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Development of a Rapid Spectrophotometric Method for Detecting Bacterial Mucinase Complex

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Abstract A rapid spectrophotometric method for detecting the mucinase complex was developed. Bovine submaxillary mucin is cleaved by commercial mucinase between the oligosaccharide chain and the side chain of peptide linkage, thereby liberating the N-acetyl neuraminic acid (NANA). The release of NANA resulted in an increase of absorbance at 280 nm. The susceptibility to NANA by the new method was found to be at least 10-fold more sensitive than the thiobarbituric acid method. Moreover, the quantification of NANA released from mucin by commercial neuraminidase and partially purified *Vibrio parahaemolyticus* mucinase showed a good linear correlation in proportion to the concentration of the enzyme used. These results demonstrate that the rapid identification of mucin degradation can be determined by a spectrophotometric assay, thereby providing a new, fast, and sensitive method for assaying the bacterial mucinase complex.

Key words: Mucin, mucinase, N-acetyl neuraminic acid, *Vibrio parahaemolyticus*

Epithelial mucous glycoproteins, which line the respiratory and intestinal tracts of mammals, consist of repeating polypeptide sequences that are modified by oligosaccharide chains attached to serine or threonine residues [6]. Mucin-producing cells often present easy-to-see borders, since the carbohydrate-rich pale-staining mucin contrasts with the protein-rich pink-staining cell surfaces [3]. It has been often reported that some correlation between the production of extracellular enzymes and the toxicity among various *Vibrio* species are being observed [8-9]. In a previous study, there appeared to be a strong correlation between mucin-degrading activity and pathogenicity [7]. The mucinase activity was most distinct among tested extracellular enzymes in addition to the proteases.

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Mucinase is isolated as a complex and this complex contains neuraminidase, endo-β-N-acetylhexosaminidase, nicotinamide-adenine-dinucleotidase, and proteinases [12]. Many studies have suggested that the mucin is the critical factor in the initiation of pathogenic bacterial infection. Schneider and Parker [11] suggested that the mucinase complex facilitates *V. cholerae* penetration of the mucous barrier, and it may attack the glycosidic linkage between N-acetyl galactosamine and D-galactose in the monosialoganglioside G_{M1} and increase the receptivity for *V. cholerae* enterotoxin molecules. Gascoyne and Van Heyningen [5] and Ackerman *et al.* [1] attributed the conversion of membrane gangliosides to enterotoxin-binding gangliosides to the action of neuraminidase. In paramyxovirus, neuraminidase is a surface glycoprotein that has enzymatic activity essential for the replication of influenza A and B. The enzyme catalyzes the cleavage of the α-ketosidic linkage that exists between a terminal sialic acid and an adjacent sugar residue. This action has been proven to be an important effect that enables the spread of the virus within the host respiratory tract [10]. The mucinase has also been studied for the development of an acellular cholera vaccine in view of its potential use as an immunogen [4]. Vaccines that include mucinase as an antigen may be more effective than ones that do not, and the role of mucinase as a protective antigen for humans should be evaluated.

Mucins are fragmented differently as a result of structural variability between apomucin peptides and their individual patterns of glycosylation. Therefore, the examination of mucinase activity from any source must take into consideration the nature and origin of the mucin used as substrate. Mucinase is generally assayed based on the measurement of sialic acid released from mucin. The thiobarbituric acid (TBA) assay used currently is time-consuming and not reproducible, because the procedure extracting a sialic acid through a cyclohexanone is highly unstable. In this study, a rapid and reliable method for

detecting mucinase activity using bovine submaxillary mucin was developed.

Bovine submaxillary mucin was obtained from Sigma (St. Louis, U.S.A.). N-acetyl neuraminic acid (NANA) and *V. cholerae* neuraminidase were purchased from Roche Diagnostics Corporation (Indianapolis, U.S.A.). Thiobarbituric acid and sodium arsenite were purchased from Sigma, and sodium periodate was obtained from Acros Corporation (New Jersey, U.S.A.). *Vibrio parahaemolyticus* ATCC17802 was incubated with shaking for 60 h in 5,000 ml of BHI (brain heart infusion, Difco) medium supplemented with 1.5% NaCl and 10% polypeptone at 37°C. Cells were removed from culture fluids by centrifugation at 4°C. The supernatants were brought to 80% saturation with ammonium sulfate and allowed to stand for 18 h at 4°C. The ammonium sulfate precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4°C and dissolved in 80 ml of 20 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed against the same buffer. The dialysate was loaded onto a Q Sepharose column (2.5×10 cm, Pharmacia) equilibrated with the same buffer, and then eluted with a step gradient of 1 M NaCl. The fractions containing the mucinase activity were pooled, concentrated, and used as the enzyme solution for further analysis.

