Carbohydrate Research, 61 (1978) 447–455

2-ACETAMIDO-2-DEOXY-α-D-GALACTOSIDASES OF *Clostridium perfringens*. SEPARATION AND CHARACTERIZATION OF AN EXOGLYCOSIDASE AND AN OLIGOSACCHARIDASE*

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(Received June 27th, 1977; accepted for publication in revised form, September 25th, 1977)

ABSTRACT

Two distinct 2-acetamido-2-deoxy-α-D-galactosidases have been separated from filtrates of cultured *Clostridium perfringens* by electrophoresis in 6.5% poly(acrylamide) gels. One of the enzymes had a mobility of 0.32–0.36 (relative to Bromophenol Blue) and was identified as the exoglycosidase, 2-acetamido-2-deoxy-α-D-galactosidase. It appears to be the same enzyme as that reported in 1972 by McGuire *et al.* The second of the two enzymes, having a relative mobility of 0.42–0.46, corresponds to the oligosaccharidase reported in 1972 by Huang and Aminoff. The A-specificities of human type-A erythrocytes and of water-soluble glycoproteins having A-activity are both destroyed by incubation with the 2-acetamido-2-deoxy-α-D-galactosidase, but not on incubation with the oligosaccharidase. A concomitant rise in blood-group O(H) activity, as indicated by the use of a lectin from *Ulex europeus*, occurred in the A-erythrocytes treated with the exoglycosidase 2-acetamido-2-deoxy-α-D-galactosidase.

INTRODUCTION

Schiff[1,2] was the first to observe that culture filtrates of *Clostridium welchii* possess the ability to destroy the specific serological properties of blood-group A-active materials. Stack and Morgan[3] partially purified the enzymes from the culture filtrates of *Cl. welchii* and showed that they are effective in inactivating the A- and H-active glycoproteins from hog gastric mucins[4]. The purification of the enzymes was not extensive, in part due to the difficulties inherent in the use of an assay based on inhibition of hemagglutination to quantitate enzyme content.

We faced the same problem in our previously described attempts to isolate the H-destroying enzyme of *Clostridium*[5]. This difficulty was overcome by the use of a chemical assay for determination of liberated free fucose in the presence of the

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

glycosidically bound sugar[6]. In the course of these studies, we noted that incubation of the crude *Cl. perfringens* filtrates with submaxillary glycoproteins of blood-types A or H resulted in the release of material that reacted as free *N*-acetylhexosamine in the Morgan–Elson test[7]. On incubation of the hog submaxillary glycoproteins with the crude *Cl. perfringens* filtrates, a disaccharide, characterized as 2-acetamido-2-deoxy-3-*O*-β-D-galacto-pyranosyl-D-galactose, was released. The isolated disaccharide gave an augmented color-response in the Morgan–Elson assay, a property characteristic of 3-substituted *N*-acetylhexosamines[8,9], and was responsible for the anomalous kinetics observed in the quantitation of the enzyme. These results suggested that the 2-acetamido-2-deoxy-α-D-galactosidase activity in *Cl. perfringens* was an oligosaccharidase[10].

Subsequently, the detection and isolation of glycosidases in *Cl. perfringens*, among which 2-acetamido-2-deoxy-α-D-galactosidase was identified, were reported[11]. This enzyme reacted with both phenyl 2-acetamido-2-deoxy-α-D-galactoside, a substrate of low molecular weight, and the high molecular weight asialo-ovine submaxillary mucin (A-OSM). Fractionation of the *Cl. perfringens* enzymes by the procedure of McGuire *et al.*[11] gave a preparation that showed no indication of excess *N*-acetylhexosamine release, as previously reported[10].

This communication is a preliminary report of the effective separation of two 2-acetamido-2-deoxy-α-D-galactosidases[10,11] by the use of poly(acrylamide)-gel electrophoresis, and a characterization of their actions on suitable substrates. The serological changes effected by these enzymes on red cells and blood-group-active glycoproteins are also reported.

