Bull. Inst. Zool., Academia Sinica **28**(4): 265-273 (1989)

A HUMAN PLACENTAL SIALIDASE WITH SELECTIVE DESIALYATION ON 5′-NUCLEOTIDASE AND ALKALINE PHOSPHATASE[1]

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(Accepted May 29, 1989)

Nin-Nin Chuang (1989) A human placental sialidase with selective desialyation on 5′-nucleotidase and alkaline phosphatase. *Bull. Inst. Zool., Academia Sinica* **28**(4): 265-273. An acidic sialidase (EC 3.2.1.18) has been purified from human placenta via successive procedures including detergent extraction, acetone precipitation, hydroxylapatite and *p*-aminophenylthio-β-D-galactoside-CH-sepharose affinity chromatography. On sodium dodecyl sulphate-polyacrylamide gel electrophoresis, the purified enzyme gave two protein bands with molecular weights of 66,000 and 60,000. The preparation retains residual β-galactosidase activity. The purified enzyme liberated sialic acid residues from glycoproteins and gangliosides. In particular, the sialidase desialyated 5′-nucleotidase and alkaline phosphatase differentially. The 5′-nucleotidase from human placenta was desialyated and was transformed, changing its molecular size from dimer to monomer. The final desialyated monomeric placental 5′-nucleotidase is indistinguishable from the enzyme in human milk through polyacrylamide-gel electrophoresis under non-denaturing conditions. On the contrary, the alkaline phosphatase from human placenta was desialyated to a negligible extent and no size transition was discernible. It is suggested that the presence of highly negative surface charge on the phosphatidylinositol attachment site shielded the enzyme from the attack by placental sialidase.

Key words: Sialidase, Alkaline phosphatase, 5′-Nucleotidase, Sialic acid, Phospholipase C.

Turnover of cell surface glycoconjugates in metazoans of the deuterostomate lineage is initiated by the cleavage of terminal sialic acids. The enzyme responsible (sialidase=neuraminidase, EC 3.2.1.18), obligatory in this phylogenetic branch, is also expressed in some viruses, bacteria and protozoa, which have close contact with animal hosts (Schauer, 1982). In prokaryotes, a functionally complete gene and amino acid sequence of a *Clostridium perfringens* sialidase has been elucidated (Roggentin *et al.*, 1988). However, very little is known about the molecular function and structure of human sialidase. One of the major reasons is that the labial and membrane-bound character of human sialidase have complicated its purification and characterization. Recently, the obervation that a human placental sialidase can be activated, stabilized and copurified with β-galactosidase (Verheijen *et al.*, 1985; 1987; Hiraiwa *et al.*, 1987), pointed out a complex of these two

1. Paper No. 237 of the Journal Series of the Institute of Zoology, Academia Sinica.

enzymes and a possibility to purify sialidase from human placenta by using the complex formation with β-galactosidase.

In the present study, in order to obtain more information of the eukaryotic sialidase, I started the purification on human placental sialidase and investigations on its desialyation effect. Two membrane glycoproteins, alkaline phosphatase and 5′-nucleotidase, both enzymes are anchored to the membrane via a covalent attachment to phosphatidylinositol (Low *et al.*, 1986), were applied in the study.

MATERIALS AND METHODS

All regents used were of the highest grade available commercially. Sialidase (*Clostridium perfringens*) and Sulphobetaine 14 [Zwittergent TM_{3-14}, 3-(N, N-dimethyl-N-tetradecyl) ammoniopropane-1-sulphonate] were obtained from Calbiochem, U. K. Fetuin, orosomucoid, sialyllactose, submixillary mucin, gangliosides and Triton X-100 were from Sigma, U. S. A. Phospholipase C was supplied by Boehringer, Germany. N-acetyl-N,N,N-trimethylammonium bromide and sarcosinate came from Merck, Germany. Reagents for polyacrylamide-gel electrophoresis were as previously described (Chuang *et al.*, 1984).

