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Serological study using α-*N*-acetylgalactosaminidase from *Acremonium* sp.

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α-*N*-Acetylgalactosaminidase, produced by *Acremonium* sp. destroyed blood group A active substance. This enzyme converted human erythrocytes from group A to O(H). The enzyme was ascertained to liberate *N*-acetylgalactosamine from group A erythrocytes membranes accompanying a decrease in hemagglutination inhibition activity of A erythrocyte-anti-A serum by the erythrocyte membranes. Glycoproteins and glycolipids extracted from A erythrocytes reduced their blood group A activity after the enzyme treatment. The enzyme also worked on Forssman hapten glycolipid.

Introduction

It is well known that a specific determinant for blood group A is terminal α-linked *N*-acetylgalactosamine of sugar chains in glycoproteins and glycolipids on the blood cell membrane. α-*N*-Acetylgalactosaminidase (EC 3.2.1.49) can cleave the α-linkages of the terminal GalNAc from sugar chains of various complex carbohydrates including blood type A substances. The enzyme has been found in several animal tissues [1–4] and microbial cultures of *Aspergillus niger* [5] and *Clostridium perfringens* [6]. The serological conversion of erythrocytes from group A to O(H) has been attempted using chicken liver α-GalNAc-ase [7]. A detailed serological study using the microbial enzymes from *A. niger* and *C. perfringens* has not been done, because the former does not act on natural substances and the latter is not easily obtainable in large amounts from the cultures of this anaerobic and pathogenic bacterium.

Recently, we found that *Acremonium* sp. isolated from soil produced a high level of α-GalNAc-ase, and reported the purification and characterization of the enzyme [8]. In this report, we describe a serological study concerning the conversion of erythrocytes from group A to O(H) using this enzyme.

Materials and Methods

Cultivation of *Acremonium* sp. and purification of the enzyme were done as described previously [8] with a small modification. The purified enzyme preparation used was ascertained to have no proteinase activity by determining the proteolytic activity with the casein digestion method [9].

Results and Discussion

To investigate the effect of the enzymatic treatment on blood group A activity of type A_1 (normal) erythrocytes (Organon Tecknika), 20 μl of 4% erythrocytes were incubated with the enzyme (140 units) in 50 mM acetate buffer (pH 5.5) containing 0.9% NaCl for 1 h at 37°C. One unit of enzyme was defined as the amount of enzyme which hydrolyzes 1 μmol of *p*-nitrophenyl-α-GalNAc to *p*-nitrophenol per min in 50 mM citrate buffer (pH 4.5). After washing the erythrocytes with phosphate-buffered saline (pH 7.2), agglutination of

Abbreviations: GalNAc, *N*-acetylgalactosamine; α-GalNAc-ase. α-*N*-acetylgalactosaminidase; TLC, thin-layer chromatography.

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the erythrocytes against anti-A serum and anti-H lectin, *Ulex europaeus* (Hohnen Oil), was tested. Enzyme-treated A_1 erythrocytes showed little hemagglutination against anti-A serum (agglutination titer, 1:16), although untreated A_1 erythrocytes showed strong hemagglutination (titer, 1:512). Moreover, enzyme-treated erythrocytes showed stronger hemagglutination against anti-H lectin (titer, 1:128) than untreated erythrocytes (titer, 1:32). This result indicated that group A erythrocytes were converted into group O erythrocytes by *Acremonium* α-GalNac-ase.