MATERIALS

The following materials were obtained from the sources indicated: acrylamide, bis(acrylamide), 1,4-dithiothreitol, and gel slicer (Bio-Rad Corporation), *N,N,N',N'*-tetramethylethylenediamine (Aldrich Chemical Company), typing antisera (Ortho Diagnostics), microtiter system (Cooke Engineering Company), 589 Blue Ribbon chromatography paper (Schleicher and Schuell), Whatman No. 1 and No. 541 filter paper (Reeve Angel Corporation), human type A, B, and AB blood (University of Michigan Blood Bank), Todd–Hewitt broth, cooked-meat medium (Difco Laboratories), 2-acetamido-2-deoxy-D-galactose (Sigma Chemical Company), *N*-acetylneuraminic acid (Koch–Light Laboratories), *p*-nitrophenyl α- and β-D-galactopyranosides (Koch–Light Laboratories), *Clostridium perfringens* (University of Michigan Department of Microbiology)[6], phosphate-buffered saline, pH 7.2, and *Ulex europeus* anti-H lectin[14].

Ovine-submaxillary mucin (OSM) and A-active hog-submaxillary mucin (SGA) were prepared by a procedure described elsewhere[6,12]. Asialo-ovine submaxillary mucin (A-OSM) was prepared by the procedure of Aminoff[13], involving hydrolysis for 45 min with 0.01M hydrochloric acid followed by exhaustive dialysis to remove the sialic acid released.

METHODS

Seed cultures of *Clostridium perfringens* were grown in cooked-meat medium for 24 h. Aliquots (2 ml) of this culture were introduced into each of 19 screw-capped erlenmeyer flasks (2 liters) containing 1500 ml of medium. Growth medium consisted of 35 g of Todd–Hewitt broth and 4.8 g of potassium dihydrogenphosphate per liter. Cultures were incubated for 64 h at 37°. The cells were sedimented at 4° in a Sharples centrifuge. Solid ammonium sulfate was added to the supernatant solution until 80% saturation was achieved. The suspension was stirred overnight at 4° and the precipitate collected by filtration through Whatman No. 541 filter paper. The precipitate was dissolved in 0.01M Tris–HCl buffer (pH 8.0) and 0.1mM 1,4-dithiothreitol (Tris–DTT buffer). The suspension was dialyzed against two changes of 4 liters of Tris–DTT buffer.

Hemagglutination and hemagglutination inhibition. — Hemagglutination and hemagglutination-inhibition assays were performed with a microtiter system with phosphate-buffered saline as diluent.

Enzyme purification. — 2-Acetamido-2-deoxy-α-D-galactosidase was purified as described by McGuire *et al.*[11], and the results of the fractionation are given in Table I.

Poly(acrylamide)-gel electrophoresis. — Gels were prepared following the protocol described by Maizel[15] for high pH (pH 8.9), discontinuous poly(acrylamide)-gel electrophoresis. The current flow through the gel cylinders was 2 mA per gel cylinder until the Bromophenol Blue had migrated through approximately 75% of the resolving gel. The gels were removed and frozen at −70° until used for enzyme assays. The frozen gels were cut into 1-mm slices by using a Bio-Rad gel slicer. The enzymic activities in the slices were then determined.

TABLE I

PURIFICATION OF 2-ACETAMIDO-2-DEOXY-α-D-GALACTOSIDASE ACCORDING TO THE PROCEDURE OF MCGUIRE *et al.*[11]

Step	*Specific activity (units/mg)*	*Purification factor*	*Recovery (%)*
Culture fluid + 0–80% saturation with $(NH_4)_2SO_4$	0.28	1.0	100
Sephadex G-150	1.38	4.96	68.3
DEAE-Cellulose I, 0.15 → 0.4M KCl, concave gradient	6.15	22.1	64
DEAE-Cellulose II, 0.14 → 0.22M KCl, stepwise gradient	12.18	43.8	40.6
DEAE-Cellulose II, concentration dialysis	10.9	39.2	36.5

Localization of enzymic activities in poly(acrylamide) gels. — Slices (1 mm) of the 6.5% acrylamide gel were added sequentially to reaction tubes and tested for the following activities:

1. *2-Acetamido-2-deoxy-α-D-galactosidase.* The reaction mixture contained a slice of the gel, 250 μg of asialo-OSM or A-active hog submaxillary mucin, and 2.5 μmol of sodium phosphate buffer, pH 6.3, in a total volume of 150 μl. The mixtures were then assayed for free *N*-acetylhexosamine by a modification[16] of the Morgan–Elson assay[7].