Enzyme assays

Sialidase activities toward glycoproteins and gangliosides were measured according to Caimi *et al.* (1979). Sialidase was assayed in a reaction mixture containing 100-200 nmol substrate as bound sialic acid, 10 μmol sodium acetate (pH 4.5) and 0.1 ml enzyme. When mixed gangliosides was applied as the substrate, the reaction mixture also contained 0.1% (w/v) Sulphobetaine 14. After incubation at 37°C for 1 h, the reaction was stopped by immediate freezing and the sialic acid released was determined by modified Warren's thiobarbituric acid method (Kattermann and Krieger, 1981). Blanks for enzyme (with added no substrate) and substrate (with heat-in-activated enzyme) were simulaneously conducted and the values obtained were substracted from the experimental figures. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid per h.

5′-Nucleotidase and alkaline phosphatase activities were measured as reported earlier (Chuang *et al.*, 1984; Chuang, 1987). β-Galactosidase activity was determined with O-nitrophenyl-β-D-galactopyranoside as a substrate (Van Diggelen *et al.*, 1980).

Enzyme purification

Enzymes were partially purified from human placenta removed from the body within 1 h after delivery, and stored for up to 1 month at −70°C before use.

Placental sialidase was purified with essentially the same procedures as described by Srivastava and Farooqui (1980), but further improved with slight modifications. The placenta was homogenized in 400 ml of buffer A (10 mM KCl, pH 4.4, 0.2% Triton X-100, 0.2% N-acetyl-N, N, N-trimethylammonium bromide) for 5 min with a Polytron unit and the homogenate was centrifuged at 3,000×g for 30 min. Following this, two volumes of ice-cold acetone (−20°C) was added and the mixture was left in cold (−20°C) for 5 min. After centrifugation at 15,000×g for 30 min, the supernatant was discarded, the precipitate was dissolved in distilled H_2O and, after dialysis for 8-10 hours against distilled H_2O, centrifuged at 15,000×g for 30 min. The resuspended pellet was digested with lysozyme at 1.2 mg/ml for 30 min to remove nucleic acid, and centrifuged at 15,000×g for 30 min. The recovered solution was fractionated by adding ammonium sulphate. Protein

precipitating between 35 and 50% ammonium sulphate was sedimented, resuspended in 1 mM potassium phosphate buffer (pH 6.8) and dialyzed against the same buffer. The dialyzate after the addition of solid $CaCl_2$ (final concentration 0.5%), was rapidly heated to 50°C for 10 min, then cooled to 0-2°C. Subsequently, the turbid mixture was centrifuged at 15,000 ×g for 30 min, and the recovered solution was applied to hydroxylapatite gel (Pharmacia) equilibrated in the 1 mM potassium phosphate buffer, pH 6.8. The enzyme was then eluted with 150 mM potassium phosphate buffer, pH 6.8 and bound to p-aminophenylthiogalactoside-CH-sepharose (Van Diggelen *et al.*, 1981). Finally, after elution with 10 mM sodium acetate, 0.5 mM NaCl, 100 mM r-galactonolactone, pH 5.0, the pooled sialidase fractions were concentrated using a 50 ml Minicon filter (Amicon).

5′-Nucleotidase was partially purified essentially as described for human liver by Chuang *et al.* (1984), resulting in a yield relative to homogenate of 10% and a specific activity of 86 units/mg of protein. Partially purified alkaline phosphatase (6,700 units/mg of protein) was prepared in the way specified previously (Chuang, 1987). Protein concentration was measured in accordance to Lowry *et al.* (1951) with bovine serum albumin as the standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis under non-denaturing condition was carried out with human placental 5′-nucleotidase, using buffer system F of Newby *et al.* (1982), and with human placental alkaline phosphatase, using buffer system 4229 of Jovin *et al.* (1980). Electrophoresis was proceeded at 4°C. 5′-Nucleotidase or alkaline phosphatase activity was determined in 2 mm gel slices and recovery of the enzyme after electrophoresis exceeded 90% unless otherwise stated.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was conducted on slab gels containing 10% (w/v) acrylamide with 0.27% (w/v) N,N′-methylenebis-acrylamide. Samples were reduced and alkylated (Lane, 1978) before application to the gels. Gels were silver stained according to the method of Merril *et al.* (1981).

