Blood Group Antigenicity of Purified Human Intestinal Disaccharidases*

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SUMMARY

1. Human intestinal maltase and sucrase contain 30 to 40% carbohydrate by weight and are resistant to proteolytic digestion by papain. The major carbohydrates associated with these enzymes are fucose, galactose, and hexosamines.

2. The purified disaccharidases demonstrate blood group antigenicity of great potency, as little as 8 × 10^{-9} to 1.5 × 10^{-7} M inhibiting red cell agglutination.

3. The blood group reactivity is not the result of contamination by blood group substance because: (a) type specific antibody causes a shift of enzyme activity on sucrose gradients; (b) antibody changes the Rf of the enzyme band on acrylamide electrophoresis; and (c) enzyme activity is selectively retained by type-specific antibody bound to Sepharose.

4. Rat intestinal disaccharidases contain 15 to 20% carbohydrate and do not have blood group reactivity, yet are papain-resistant.

5. Alkali treatment in the presence of sodium borohydride produces heterogeneous protein fragments without carbohydrate (18,000 to 25,000 mol wt) and a carbohydrate fragment with a molecular weight of about 1,800.

6. We conclude that human intestinal disaccharidases are glycoproteins and that blood group reactivity is contained in the oligosaccharide side chain covalently linked to the enzyme. This is the first demonstration of blood group antigenicity associated with a functioning protein molecule. The disaccharidases may account for some of the insoluble blood group reactivity found in intestinal tissue.

The intestinal disaccharidases are located on the external surface of the enterocyte membrane, thus being an integral part of the intestinal brush border microvilli (1, 2). Overlying the brush border is the glycocalyx which is an amorphous covering rich in complex carbohydrates of various types (3). We recently purified human maltase almost to homogeneity and found it to contain 32 to 38% carbohydrate, with the predominant sugars being fucose, galactose, and hexosamines (4). We now report that the carbohydrate moieties of the enzyme have potent ABO blood group reactivity. Evidence is also presented which eliminates contaminating blood group substance as the source of this blood group activity.

EXPERIMENTAL PROCEDURE

Enzyme Preparation—The proximal small intestine was obtained within 6 hours of death from six patients at the time of postmortem examination. The age, race, sex, cause of death, and blood group type were the following: R.W. 46, white, female, myocardial infarction, type B; S.H. 36, white, female, myocardial infarction, type A; W.R. 68, white, male, coronary artery disease, type A; J.N. 78, white, male, cerebrovascular accident, type B; M.M. 57, white, female, cardiac arrest, type B; and S.R. 65, white, female, carcinoma of the breast, type A. The intestine was opened from the ligament of Treitz to the mid-small bowel, rinsed very gently with 0.9% NaCl solution, and carefully blotted. Mucosa was obtained by scraping with glass slides. Maltase was purified as described previously (4), using ethanol precipitation, chromatography on DEAE-cellulose, gel filtration on Sephadex G-200, and preparative polyacrylamide electrophoresis to obtain a preparation which was over 95% homogeneous by analytical polyacrylamide electrophoresis. Human and rat sucrase were purified as described by others (5) until each gave a single protein band on polyacrylamide electrophoresis, using three different pH systems.

Three per cent SE-30 on Chromosorb Q was obtained from Applied Science Laboratories, Inc. (State College, Pa.). Sepharose 4-B was a product of Pharmacia, Inc. (Ficataway, N. J.).

Chemicals—Sodium [3H]borohydride (100 μCi per μmole) was a product of New England Nuclear (Boston, Mass.). All sugars used were of highest purity available and contained only traces of contamination on gas-liquid chromatography. Arabinotol, mannitol, xylose, and fucose were obtained from Pfannstiel Laboratories, Inc. (Waukegan, Ill.). Mannose and galactose were purchased from Fisher Chemical Co. (Pittsburgh, Pa.). Hexosamines were obtained from Calbiochem (La Jolla, Calif.). All other chemicals were of the highest grade available. Anti-A and anti-B antibody were purchased from Ortho Pharmaceutical Corp. (Raritan, N.J.). Catalase was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Carbohydrate Composition—Carbohydrate analysis was performed by gas-liquid chromatography using a modification of the method of Clamp et al. (6) as described by Reinhold (7).

* This work was supported in part by Grants AM 14038 and AM-05280 from the National Institutes of Health.