2. *Enzymes destroying blood-group A specificity.* A 5% suspension of red blood cells in 0.4 ml of phosphate-buffered saline was added to each slice. The suspensions were incubated for 18 h at 37° and the cells were washed with the buffer. The packed cells were then resuspended in phosphate-buffered saline and separated from the gel slice to give a 1% suspension. The enzyme-treated cells were then titrated against group A or group B typing antiserum or *Ulex* anti-H lectin.

3. *Enzymes destroying the hemagglutination inhibition activity of water-soluble, blood-group substances.* The mixture contained a slice of the gel, 125 μg of the A-active hog submaxillary mucin, and 2.5 μmol of phosphate buffer, pH 6.3, contained in a total volume of 150 μl. After 18 h at 37°, 50 μl of the suspension was added to 50 μl of anti-A typing serum and the mixture incubated for 0.5 h at room temperature. The mixture was then titrated against a 1% suspension of human type-A erythrocytes in phosphate-buffered saline.

4. N-*Acetylneuraminic acid aldolase.* Gel slices were added sequentially to tubes containing 2.5 μmol of phosphate buffer (pH 6.3) and 0.05 μmol of *N*-acetylneuraminic acid contained in a total volume of 150 μl. Mixtures were incubated for 18 h at 37° and the *N*-acetylhexosamine released was determined by the modification[16] of the Morgan–Elson assay[7].

5. *Sialidase.* Gel slices were added sequentially to tubes containing 25 μmol of acetate buffer (pH 5.5) and 250 μg of OSM contained in 150 μl. The mixtures were incubated for 18 h at 37° and assayed for free sialic acid by the thiobarbituric assay of Aminoff[13].

6. *β-Galactosidase.* Gel slices were added sequentially to tubes containing 25 μmol of citrate buffer (pH 5.5) and 0.01 μmol of *p*-nitrophenyl β-D-galactopyranoside contained in 150 μl. After 18 h at 37°, 1 ml of 0.2M sodium carbonate was added per tube and the absorbance at 420 nm was recorded.

Chromatography and high-voltage electrophoresis of products of 2-acetamido-2-deoxy-α-D-galactosidase action on asialo-A-active hog submaxillary mucin. — Gel slices were incubated with A-active hog submaxillary mucin as just described. After 18 h at 37°, the protein and salts were precipated by the addition of abs. ethanol to a concentration of 80% and stored for 24 h at −20°. The alcoholic suspensions were then centrifuged at 40,000*g* in a Sorvall refrigerated centrifuge, and the supernatant solutions were evaporated on a rotary evaporator. The residue was then resuspended in 80% ethanol in a volume equivalent to one-tenth of the original volume. An aliquot was spotted on Schleicher and Schuell 589 blue-ribbon paper and examined

by paper chromatography, or onto Whatman No. 1 paper for high-voltage electrophoresis. The papers were developed by descending chromatography with 6:4:3 (v/v/v) butanol–pyridine–water for 48 h, or by high-voltage electrophoresis in 1% potassium borate (pH 9.2) for 1.5 h at 280 mA, constant current. The spots of *N*-acetylhexosamines, disaccharide, and sialic acid were detected by the spray procedure of Partridge[17].

Descending paper chromatography was also performed as above for the quantitative determination of the distribution of 2-acetamido-2-deoxy-D-galactose and oligosaccharide in the various incubation products. The areas corresponding to the 2-acetamido-2-deoxygalactose and disaccharide were cut out, and analyzed by the Morgan–Elson assay for *N*-acetylhexosamines.

RESULTS

Poly(acrylamide)-gel electrophoresis of Clostridium perfringens *filtrates.* — A preparation of an extensively purified fraction obtained by the procedure of McGuire[11] was subjected to gel electrophoresis[15]. The results obtained with asialo-OSM as substrate are shown in Fig. 1A, which shows a major peak of 2-acetamido-2-deoxy-α-D-galactosidase activity having an R_e value of 0.34 and a subsidiary activity having an R_e value of 0.44 (R_e is the electrophoretic mobility of the enzyme relative to that of the indicator band of bromophenol blue).