Thiobarbituric acid (TBA) assay was performed according to the procedure described previously [13]. Bovine submaxillary mucin (20 mg) was dissolved in 5 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.1% $CaCl_2$ and 1% NaCl. *V. parahaemolyticus* mucinase solution (0.1 ml) was added to 0.4 ml solution of bovine submaxillary mucin. The volume was adjusted to 1 ml with buffer and the mixture was incubated at 37°C for 30 min. Subsequently, the mixture was heated at 100°C for 5 min (or 1 ml of phosphotungstic acid was added and the mixture was centrifuged) and 0.1 ml of periodate solution was added. After 20 min at room temperature, 1 ml of arsenite solution was added and the mixture was shaken vigorously to reduce the yellow colored iodine. Three ml of thiobarbituric acid solution were added to the tubes, and the mixture was shaken, and the hydrolysis was stopped by heating at 100°C for 15 min before cooling rapidly to room temperature. Three ml of cyclohexanone were added to the mixture and centrifuged for 3 min in order to separate into two layers. Finally, the absorbance of the upper cyclohexanone layer was measured at 549 nm. The amount of NANA released from the mucin was determined by reference to a standard curve of NANA.

For the new spectrophotometric method, bovine submaxillary mucin (0.5 g) was dissolved in 50 ml of 0.02 M Tris-HCl buffer, pH 5.5. The partially purified *V. parahaemolyticus* mucinase (1 ml) was added to 1 ml of the mucin solution and incubated at 37°C for 30 min. The reaction was stopped by adding 3 ml of 10% (w/v) trichloroacetic acid and incubated at 45°C for 15 min. Undigested mucin was removed by centrifugation at 1,200 ×g for 10 min and the absorption of the supernatants at 280 nm were recorded. The amount of NANA released from the mucin was determined according to a standard curve of NANA. Positive controls were *V. cholerae* neuraminidase (Roche). A unit of enzyme is defined as the amount that releases 1 micromole of NANA from mucin per minute under the described conditions. The protein concentration was quantified by Bradford's method [2].

In order to evaluate the usefulness and effectiveness of the new spectrophotometric method for measuring the release of NANA from the substrate mucin, the standard NANA solution was prepared and the change of absorbance at 280 nm was plotted against the known NANA concentration. The experiments are performed in triplicates. The absorbance change increased in proportion to NANA over the concentration range from 0 to 1,000 μmol. In the TBA method, the change in absorbance at 549 nm was not very significant in proportion to the increase of the NANA concentration, whereas marked absorbance changes were observed in the new spectrophotometric method (Fig. 1). These absorbance changes observed in the TBA method greatly affect the accuracy of the measurement. The data followed a simple linear regression fit in both methods. A correlation coefficient (r value) for the new spectrophotometric and the TBA method was 0.994 and 0.981, respectively. The linear range of the absorbance change against NANA concentration was 60 to 700 μmol in the new method. The lowest detection limit was approximately 30 μmol. The susceptibility to NANA was nearly 10-fold more sensitive than with the TBA method. This result indicates that the TBA method is not a desirable method for accurate and reliable estimation of the NANA produced from the mucin degradation.

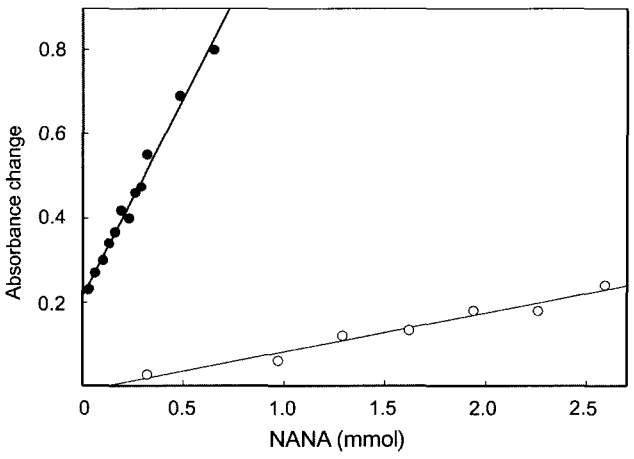

Fig. 1. Linearity of the absorbance changes and the corresponding concentrations of NANA by TBA (○) and a new spectrophotometric method (●).
The absorbance was measured at 280 nm (new spectrophotometric method) and 549 nm (TBA method), respectively.

