Assay for Sialidase Using Erythrocytes and Peroxidase-Labeled Peanut Lectin

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A rapid and sensitive sialidase assay method based on peroxidase-labeled peanut lectin (PNA) binding to desialylated erythrocytes is described. Formalinized sheep erythrocytes were used both as a stable substrate for sialidase and as a target for the lectin. In the case of sialidases from *Vibrio cholerae* and *Arthrobacter ureafaciens*, a linear relationship was observed between the amount of peroxidase-labeled PNA bound to erythrocytes and the enzyme amount. Binding of the lectin to sialidase-treated erythrocytes was completely prevented in the presence of 25 mM lactose and galactose. The method is particularly useful as a selective assay for sialidase which is active towards gangliosides or sialoglycoproteins, because a mammalian sialidase which is preferentially active towards sialooligosaccharides and sialoglycopeptides is not able to remove sialic acid from erythrocytes.

Keywords sialidase; neuraminidase; *Vibrio cholerae*; *Arthrobacter ureafaciens*; mouse liver; peanut lectin; peroxidase

Sialidase [EC 3.2.1.18, neuraminidase] catalyzes the removal of sialic acid residues from sialoglycoconjugates. Several important biological reactions,[1] such as clearance of serum glycoproteins and erythrocytes, antigenic expression and recognition by receptors, are associated with this removal.

The thiobarbituric acid (TBA) procedure,[2] which has been generally used for sialidase assay, is relatively insensitive and is interfered with endogenous substances. Methods using a fluorometric substrate[3] are sensitive, but such a substrate is not effectively cleaved by sialidase for gangliosides.[4] The assay of sialidase using radioactive substrates[5] is extremely sensitive and less subject to interference by biological materials, but requires special precautions.

Pereira described a rapid and sensitive assay for sialidase using peanut lectin (PNA) hemagglutination,[6] and this method is useful in the sialidase analysis of multiple samples, such as for screening of hybridoma antibodies against sialidase. However, hemagglutination assay is difficult to use for quantitative assay of sialidase. The present paper describes a modification of the sialidase assay described by Pereira.

Materials and Methods

Materials *Vibrio cholerae* sialidase (Koch-Light Ltd., Haverhill, Suffolk, England) was purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan, and *Arthrobacter ureafaciens* sialidase (Marukin Shoyu Co., Ltd., Kyoto, Japan) was from Nacalai Tesque Co., Ltd., Kyoto, Japan. One unit of sialidases was defined as the amount of enzyme which catalyzed the release of 1 μmol of sialic acid per min at 37 °C from human α_1-acid glycoprotein at pH 5.5 (*V. cholerae* sialidase) or sialyllactose at pH 5.0 (*A. ureafaciens* sialidase). Sialic acid released was determined by the TBA method.[2b] PNA from *Arachis hypogaea* and peroxidase-labeled PNA were purchased from Hohnen Oil Co., Ltd., Tokyo, Japan. Sheep erythrocytes in Alserver's solution were obtained from Nippon Bio-Test Laboratories Inc., Tokyo, Japan. The erythrocytes were formalinized by the method of Butler.[7] Formalinized cells were stable at 4 °C for several weeks.

Peroxidase-Labeled PNA Binding Assay of Sialidase Activity The standard assay mixture contained 200 μunits of *V. cholerae* sialidase, 3.3% formalinized sheep erythrocytes in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) buffer (pH 7.0) containing 0.15 M NaCl and bovine serum albumin (BSA) (0.2 mg) in total volume of 150 μl. Unless otherwise indicated, a reaction mixture without enzyme served as the control. After incubation for 15 min at 37 °C in a shaker, the mixture was cooled in an ice-water bath. The erythrocytes in each tube were washed three times with 0.5 ml of 0.15 M NaCl, 10 mM K_2HPO_4–KH_2PO_4 (pH 7.2) (PBS) containing 2 mg/ml of BSA, centrifuged at 3500 rpm for 2 min, and resuspended in 250 μl of the same solution (2% suspension), then the 20 μl of the suspension was added to each of triplicate wells of Microtiter "V" plates (Sanko Junyaku Co., Ltd.) containing 20 μl per well of PNA conjugated with peroxidase (0.02 mg/ml). The plates were covered with transparent tape, shaken, and incubated for an additional 1 h at room temperature, followed by centrifugation at 2000 rpm for 20 min. The peroxidase activity of the supernatant (10 μl) in another plate was measured by adding 190 μl per well of 4-aminoantipyrine (0.05 mg/ml) containing 1% phenol (saturated with water) and 0.05% H_2O_2. The mixtures were incubated for 10 min at room temperature and the absorbance was measured at 492 nm on a microplate reader, model MPR-A4 (Tosoh Co., Ltd., Tokyo, Japan). The percent of peroxidase bound to erythrocytes was calculated as follows:

$$(A-B)/A \times 100\ (\%)$$

A: absorbance at 492 nm in control (without enzyme).
B: absorbance at 492 nm in complete reaction mixture.