Analysis of less-pure fractions, 0–80% ammonium sulfate precipitate, of *Clostridium perfringens* filtrate by poly(acrylamide)-gel electrophoresis, using both asialo-OSM and SGA (see Materials) as substrates, gave rise to two principal peaks of *N*-acetylhexosaminidase activity, the first located at $R_e = 0.34$ and the second at $R_e = 0.44$ (Fig. 1B). However, the apparent activities differed according to the substrate used. With A-OSM as substrate, a greater response was obtained with the enzyme at $R_e = 0.34$, whereas with SGA as substrate, the response was greater with the enzyme at $R_e = 0.44$ (Figure 1, A and B).

Sialidase activity was found in a number of places (Fig. 2H). Gels assayed for *N*-acetylneuraminic acid–aldolase activity gave a single peak at $R_e = 0.72$–0.75 (Fig. 2I). Gels assayed for β-D-galactosidase showed activity at $R_e = 0.16$–0.20 and 0.32–0.36 (Fig. 2J), with the zone of greatest activity overlapping the two 2-acetamido-2-deoxy-α-D-galactosidase activities.

Action of Clostridium perfringens *enzymes, separated by poly(acrylamide)-gel electrophoresis, on A-active hog-submaxillary mucin.* — The hemagglutination-inhibitory activity of hog A-active submaxillary mucin was destroyed by the action of both enzymes ($R_e = 0.34$ and 0.44); however, the magnitude of the response was much greater with the first band ($R_e = 0.32$–0.36) of enzymic activity (Fig. 2C).

Action of Clostridium perfringens *enzymes, separated by poly(acrylamide)-gel electrophoresis, on human erythrocytes.* — The results of the action of the enzymes separated by poly(acrylamide)-gel electrophoresis on red cells of types A and AB are summarized in Fig. 2, D–G. The 2-acetamido-2-deoxy-α-D-galactosidase having

