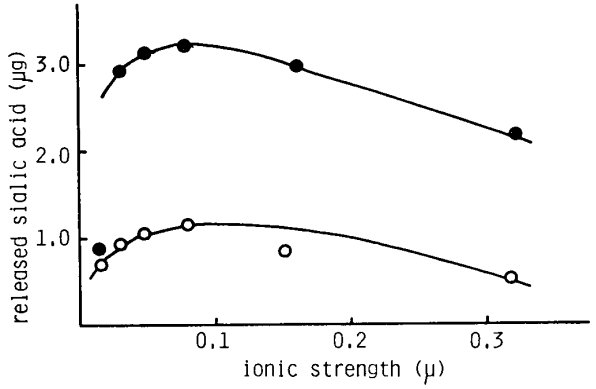

Fig. 10. Effect of ionic strength on neuraminidase activity solubilized from CAM. Colominic acid and mucin were used as substrates. ●—●, colominic acid; ○—○, mucin.

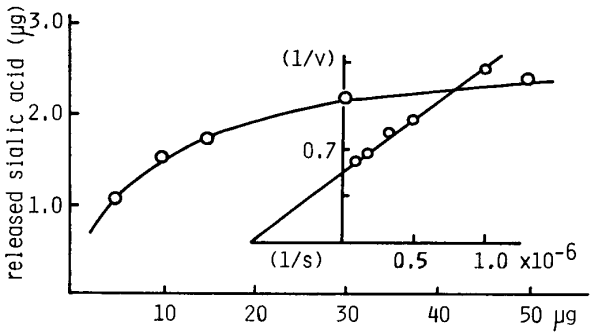

Fig. 11. Effect of the concentration of colominic acid on the velocity of hydrolysis.

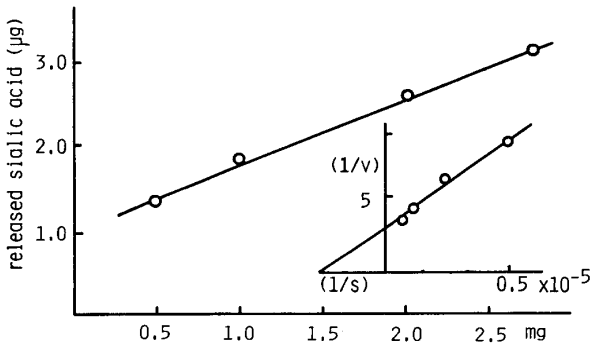

Fig. 12. Effect of the concentration of fetuin on the velocity of hydrolysis.

for mucin showed a broad peak between 4.3 and 4.5.

The time course of the reaction in neuraminidase activity followed a straight line with in the time untill 4 hr. Also, a linear relationship was observed between protein concentration and the sialic acid release. The both reactions were assayed with colominic acid as substrate.

Table 2. Effect of various cations on the neuraminidase activity solubilized from CAM

cation	activity (%)
Fe^{2+}	110
Co^{2+}	120
Ni^{2+}	100
Zn^{2+}	80
Ca^{2+}	80
Mg^{2+}	70
Cu^{2+}	20
Hg^{2+}	0
Ag^{+}	60

The cations were added to the reaction mixture at the final concentration of 5×10^{-3}M, and the remaining enzyme activity after the reaction was expressed compared with the initial enzyme activity (100%).

Heat stability of neuraminidase was measured, preincubating for 60 min at 30°C to 80°C prior to the assay. As shown in Fig. 9, a marked loss of the activity was observed when the enzyme was heated above 60°C. The enzyme experienced a progressive loss of activity during the prolonged incubation and about 50% of the initial activity was lost after 12 hrs' incubation at 37°C, pH 4.3. Solubilized membrane neuraminidase were stable for 24 hrs' incubation at various pH ranged from 3 to 8 at 37°C. Effect of ionic strength on the enzyme activity was determined using colominic acid and mucin. In the both cases, the slow down in the enzyme activity was recognized at high ionic concentration. (Fig. 10)

The interaction of substrate concentration and reaction rate are demonstrated in Fig. 11 and 12. With the use of the Lineweaver and Burk plot, calculted Km values were 1.6×10^{-6} M and 2.2×10^{-5} M for colominic acid and fetuin and Vmax were 0.2 μg released sialic acid/mg protein/min for both substrates.