(Received for publication, April 27, 1973)
Analysis of the trimethyl silyl sugar derivatives was performed on a two-meter, U-shaped column (inner diameter 3 mm) packed with 3% SE-30 on Chromosorb Q. Five micrograms of enzyme hydrolyzed with methanolic HCl were applied, using a flow rate of helium of 50 ml per min and a temperature gradient of 1° per min, from 140–200°. Experimentally determined molar adjustment factors were as follows: fucose 1.24, xylose 1.33, mannose 0.97, galactose 0.93, N-acetylgalactosamine 1.23, and N-acetylglucosamine 1.50. Internal standards used were arabinitol and mannitol. The area under each curve was determined by tracing the curve, cutting out each peak, and weighing the resulting portions of the curve.

**Blood Group Antigenicity**—Blood group testing was performed by a hapten inhibition test, as described by Kabat (8) using a microtitration system. Dilutions of antigen from 1/1-1/4096 were made using a microtitrator (Cooke Engineering Co., Alexandria, Va.). Each well contained 25 μl of 0.9% NaCl solution, and 25 μl of washed red blood cells. The red cell suspensions were then incubated at 37° for 1 to 2 hours. The end point for hapten titer was that dilution causing complete agglutination of cells. All samples assayed used a 2-fold excess of antiserum required to completely agglutinate the red cells.

**Alkaline Borohydride Treatment**—Purified maltase or sucrase (0.75 mg) was added in 2 ml to 2 ml of 0.4 m sodium [PH]borohydride (2 μCi per ml) in 0.2 x NaOH. The tube was flushed for 1 min with N2, stoppered, and left for 24 hours at room temperature in the dark. The solution was neutralized with 2 ml of HCl (approximately 0.4 ml) and lyophilized. The lyophilized material was resuspended in 0.2 ml of 100 mM KPO4 buffer, pH 7.0, and chromatographed on a Sephadex G-200 column (0.7 x 20 cm).

**Preparation of Sepharose-linked Anti-A and Anti-B Antisera**—The antisera were conjugated to Sepharose by the method of Cantreels (9). Seven and one-half milliliters of Sepharose 4-B and 7.5 ml of H2O were stirred with 0.75 g of cyanogen bromide. The pH was adjusted to 10 with 20 μl of NaOH and stirring continued for 10 to 12 min. The Sepharose was filtered rapidly and washed with 100 mM NaHCO3. The activated Sepharose was resuspended in 7.5 ml of NaHCO3, 15 mg of type-specific antiserum were added, and the mixture shaken overnight at 4°. The Sepharose was washed extensively with 100 mM NaHCO3 and then equilibrated with 50 mM KPO4 buffer, pH 6.0. Eighty-four per cent of the added protein was coupled to the Sepharose. A column (0.5 x 14 cm) was poured, with a void volume of 2.5 ml. Elution was performed using 50 mM KPO4 buffer, pH 6.0.

**Molecular Weight Determination of Oligosaccharides**—Gel filtration on Sephadex G-50 for determination of oligosaccharide molecular weight was carried out as described by Bhatti and Clamp (10). A column (0.9 x 30 cm) was equilibrated with 50 mM KPO4 buffer, pH 7.0. Standards used were glucose, [14C]sucrose (Amersham-Searle, Arlington Heights, Ill.), maltopentaose (purified as described by Gibson et al. (11)), and a glycopeptide of molecular weight 1919 prepared from an immunoglobulin myeloma protein (kindly supplied by Dr. S. Kornfeld). Glucose was assayed by the glucose oxidase reaction (12), sucrose by radioisotopic counting in Bray's solution (13). Maltopentaose and the IgG glycopeptide were detected by the phenol-sulfuric acid method (14). Glucose, sucrose, maltopentaose, and the carbohydrate residue derived from alkaline borohydride hydrolysis, labeled with 3H, were chromatographed simultaneously, while the IgG glycopeptide was analyzed separately. The column void volume was 17 ml, and the fractions of 0.25 ml were collected.

Sucrose gradient centrifugation was performed by the method of Britten and Roberts (15).

**Biochemical Determinations**—Disaccharidases were measured by the method of Dahlqvist (16). Protein was determined by the method of Lowry et al. (17). Polyaclaramide electrophoresis was performed using a standard Tris-glycine system, pH 9.5, imidazole-HCl, pH 8.6, and glycine-KOH, pH 6.6 (18).