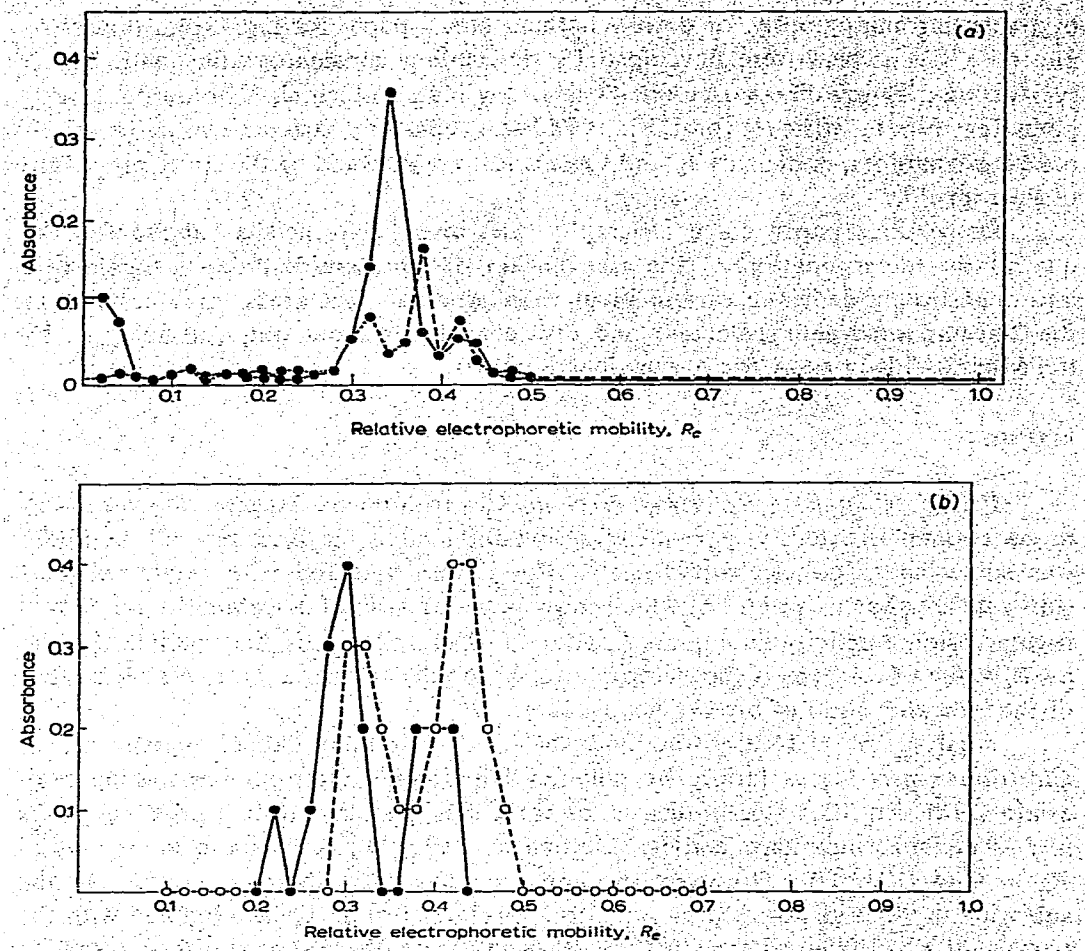

Fig. 1. The 2-acetamido-2-deoxy-α-D-galactosidase profiles for *Cl. perfringens* filtrates (*a*) purified by McGuire's procedure[11], and (*b*) crude filtrate precipitated with ammonium sulfate (0–80% saturated) as tested with asialo-OSM (.—.) and A-active hog submaxillary glycoprotein (.- - - .). R_e is the electrophoretic mobility of the enzyme relative to that of the indicator band of Bromophenol Blue. Duplicate gels gave a variation of ±0.02 in relative mobility.

an R_e value of 0.32–0.36 destroys the A-activity of A and AB cells. It has no effect on B cells. The A cells so treated developed H reactivity only where there was a loss of A activity. Moreover, a small but significant amount of Morgan–Elson positive material was liberated from the erythrocytes by the action of the enzyme.

Identification of the products produced from A-active, hog-submaxillary mucin when treated with Clostridium perfringens *enzymes.* — Products of the action of the 2-acetamido-2-deoxyhexosidases (R_e = 0.34 and 0.44) on hog A-active submaxillary mucin were subjected to descending chromatography with 6:4:3 (v/v/v) butanol–pyridine–water as solvent. 2-Acetamido-2-deoxy-D-galactose was the principal product obtained with the enzyme having R_e 0.32–0.36, whereas the enzyme migrating

at R_e = 0.42–0.46 produced only slower-migrating oligosaccharides (Fig. 3).

Quantitation of the products released from asialo-A-active hog submaxillary glycoprotein when treated with Clostridium perfringens *enzymes.* — High-voltage electrophoresis gave a very effective separation of the 2-acetamido-2-deoxy-D-mannose from 2-acetamido-2-deoxy-D-galactose (18.3 and 7.8 cm anodic migration from the origin,