RFSULTS

Purification of sialidase

The purification scheme used for the isolation of human placental sialidase is summerized in Table 1. The enzyme preparation obtained on hydroxylapatite gel absorption was activated due to the removal of major protein contaminants as previously described (Hiraiwa *et al.*,

Table 1
Purification of sialidase from human placenta.
Fresh placenta was used for purification, and sialidase activity was assayed with gangliosides as substrate

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield of β-galactosidase act. (%)
Acetone precipitation	10,100	1,800	0.18	100
Heat inactivation	522	200	0.38	1.9
Hydroxylapatite gel	9	4,200	467	7.9
p-aminophenylthiogalactoside-CH-sepharose	0.035	1,750	50,000	0.3

1987; Verheijen *et al.*, 1985) and then applied to a p-aminophenylthiogalactoside-CH-sepharose column, β-galactosidase specific. This step was a good method and the specific activity of sialidase was increased 100 times. The final preparation was still accompanied with a little β-galactosidase as mentioned before (Verheijen *et al.*, 1985, 1987; Hiraiwa *et al.*, 1987).

When purified sialidase was subjected to denaturing SDS-polyacrylamide gel electrophoresis to investigate the subunit composition of the enzyme. As shown in Fig. 1, the sialidase exhibited two protein bands with molecular weights of 66,000 and 60,000. The results agree well with the findings of McNamara *et al.* (1981). A report that a protective protein subunit with a relative Mr 32,000 was retained in the final sialidase preparation to protect enzyme from proteolytic degradation could not be confirmed in the present experiments. However, it is not inconceivable that the sialidase purified and compared is different.

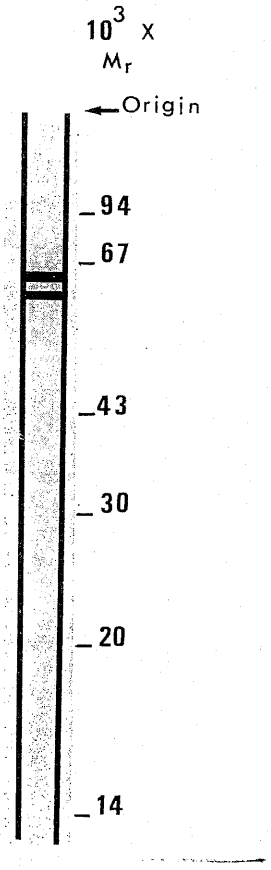

Fig. 1. Sodium dodecyl sulfate/polyarylamide gel electrophoresis of the purified placental sialidase preparation. The affinity purified sialidase preparation from human placenta was electrophoresed into 10% (w/v) polyacrylamide gels in the presence of SDS. Protein was silver stained. Molecular weights were identified by a series of standard proteins.

Substrate specificity

The sialidase liberated sialic acid from gangliosides and glycoproteins (Table 2). However, the enzyme showed neither a high activity toward gangliosides nor sialoglycoproteins. In order to obtain information on the substrate specificity, the membrane glycoprotein, 5′-nucleotidase and alkaline phosphatase

Table 2
Substrate specificity of human placental sialidase

Substrate	Salic Acid		% Hydrolysis
	Bound (nmol)	Released (nmol)	
Gangliosides	120	12	10.0
Sialyllactose	100	5.8	5.8
Fetuin	150	1.5	1.0
Orosomucoid	150	1.5	1.0
Submaxillary mucin	150	16.2	10.8