Hemagglutination Assay of Sialidase Activity Hemagglutination assay was carried out by a modification of the procedure described by Pereira.[6] Enzyme reactions of sialidase against formalinized sheep erythrocytes were performed in the same way as above. The agglutination end point was taken as the first dilution of the PNA at which the treated cells settled into a clearly circumscribed dot at the bottom of the well. The agglutination titer was defined as the reciprocal of the end point dilution.

Other Methods Mouse liver sialidase fractions were prepared as described previously.[8] Protein concentration was determined by the method of Lowry *et al.*[9] with BSA as a standard.

Results and Discussion

V. cholerae sialidase was incubated with formalinized sheep erythrocytes, and then degree of erythrocyte desialylation was detected by the peroxidase-labeled PNA binding method (Fig. 1). The reaction was fastest during the first 10 min and slowed down progressively thereafter. The increase of peroxidase activity bound to erythrocytes was in parallel with the increase of the hemagglutination titer for PNA. PNA binding was observed after 10 min incubation of erythrocyte suspension with 200 μunits of *V. cholerae* sialidase (Fig. 1). However, 10 munits of the enzyme and an incubation time of 60 min were required to detect released sialic acid by the TBA method (data not shown). The peroxidase-labeled PNA binding method is about 100 times more sensitive than the TBA method when erythrocytes are used as the substrate.

The hemagglutination method can estimate the sialidase activity, but not quantify the enzyme activity because of the 2-fold dilution procedure used. As shown in Fig. 2, peroxidase activity bound to *V. cholerae* or *A. ureafaciens* sialidase-treated erythrocytes was in proportional to the

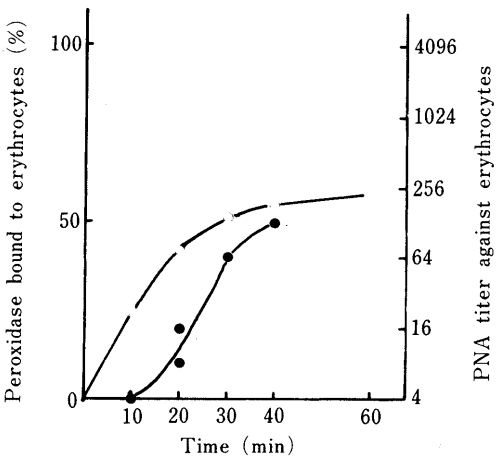

Fig. 1. Hydrolysis of Formalinized Sheep Erythrocytes with *V. cholerae* Sialidase as a Function of Time

At every time point, the erythrocytes were checked for agglutination with peanut lectin starting at a concentration of 1.0 mg/ml (●), and peroxidase-labeled PNA binding (○).

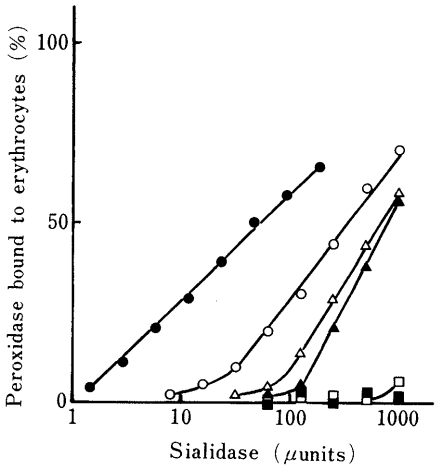

Fig. 2. Effect of Enzyme Amount on Hydrolysis of Formalinized Sheep Erythrocytes by *V. cholerae* and *A. ureafaciens* Sialidases

The indicated amount of *V. cholerae* (○) or *A. ureafaciens* (●) sialidase was incubated with the formalinized sheep erythrocytes at pH 7.0 for 15 min. The peroxidase-labeled PNA binding to the erythrocytes treated with *V. cholerae* sialidase was also performed in the presence of 25 mM lactose (□), 25 mM galactose (■), 0.2 M mannose (△) or 0.2 M glucose (▲).