Fig. 1 illustrates the time-course of the degradation of A erythrocyte membranes by the α-GalNac-ase. The erythrocyte membranes were prepared from group A erythrocytes according to Dodge et al. [10]. α-GalNac-ase (60 units) was incubated at 37°C in 45 mM citrate buffer (pH 4.5) with 110 mg of erythrocytes membranes, in a total volume of 5.5 ml. After an aliquot was taken from the reaction mixture at intervals and boiled for 3 min, it was dialyzed against distilled water and then liberated free-GalNac in concentrated dialyzate was determined according to the method of Reissig et al. [11]. As shown in Fig. 1, GalNAc was found to be liberated by α-GalNAc-ase from the erythrocyte membranes rapidly in the first 0.5 to 1 h, and thereafter slowly. These aliquots in the reaction mixture were also tested for their inhibitory effects on hemagglutination of A-erythrocytes-anti-A serum. 10 μl of anti-A serum was added to 25 μl of a 2-fold serial diluted solution of the reaction mixture: 25 μl of 512-fold diluted solution of the reaction mixture contained about 1 μg of erythrocyte membranes (dry weight). After incubation with reaction mixtures and serum at 37°C for 1 h, 5 μl of a 4% suspension of A erythrocytes was added and then the mixture was further incubated at 37°C for 1 h. Hemagglutination for anti-A serum was strongly inhibited by the erythrocyte membranes in reaction mixture at the beginning of the incubation. As the enzyme reaction proceeded, the hemagglutination inhibition was weakened: a large amount of erythrocyte membranes in the reaction mixture was needed for the inhibition of the hemagglutination. For example, after a 24 h reaction, approx. 100 μg of erythrocyte membranes was needed to obtain the same degree of agglutination inhibition as that which occurred with about 1 μg of erythrocyte membranes at the start of the reaction. This means that the ability of erythrocyte membranes for agglutination inhibition reduced to 1:100 after 24 h reaction. This suggested that the blood group A active substances on the erythrocyte membranes were destroyed by the enzyme. On the contrary, hemagglutination inhibition activity of the erythrocyte membranes against anti-H lectin increased (data not shown).

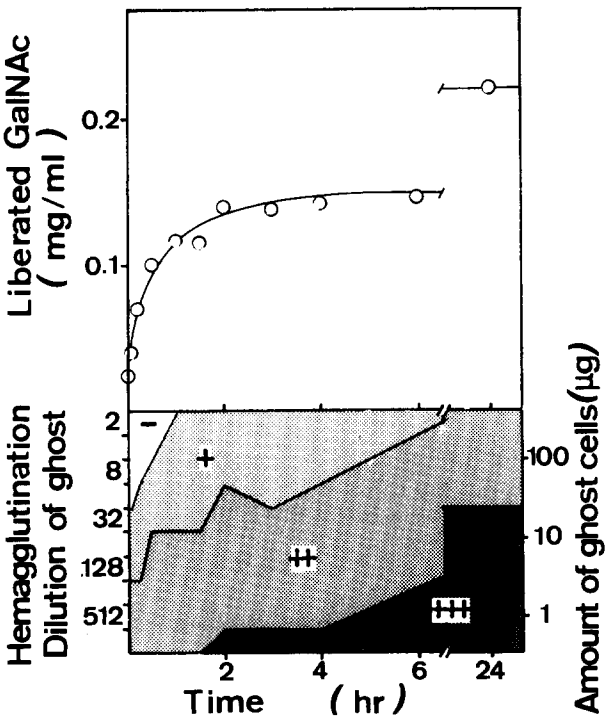

Fig. 1. Time-course of degradation of group A erythrocyte membranes by *Acremonium* α-GalNAc-ase. Details of the enzyme reaction are given in the text. The degradation of the erythrocyte membranes by the enzyme was examined in two ways. The liberation of GalNAc from the erythrocyte membranes (above) was determined by a colorimetric method [5]. The hemagglutination inhibition activity against A erythrocyte-anti-A serum (below) was tested with a dialyzed solution of reaction mixture. The amount of erythrocytes membranes was calculated from the dilution rate of the reaction mixture, –, no agglutination (strong inhibition): +, weak agglutination: + +, slightly strong agglutination: + + +, most strong agglutination (weak inhibition).

The antigenic determinant of blood group A, terminal α-linked GalNAc of sugar chains, is known to be derived from glycoproteins and glycolipids of A erythrocytes. Therefore, we investigated whether the enzyme worked on the glycoproteins or glycolipids of the erythrocytes or both. Glycoproteins were prepared from A erythrocyte membranes by both methods of chloroform/methanol extraction and pronase treatment. Chloroform/methanol (2:1, v/v) extraction was done according to Hamaguchi and Cleve [12]. Pronase treatment was done by incubating lyophilized ghost with pronase E (Kakenkagaku) at 37°C followed by gel filtration with Sephadex G-25. The fractions containing carbohydrates were concentrated. Lyophilized glycoprotein (0.24 mg) extracted from the erythrocyte membranes by chloroform/methanol (the aqueous phase) and lyophilized glycoprotein (1 mg) prepared by pronase treatment were used. Each was incubated with α-GalNac-ase (12 units) at 37°C in 45 mM citrate buffer (pH 4.5) for 12 h and the reaction was terminated by boiling for 5 min. After dialysis of the reaction mixture against water to remove GalNac, enzyme-treated glycoproteins were examined for hemagglutina-