**RESULTS**

**Carbohydrate Composition**—Human sucrase was purified to homogeneity as judged by migration on three different polyacrylamide electrophoretic systems. Purified maltase revealed one major protein band and three minor bands (Fig. 1). Since the proteins in at least one of these bands had identical enzymatic properties (4), it may be an isoenzyme, perhaps generated by the initial papain solubilization step in the enzyme preparation. When the major protein band of maltase was isolated by preparative polyacrylamide electrophoresis (4), the carbohydrate composition was similar to that described below. Thus, for most studies of maltase reported here, the preparation shown in Fig. 1 was used. Sucrase and maltase revealed large amounts of carbohydrate on gas-liquid chromatographic analysis. A representative chromatogram of sucrase is depicted in Fig. 2. The sugars present in largest amounts were fucose (Peaks 1, 2, 3), galactose (Peaks 8, 10, 11), and N-acetylgalactosamine (Peaks 15, 17, 19, 20). No glucose (Peaks 12, 13) or sialic acid (Peak 21, not shown) was detected. Quantitative analysis of sucrase and maltase from two patients is shown in Table I. In each case, sucrase contained more total carbohydrate than did maltase. Blood group type B has a terminal galactose residue, and the highest galactose content was in a type B patient. Type A has a terminal N-acetylgalactosamine and this was reflected in higher levels of this carbohydrate in the sucrase, but not maltase, from the type A patient. Large amounts of fucose were present in all samples analyzed.
Fig. 2. Gas-liquid chromatogram of sugars from human intestinal sucrase. Five micrograms of purified sucrase from type B patient R.W. were hydrolyzed as described under "Experimental Procedure" and applied to a 3% SE-30 column as described under "Experimental Procedure." Peaks correspond to the trimethylsilyl derivatives of the following sugars: 1 to 3, fucose; 4 and 5, xylose; 6, arabinose; 7, mannose; 8, galactose; and 13 and 15, glucose; 14, mannoset; 16 and 17, N-acetylglucosamine; 18, N-acetylgalactosamine; 19 and 20, N-acetylglucosamine.

<table>
<thead>
<tr>
<th>Carbohydrate composition of human intestinal disaccharidases</th>
<th>Sucrase</th>
<th>Maltase</th>
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<tbody>
<tr>
<td>Fucose</td>
<td>222</td>
<td>93</td>
</tr>
<tr>
<td>Xylose</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Mannose</td>
<td>69</td>
<td>33</td>
</tr>
<tr>
<td>Galactose</td>
<td>296</td>
<td>67</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>201</td>
<td>28</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>32</td>
<td>48</td>
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<tr>
<td>R.W.</td>
<td>R.S.</td>
<td>Sucrase</td>
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</tbody>
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<th>Relative potency of blood group substances in human intestinal sucrases</th>
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| Hemagglutination inhibition assays were performed as described under "Experimental Procedure." Serial dilutions of the various purified sucrase preparations from each intestine were made, from 1/10 to 1/2048. The last dilution which inhibited type-specific agglutination was used as the end point, and the amount of protein present was calculated from the initial concentration. Where no inhibition was noted, the undiluted sample contained 10 ng of purified enzyme.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Blood group type</th>
<th>Inhibition of type A cell agglutination</th>
<th>Inhibition of type B cell agglutination</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ng protein</td>
<td>mg protein</td>
</tr>
<tr>
<td>R.W.</td>
<td>R</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>J.N.</td>
<td>B</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>M.M.</td>
<td>B</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>S.H.</td>
<td>A</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>W.R.</td>
<td>A</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>W.S.</td>
<td>A</td>
<td>None</td>
<td>20</td>
</tr>
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</table>

| Blood Group Reactivity with the Enzyme Itself—Because of the extreme potency of blood group substance and its low protein content (20), it seemed possible that the antigenic activity found with the enzyme might represent a contaminant from mucus secretions. To rule out this possibility, enzyme activity was followed by three chromatographic methods before and after addition of antibody, to see whether type-specific antibody caused a shift in the position of enzyme activity.

| Association of Blood Group Reactivity with the Enzyme Itself—Because of the extreme potency of blood group substance and its low protein content (20), it seemed possible that the antigenic activity found with the enzyme might represent a contaminant from mucus secretions. To rule out this possibility, enzyme activity was followed by three chromatographic methods before and after addition of antibody, to see whether type-specific antibody caused a shift in the position of enzyme activity.

| Purified enzyme was first incubated with blood group antibody and applied to a surose density gradient (Fig. 3). Addition of the antibody did not affect enzyme activity. In this experiment, maltase with type A activity was first incubated with anti-A antibody. A definite shift of maltase activity to the right, or bottom, of the gradient can be seen (upper graph, Fig. 3), suggesting that enzyme-antibody complexes might be formed. The position of the enzyme was not affected by anti-B antibody (lower graph, Fig. 3).