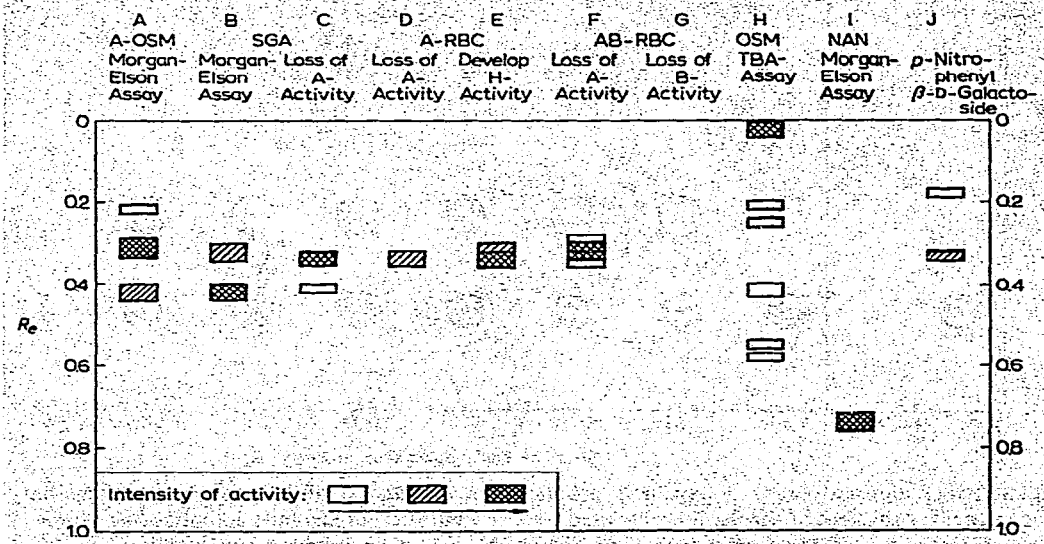

Fig. 2. Diagrammatic reconstruction of the enzymic-activity profiles for *Cl. perfringens* filtrates. Ammonium sulfate (0–80%) precipitates of *Cl. perfringens* filtrates were submitted to poly(acrylamide)-gel electrophoresis and sequential 1-mm slices were incubated with various substrates, and analyzed as indicated. See text for experimental details: *A and B.* — Asialo-ovine submaxillary mucin (A-OSM, A) and A-active hog submaxillary mucin (SGA, B) were used as substrates and the reaction products submitted to the modified Morgan–Elson assay[16]. *C.* — SGA was incubated with the various slices and the products were tested with anti-A serum in the inhibition of the hemagglutination reaction. *D and E.* Red blood-cells of type A (A-RBC) were examined for their ability to react with anti-A serum (D) and *Ulex* anti-H (E) after incubation with the gel slices. *F and G.* — Red blood cells of type AB (AB-RBC) were examined for their ability to react with anti-A serum (F) and anti-B serum (G) after incubation with the gel slices. *H.* — Ovine submaxillary mucin (OSM) was incubated with the gel slices; the amount of sialic acid released[13] is indicative of sialidase activity. *I.* — *N*-Acetylneuraminic acid as substrate and the Morgan–Elson assay[16] were used to detect the location of *N*-acetylneuraminic acid aldolase. *J.* — *p*-Nitrophenyl β-D-galactoside as substrate was used to locate the galactosidase activities.

TABLE II

COMPOSITION OF THE INCUBATION PRODUCTS OBTAINED WITH A-ACTIVE HOG SUBMAXILLARY GLYCOPROTEIN AND THE 2-ACETAMIDO-2-DEOXY-α-D-GALACTOSIDASES, RESOLVED BY POLY(ACRYLAMIDE)-GEL ELECTROPHORESIS, HAVING R_e = 0.34 AND 0.44

Enzyme fraction R_e	*2-Acetamido-2-deoxy-α-D-galactose (μmol)*	*Disaccharide (μmol)*	*2-Acetamido-2-deoxy-α-D-galactose/disaccharide*
0.34	1.98	0.51	3.88
0.44	1.02	1.62	0.63