tion inhibition activity against A erythrocyte-anti-A serum. Its activity with both glycoproteins was decreased by α-GalNAc-ase treatment: in the case of glycoproteins obtained by chloroform/methanol extraction, the agglutination titer was 1:1, compared to that without treatment (titer, 1:128); and with the glycoproteins obtained by pronase treatment, the agglutination titer was 1:1, compared to that without treatment (titer, 1:64). This result indicated that the enzyme could react with the glycoprotein of A-type erythrocytes. The enzyme protein showed no hemagglutination inhibition activity.

Next, we examined whether or not the enzyme worked on the glycolipids of A-type erythrocytes. Glycolipids were prepared from A-type erythrocyte membranes as described by Laine et al. [13]. Glycolipids (1.8 mg) were incubated with the enzyme (60 units) in 45 mM citrate buffer (pH 4.5) at 37°C for 15 h (the reaction mixture was turbid after treatment). The hemagglutination inhibition activity of enzyme-treated glycolipids was reduced compared with that of untreated glycolipids (agglutination titer of treatment was 1:2, while that without treatment was 1:32). Moreover, the action on the glycolipids with the enzyme was checked by HPTLC (high-performance thin-layer chromatography) and the formation of new glycolipids was ascertained. The enzyme treatment might reduce the solubility of glycolipids of group A erythrocytes by losing terminal α-GAlNac from sugar chains, resulting in turbidity.

To confirm that the enzyme could act on glycolipids, canine Forssman hapten (10 μg) was used as a substrate and the reaction was carried out with α-GalNAc-ase (0.5 units) in 45 mM citrate buffer (pH 4.5) containing 0.1% sodium taurodeoxycholate. The structure of Forssman hapten glycolipid is GalNAcα-GalNAcβ-Galactose α-Galactose β-Glucose-Ceramide. From a TLC analysis as shown in Fig. 2, globoside was formed. The result indicates that this enzyme can also work on sugar chains of glycolipids. Because no microbial α-GalNAc-ase has been shown to react on glycolipids, this enzyme is the first microbial α-GalNAc-ase to hydrolyze sugar chains of glycolipids.

In the previous report [8], we described that α-GalNAc-ase from *Acremonium* sp. hydrolyzed various blood group A active glycoproteins. In the present report, we showed that this enzyme could convert group A erythrocytes to group O erythrocytes and act on glycolipids including Forssman hapten. For this study, we used the hemagglutination test and obtained reproducible results, although the test is not sufficiently accurate to be regarded as quantitative. Because of the broad specificity, *Acremonium* sp. α-GalNAc-ase may be useful for the structural analysis of glycoconjugates. It might also be helpful for the serological conversion of erythrocytes.

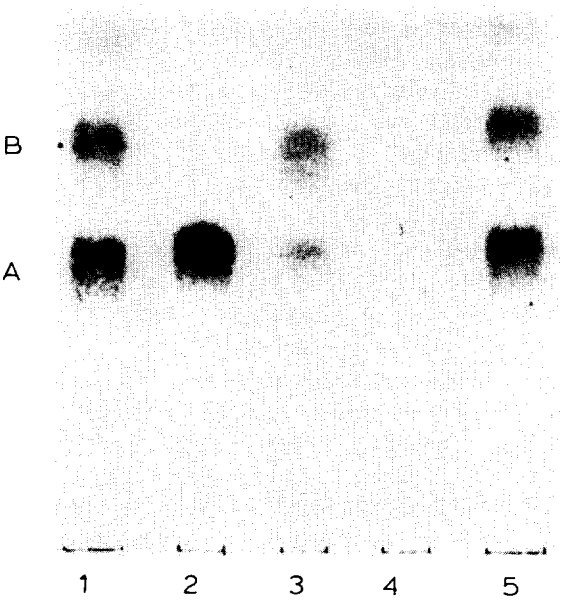

Fig. 2. Thin-layer chromatogram of α-GalNAc-ase-treated Forssman hapten. lanes, 1 and 5, standard (Frossman hapten + globoside); 2, Forssman hapten (control); 3, Forssman hapten + the enzyme; 4, the enzyme only. A, Forssman hapten: B, globoside.

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