The effect of several bivalent cations on the enzyme activity was studied. At the final concentration of 5×10^{-3} M, Cu^{2+} and Hg^{2+} caused strong inhibition of the enzyme. Other cations had very little effect on the activity. These results are shown in Table 2.

Discussion

For the purpose of clarification of the absorption mechanism of egg white sialoglycoprotein into embryo and the interaction between sialoglycoprotein and chorioallantois, sialoglycoprotein and neuraminidase were isolated from CAM. LIS, SDS, Triton X-100[8] and pyridine[11] were used for the extraction of glycoprotein from membranes. The use of Triton X-100 and pyridine was unsuccessful. Table 1 shows chemical compositions of sialoglycoprotein extracted with SDS and LIS are relatively resemble. Three protein peaks were observed by gel chromatography of isolated sialoglycoproteins solubilized by SDS on Sephadex G-50 (fine) (Fig. 1). But, the trials of rechromatography of each peak were unsuccessful. These findings show the aggregation and polymerization of membrane proteins after gel filtration.

Besides, it could not use as substrate because of inhibitory activity of SDS for the enzyme activity. It would be a point how to remove SDS from purified protein completely.

On the other hand, the sialoglycoprotein extracted with LIS was hydrolyzed by neuraminidase purified from *Arthrobacter Ureafaciens* as shown in Fig. 2, but it was hardly hydrolyzed with intact and solubilized CAM neuraminidase. As regards this glycoprotein, it is difficult to separate by column chromatography owing to insolubility of it. Perhaps, the gel filtration containing nonionic surface active agent will be effective concerning this membrane glycoprotein extraction.

Neuraminidase was isolated from chorioallantoic membrane using deoxycholic acid sodium salt and the several properties of it were studied. Substrate specificities of intact and solubilized neuraminidase were measured by the use of sialyllactose, ganglyoside, fetuin etc. These results showed poor activity of intact CAM toward fetuin and that of solubilized neuraminidase for ganglyoside. While other substrates were hydrolized by both enzymes, except sialoglycoprotein extracted from CAM.

The pH optima for the solubilized and intact CAM neuraminidase were very resemble[12]. As the coloration of 2-thiobarbituric acid was sensitive to ionic strength, it is difficult to make clear the effect of ionic strength on enzymatic activity, though we got the results in which the ionic strength effected on the activity. EDTA (10^{-3} M), Ca^{2+} (5×10^{-3} M) did not affect enzyme activity. But the results obtained by the addition of Cu^{2+} or Hg^{2+} to the neuraminidase of CAM are similar to those previously observed with the neuraminidase of liver, brain and mammary glands of rat.[13-15]

A partial purification of the enzyme was achieved by the gel filtration and ion exchange chromatography. However, the results were unsatisfactory. Though the single protein chromatographically was obtained, that enzyme was very unstable. Something to keep the enzyme protein stable might be removed by ion exchange Sephadex.

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鶏胚漿尿膜のシアリダーゼに関する研究

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要旨：孵卵17日目の鶏胚の漿尿膜（CAM）よりシアロ糖タンパク質をリチウム 3,5-ジヨードサルチル酸（LIS）やドデシル硫酸（SDS）を用いて可溶化した。LIS によって可溶化されたタンパク質は，微生物由来のシアリターゼによって加水分解されてシアル酸を遊離した。このことは CAM 自体が膜シアリダーゼの基質になっている可能性を示唆している。SDS によって可溶化されたタンパク質は，酵素の一般的阻害剤である SDS を完全に除去することが難かしく，加水分解されなかった。デオキシコール酸を用いて，CAM よりシアリダーゼを可溶化し，ゲル沪過と CM-Sephadex C-50 によるイオン交換クロマトグラフィーによって部分精製した。可溶化した酵素は CAM より単離したタンパク質，オボムコイド，ムチン，フエツイン，コロミン酸，シアリルラクトーズなどを基質として，その性質を検討した。最適 pH は4.3で60°C 以上の加熱で急に失活した。コロミン酸とフエツインに対して Km や Vmax 値を求め，また各種カチオンの効果も検討した。