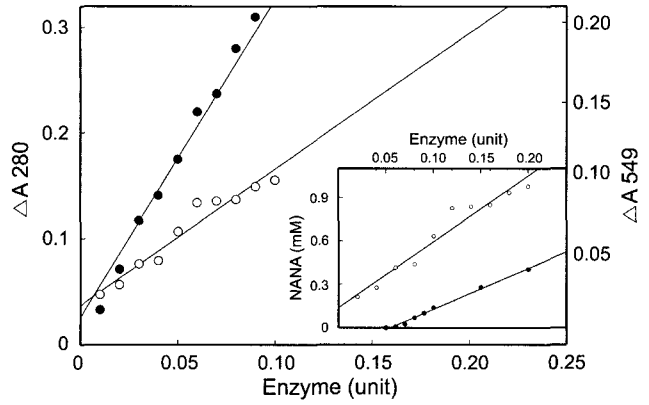

Fig. 2. Effect of the *V. cholerae* neuraminidase concentrations on the production of NANA by the TBA method (○) and the new spectrophotometric method (●).
The data are plotted according to the linear regression equation. A correlation coefficient, r=996 and 974 for the new spectrophotometric and the TBA method, was calculated from these data. The insert shows the amount of NANA increased in proportion to the enzyme concentration.

The commercially available *V. cholerae* neuraminidase and partially purified *V. parahaemolyticus* mucinase were also used to examine which method is more efficient for the measurement of actual amount of the NANA released from bovine submaxillary mucin. The enzyme-catalyzed degradation of mucin was monitored through the difference in absorbance intensity between the substrate mucin and the production of NANA. The increase of the absorbance (indication of the increase of NANA concentration - see the insert in Fig. 2) in proportion to the increase of the *V. cholerae* neuraminidase was well-fitted to the linear regression equation. The correlation coefficient (r=0.996) obtained from the new method was higher than that acquired from the TBA method (r=0.974) (Fig. 2). This phenomenon was also observed when the culture supernatant of *V. parahemolyticus* was used (Fig. 3). Therefore, the comparison of a correlation coefficient for the production of NANA revealed by the new method was more efficient than the TBA method.

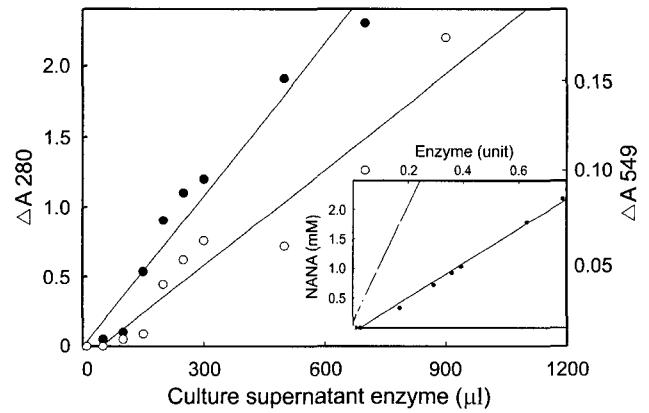

Fig. 3. Effect of the *V. parahemolyticus* mucinase concentrations on the production of NANA by the TBA method (○) and the new spectrophotometric method (●).
The data are plotted according to the linear regression equation. A correlation coefficient, r=0.978 and 0.963 for the new spectrophotometric and the TBA method, was calculated from these data. The insert shows the amount of NANA increased in proportion to the enzyme concentration. The culture supernatant of *V. parahemolyticus* was used as a crude enzyme solution.

A new method was further tested to see whether it is possible to take advantage of this method to purify the mucinase from *V. parahaemolyticus*. The mucinase from *V. parahaemolyticus* was partially purified by ammonium sulfate precipitation and then by Q-Sepharose column chromatography. In the TBA method, the mucinase activity was spread over the fractions. However, the mucinase activity was narrowed down to 0.1–0.2 M NaCl eluate when the new spectrophotometric method was used (data not shown). The broad distribution of the enzyme activity in more than one peak is due to the presence of the mucinase in various enzyme fractions such as neuraminidase and proteinases [12]. It also indicates that the new spectrophotometric method is an easy way to isolate and purify the mucinase from the other proteins, compared to the TBA method.

Therefore, it is likely that the method proposed here is valuable and useful for rapid determination of mucinase. Recent studies strongly imply that the mucinase is highly involved in the pathogenesis of various pathogens. The new method, along with the importance of this enzyme, can be effective in monitoring the enzyme in biological samples and useful in the screening of bacterial pathogens.

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