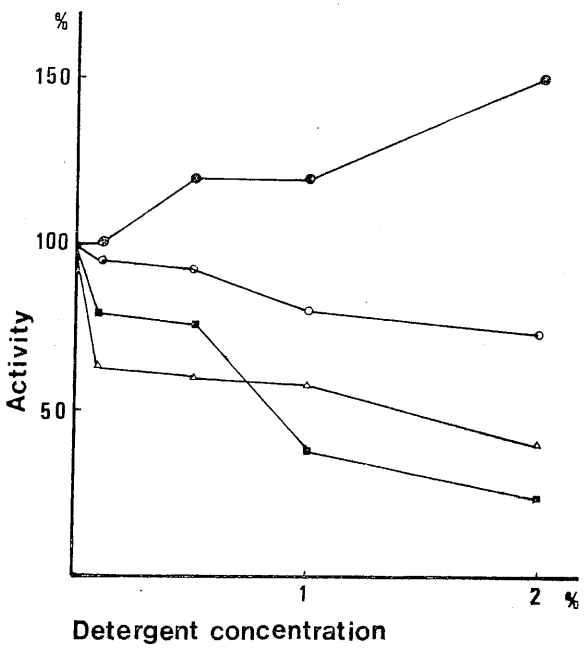

Fig. 2. Effect of different detergents on the activity of placental sialidase. The control mixture did not contain detergents. The enzyme was assayed with gangliosides as substrate in the presence of various amounts of detergent for 1 h. ●, Sulphobetaine 14; ○, N-acetyl-N, N, N-trimethylammonium bromide; △, Sarcosinate; ■, Triton X-100.

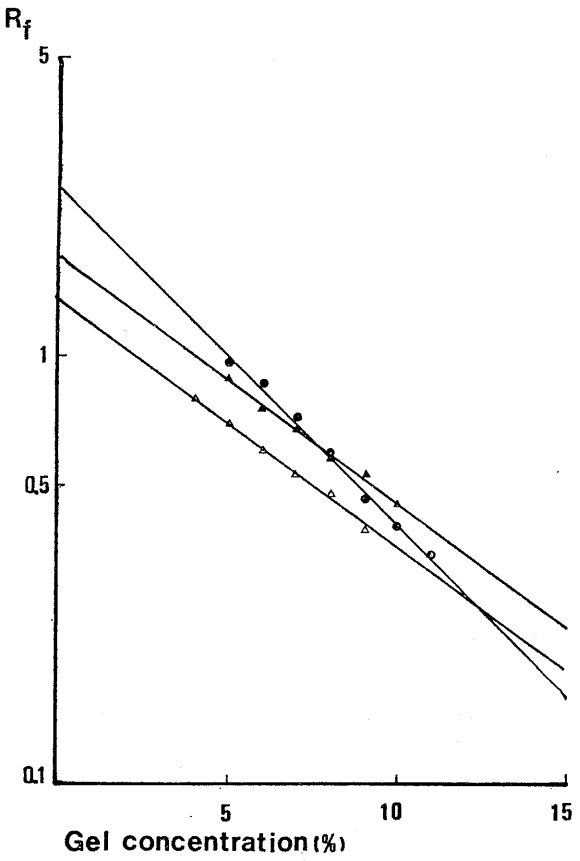

Fig. 3. Ferguson plot analysis of 5′-nucleotidase. Partially purified 5′-nucleotidase from human placenta was untreated (●) or treated (▲) with the purified placental sialidase and subsequently electrophoresed on non-denaturing tube gels over different concentrations in the presence of Sulphobetaine 14 (0.1%). For comparison, the enzyme treated with the sialidase of *Clostridium perfringens* (△) was included. For each gel concentration duplicate gels were measured, and the plot of R_f (logarithmic scale) versus gel concentration (%) was constructed from the results for three samples using least-squared linear regression.

were prepared and served as substrates.

Since membrane glycoprotein would require detergent to be present in the reaction medium, the detergent effect on the sialidase was examined with increasing concentrations of different detergents (Sulphobetaine 14, Triton X-100, N-acetyl-N, N, N-trimethylammonium bromide, Sarcosinate). As shown in Fig. 2, Sulphobetaine 14 activated the activity of placental sialidase; all the other detergents exhibited a more or less marked inhibitory effect which increased with detergent concentration, the findings being in agreement with Venerando *et al.* (1987). On this basis Sulphobetaine 14 was employed at the concentration of 0.1% to study the desialyation effect of placental sialidase on 5′-nucleotidase, and Triton X-100 was employed at the concentration of 0.01% in the study with alkaline phosphatase. This is due to at the above mentioned detergent concentration, the

micelle effect of detergent on sialidase activity is least.