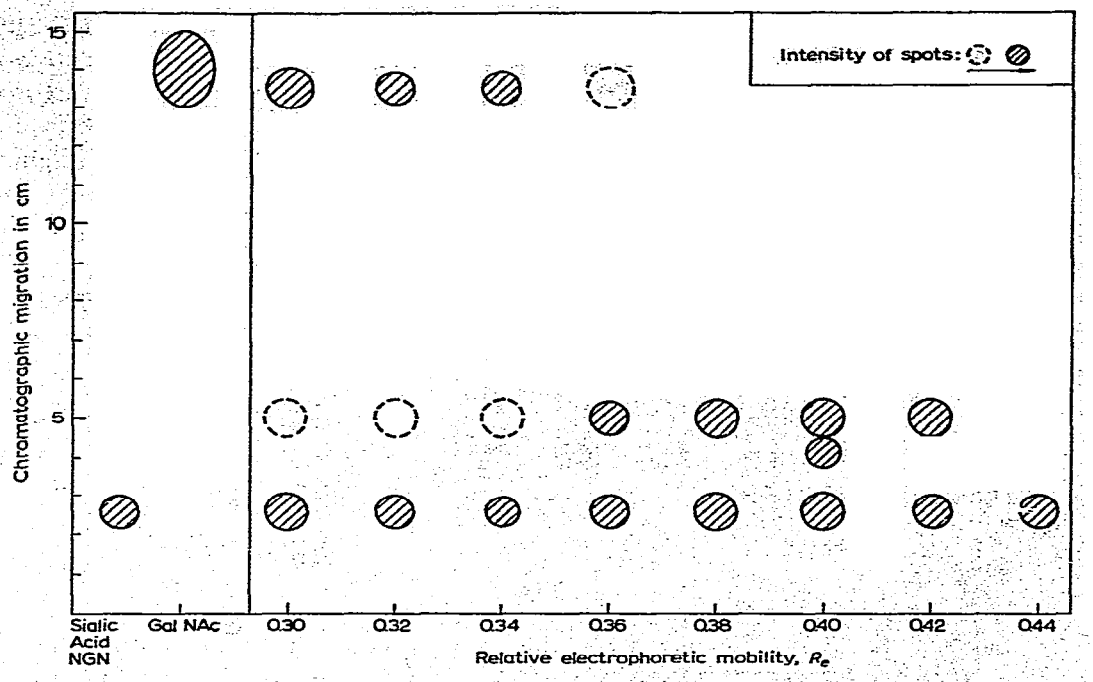

Fig. 3. Paper chromatogram of products produced on incubation of A-active hog submaxillary glycoprotein with slices of acrylamide gels used for the separation of enzymic activities of cultures of *Cl. perfringens*. See text for experimental details. NGN = *N*-glycoloylneuraminic acid.

respectively). This procedure, therefore, is excellent for demonstrating the absence of 2-acetamido-2-deoxy-D-mannose in the incubation products, whereas paper partition chromatography was used for the quantitation of the 2-acetamido-2-deoxy-D-galactose and oligosaccharide released. The variable composition of the products obtained with the different enzyme preparations as separated by disc-gel electrophoresis is shown in Table II.

DISCUSSION

The following evidence is submitted in support of our contention that there are at least two 2-acetamido-2-deoxy-α-D-galactosidases in the culture filtrates of *Clostridium perfringens* that are separable by poly(acrylamide)-gel electrophoresis in 6.5% gels. These two enzymes have relative mobilities, R_e = 0.32–0.36 and R_e = 0.42–0.46:

1. The crude preparation of the enzyme, when submitted to analysis by poly(acrylamide)-gel electrophoresis gives two distinct zones of activity with asialo-OSM as substrate. The more-highly purified enzyme derived from this crude preparation of the enzyme concentrates only one of these enzymes, that having R_e 0.32.

2. These same two preparations of enzyme, crude and partially purified, each resolved by poly(acrylamide)-gel electrophoresis show different relative reactivity

depending on the substrates used, asialo-OSM or A-active hog submaxillary glycoproteins.

3. Quantitative analysis of the reaction products obtained from the gel slices with either R_e 0.34 or R_e 0.44 indicates the release of different amounts of disaccharide and free 2-acetamido-2-deoxy-D-galactose when incubated with the more-complex, A-active hog submaxillary glycoprotein as substrate.

The purification scheme of McGuire *et al.*[11] favors the isolation of the exoglycosidase 2-acetamido-2-deoxy-α-D-galactosidase with but minor contamination by the oligosaccharidase[10]. The enzyme having the lower mobility, R_e = 0.32–0.36, would appear to be the exoglycosidase described by McGuire *et al.*, releasing 2-acetamido-2-deoxy-D-galactose from asialo-OSM and A-active hog submaxillary mucin. The action on the latter substrate is accompanied by loss of blood-group A activity. Likewise, it is this enzyme that is effective in removing the blood-group A activity on A and AB cells, concomitantly changing the specificity to H. It has no action on blood-group B specificity.

The enzyme having mobility 0.42–0.46 corresponds to the oligosaccharidase of Huang and Aminoff[10], and, although it decreases the A-activity of water-soluble glycoproteins, the enzyme has no effect on the A-specificity of A or AB cells. This apparent contradiction may be explained by the fact that the oligosaccharides cleaved from the glycoproteins are not such potent inhibitors of anti-A serum as the intact glycoproteins.

ACKNOWLEDGMENTS

This project was supported, in part, by Grant HL-17881 from the National Institutes of Health and, in part, by Contract ONR N00014-76-C-0296 from the Office of Naval Research.

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