logarithm of amount of enzyme. These results indicate that the peroxidase-labeled PNA binding method is useful for quantification of microbial sialidase activity. This peroxidase-labeled PNA binding was completely blocked in the presence of 25 mM lactose and galactose, but other sugars tested such as 0.2 M glucose and mannose had less effect on the binding. PNA is highly specific for the disaccharide D-Gal-β-(1→3)-D-GalNAc, but also binds D-Gal-β-(1→4)-D-GlcNAc to a lesser extent.[10] These structures are commonly found in the complex carbohydrates located on the cell surface, but the disaccharides are usually sialylated and are not available for lectin binding unless the cells are treated with sialidase to expose the lectin receptor sites. These results indicate that the peroxidase-labeled PNA binding method is very specific for the assay of sialidase activity. Pereira had used native erythrocytes as a substrate for sialidase,[6] so the assay could not be performed over a wide pH range, whereas formalinized erythrocytes could be used at a wide pH range.

The sialidase activities of lysosomal and microsomal fractions from mouse liver homogenate were examined by the hemagglutination method, but insoluble materials prevented measurement of the hemagglutination. When the $1000 \times g$ supernatant fraction of mouse liver homogenate was incubated at 37 °C in citrate-phosphate buffer (pH 3.8) containing 0.7% galactose to inhibit endogenous galactosidase activity, an increase of peroxidase activity bound to erythrocytes, dependent on both the incubation time and enzyme amount, was observed (data not shown), but only 26% of the peroxidase-labeled PNA binding was blocked in the presence of 0.2 M lactose. These results indicated that most of this binding is due to non-specific binding, and this assay method is not applicable to the insoluble enzymes.

Previously we have reported the solubilization[8] of mouse liver lysosomal sialidase fraction. This solubilized fraction was preferentially active toward sialooligosaccharides and sialoglycopeptides but not gangliosides.[8] Sialidase activity against formalinized erythrocytes could not be detected in this fraction by the hemagglutination method or TBA method. Recently, Usuki *et al.* reported that a sialidase for G_{M3} is present in the medium of cultured human fibroblasts, but it could not be detected by the use of sialyllactitol as a substrate.[11] These observations suggest that the peroxidase-labeled PNA binding method may be also useful as a selective assay of sialidase for ganglioside.

Acknowledgement We wish to thank Ms. K. Unezawa for her technical assistance.

References

1) R. Schauer, *Adv. Carbohydr. Chem. Biochem.*, **40**, 131 (1982); W. Reutter, E. Köttgen, C. Bauer and W. Gerok, "Cell Biology Monographs," Vol. 10, ed. by R. Schauer, Springer-Verlag, Vienna-New York, 1982, pp. 263—305.
2) *a)* L. Warren, *J. Biol. Chem.*, **234**, 1971 (1959); D. Aminoff, *Biochem. J.*, **81**, 384 (1961); *b)* I. E. Horgan, *Clin. Chim. Acta*, **116**, 409 (1981).
3) M. Potier, L. Mameli, M. Belisle, L. Dallaire and S. B. Melançon, *Anal. Biochem.*, **94**, 287 (1979); R. W. Mayers, R. T. Lee, Y.-C. Lee, G. H. Thomas, L. W. Reynolds and Y. Uchida, *ibid.*, **101**, 166 (1980).
4) J. Sagawa, T. Miyagi and S. Tsuiki, *Jpn. J. Cancer Res. (Gann)*, **79**, 69 (1988).
5) V. P. Bhavanandan, A. K. Yeh and R. Carubelli, *Anal. Biochem.*, **69**, 385 (1975); A. Frisch and E. F. Neufeld, *ibid.*, **95**, 222 (1979).
6) M. E. A. Pereira, *J. Immunol. Methods*, **63**, 25 (1983).
7) W. T. Butler, *J. Immunol.*, **90**, 663 (1963).
8) T. Nagai and H. Yamada, *Chem. Pharm. Bull.*, **36**, 4008 (1988).
9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) M. E. A. Pereira, E. A. Kabat, R. Lotan and N. Sharon, *Carbohydr. Res.*, **51**, 107 (1976).
11) S. Usuki, S.-C. Lyu and C. C. Sweeley, *J. Biol. Chem.*, **263**, 6847 (1988).