With the technique of polyacrylamide-gel electrophoresis under non-denaturing conditions, the molecular form of membrane enzyme, 5′-nucleotidase or alkaline phosphatase, from human placenta was compared with that of the enzyme after incubation with the purified sialidase preparation.

After treatment with placental sialidase, in addition to the expected difference in surface charge due to the removal of the highly negatively charged sialic acid from the enzyme, the size of 5′-nucleotidase alters as well (Fig. 3). The placental 5′-nucleotidase, before the loss of sialic acid, has a geometric mean radius of 3.4 nm and an apparent Mr of 140,000, whereas its desialyated form has a geometric mean radius of 2.5 nm and an apparent Mr of 70,000 (Table 3). That is, the action of sialidase on the placental membrane 5′-nucleotidase induces a transition of the enzyme molecules from dimers to monomers, which possesses electrophoretic characteristics identical to the enzyme in human milk (Chuang, 1987; Table 3). No additional molecular form has been detected. This indicates the desialyation effect of placental sialidase on purified 5′-nucleotidase is complete.

In the study on alkaline phosphatase, neither molecular size transition, as noticed in the study with 5′-nucleotidase, nor charge shifting, as expected from loss of negative charged sialic acid residues, has been discovered with the major fast-moving form of the enzyme (Fig. 4). The treatment with placental sialidase changes the electrophoretic mobility of the enzyme negligibly.

In a test on whether the glycosylated c-terminal membrane binding domain was lost during enzyme purification, alkaline phosphatase was treated with either phospholipase C or sialidase (*Clostridium perfringens*). Both enzyme treatments resulted in a pronounced gain of surface charge on the enzyme molecules, and a significant charge-shift was noticed on the Ferguson plot analysis (Fig. 4). It indicated that the alkaline phosphatase partially purified from phase-separating detergent is with a whole glycosylated c-terminal membrane-binding domain, inclusive of sialic acid and phosphoinositol attachment sites (Low *et al.*, 1986).

Table 3
Properties of human placenta and milk 5′-nucleotidae. Data are presented as means±S.E.M. for three determinations unless stated otherwise

	Placenta			Milk
	Control	Placental sialidase	Clost. Perf. sialidase	
5′-Nucleotidase activity	90±20 units/mg of protein			1-10 m units/ml
Sample size	10			179
Inhibition (%) by:				
[αβ-Methylene]ADP (200 μM)	98±2			90±2
Rabbit antiserum (1:10)	95±3			94±2
Ferguson-plot analysis:				
Y_0	2.5±0.2	1.7±0.2	1.4±0.2	1.7±0.2
K_r	0.090±0.003	0.070±0.002	0.070±0.002	0.070±0.003
Electrophoretic forms in presence of Sulphobetaine 14	S	F	F	F

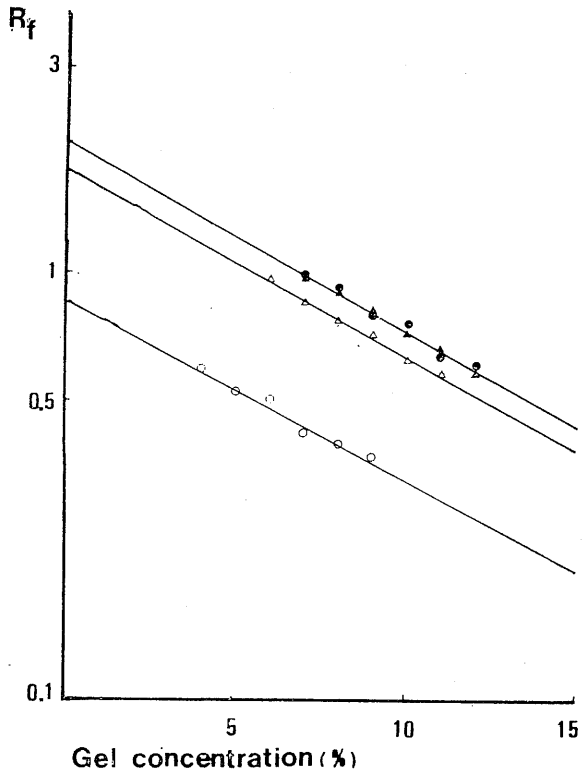

Fig. 4. Ferguson plot analysis of placental alkaline phosphatase. The major placental alkaline phosphatase fraction was untreated (●) or treated (▲) with the purified placental sialidase and electrophoresed on non-denaturing tube gels over different concentrations in the presence of Triton X-100 (0.01%). For comparison, alkaline phosphatase treated with the sialidase of *Clostridium perfringens* (△) or with phospholipase C (○) was included.

DISCUSSION

By appling the partially purified sialidase from human placenta to study the effect of desialyation on membrane sialoglycoproteins in the presence of detergent, a contrastly different result was found between 5′-nucleotidase and alkaline phosphatase. The desialyated form of the 5′-nucleotidase had characteristics indistinguishable from the enzyme found in human milk (Chuang, 1987) and the alkaline phosphatase appeared to be inert to the action of placental sialidase. Some evidence for a contrasting result of these two ectoenzymes was previously reported after the digestion by phospholipase C (Shukla *et al.*, 1980).

The desialyated 5′-nucleotidase appeared to be half the molecular size of the sialyated form but with less negative charge. Indirect evidences from alkaline phosphatase, such as ineffective digestion by placental sialidase and the preservation of an intact c-terminal membrane anchoring domain, suggest that the sialidase preparation does not contain proteolytic activity and that the monomeric 5′-nucleotidase is most probably a monomer derived from the plasma-membrane dimer without the involvement of proteolytic cleavage. Some evidences for a monomeric form of the ectoenzyme were previously reported after N-dodecylsarcosinate treatment of liver membranes (Evans and Gurd, 1973), and in either serum or milk without any treatment (Chuang *et al.*, 1984; Chuang, 1987).

Alkaline phosphatase and 5′-nucleotidase are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Previously, polyacrylamide-gel electrophoresis under nondenaturing conditions in the presence of detergent plus inhibition by antiserum has provided evidence that both enzymes in human milk are derived from membrane (Chuang, 1987). But the sialyation of these enzymes in milk has completely contrasting results. All the 5′-nucleotidase in human milk was found to be totally without sialic acids but alkaline phosphatase was still complexed with sialic acids. This suggests that a sialidase may have a selective desialyation on these two membrane enzymes.

In the present study, I discover the existence of a placental sialidase with differential activity toward 5′-nucleotidase and alkaline phosphatase. The purified

enzyme liberates sialic acid residues from membrane glycoproteins with selection. However, the possibility of complex mechanisms, such as the inactivation of sialidase by membrane lipids (Venerando *et al.*, 1987), cannot be ignored. The detergent purified membrane enzymes, such as alkaline phosphatase, may retain some specific lipid domain in the micelles. Further investigations on the possible functional role of membrane lipids on the regulation of ectoenzyme activity are being undertaken.

Acknowledgements: I thank Dr. F. K. Chuang and Dr. T. B. Tang for valuable advice and discussion. I thank Municipal Maternity Hospital for providing placenta and Ms. S. L. Shih for her assistance. The work was supported by the National Science Council (NSC-77-0211-B011-44).

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五-核苷酸酶與鹼性磷酸酶的差異去涎酸反應

莊 寧 寧

由人胎盤純化出的涎酸酶，仍含有極微量的 β-半乳糖苷酶。經非活性電泳分析 (SDS/PAGE) 測定分子量，知由兩個蛋白單元分子組成 (Mr 66,000 與 Mr 60,000)，此涎酸酶對膜蛋白，五-核苷酸酶，與鹼性磷酸酶的作用迥異，在五-核苷酸酶失去涎酸可導致分子大小的變化，即有二聚體至單聚體的轉型。對鹼性磷酸酶無此分子的改變，且不易失去涎酸。