Next, maltase from a blood group type A patient was incubated with anti-blood group antibody and subjected to elec-
Sucrese density gradient centrifugation of human intestinal sucrase. Fifty micrograms of purified maltase from type A patient R.S. were added to 50 μl of anti-A or anti-B antibody (containing about 100 μg of protein), and incubated for 1 hour at 37°, then for 6 hours at 4°. Two hundred microliters of catalase were layered on a 5 to 20% sucrose gradient of 12.5 ml. Centrifugation in an SW 36 Beckman rotor was performed for 16 hours at 67,500 × g in a Spinco model L ultracentrifuge. Samples were harvested using an Autodensiflow apparatus (Buchler Instruments, Fort Lee, N. J.), and maltase activity was determined.

Maltase was chosen because the electrophoretic mobility of sucrase was identical with the slowest moving protein in the antibody preparation, whereas maltase had a slower RF and could be readily detected in the presence of the antibody preparation. When anti-A antibody was added prior to electrophoresis (middle gel), the major maltase band could still be seen, although somewhat less intense than in the absence of any antibody. However, this may be related to the length of staining time for protein, since an identical unstained gel, sliced and assayed for enzyme activity, revealed 86% of initial activity migrating with the maltase band (middle graph, Fig. 4). When type-specific anti-A antibody was added (bottom gel), no maltase band was detected, and enzymatic analysis of an unstained gel revealed 21% of the initial enzyme activity migrating into the gel.

Most direct proof of the attachment of blood group reactivity with the disaccharidases themselves was obtained by covalently attaching anti-blood group antibody to an insoluble matrix, Sepharose 4-B. When blood group type B sucrase was tested, virtually all of the activity passed through a column containing anti-A antibody (Fig. 5), whereas almost all of the activity was retained by a column made with anti-B antibody. Similar specificity was found using a type A enzyme. Moreover, virtually all of the type B enzyme activity could be eluted from the column by 1 M galactose (Fig. 5). Furthermore, when rat sucrase was used, no enzyme activity was retained. This was not surprising, since blood group reactivity was not found in purified rat sucrase preparations.

Nature of Carbohydrate-Protein Linkage—To prove that the carbohydrate and protein portions of the intestinal disaccharidases were linked covalently, the purified enzymes were subjected to alkaline borohydride treatment, which releases carbohydrate chains linked to serine and threonine residues. Sodium [3H]borohydride was added in order to label the reducing end of the oligosaccharides released by the alkaline treatment. Fig. 6 shows Sephadex G-200 chromatography of a type B sucrase preparation treated with alkaline borohydride. The untreated enzyme (not shown) was eluted in the void volume. Protein from the treated enzyme was eluted as a broad peak, with an estimated molecular weight of 18,000 to 25,000, whereas the labelled carbohydrate was eluted later. The peak of radioactivity coincided with the peak of carbohydrate determined chemically and corresponded to a low molecular weight. Fractions 12 to 17 were pooled and rechromatographed on Sephadex G-50 (10) to determine molecular weight more precisely (Fig. 7). The chromatographic profile of radioactivity and total carbohydrate corresponded to a population of molecules heterogeneous in size. The peak of the released carbohydrate was used to estimate molecular weight, and corresponded to approximately 1,800 (inset, Fig. 7). This would correspond to an average of about 9 sugar residues per carbohydrate chain.

**DISCUSSION**

These experiments have demonstrated a high carbohydrate content of human intestinal disaccharidases, with fucose, galactose, and the hexosamines as the major constituents. Moreover, the purified enzymes manifested blood group reac-
Fig. 5. Antibody-Sepharose chromatography of human intestinal sucrase. Thirty micrograms of sucrase from type B patient R.W. were added to Sepharose antibody column (0.5 × 14 cm) prepared as described under "Experimental Procedure." Each fraction contained 0.5 ml, with a V₀ for the column of 2.5 ml. After 15 void volumes had passed through the column, enzyme activity was eluted with 1 M galactose in 50 mM potassium phosphate buffer, pH 6.0.

Fig. 6. Sephadex G-200 chromatography of alkaline borohydride-treated sucrase. Two hundred micrograms of purified sucrase from type B patient R.W. were treated with alkaline borohydride as described under "Experimental Procedure"; and then added to a Sephadex G-200 column prepared as described under "Experimental Procedure." Carbohydrate was analyzed by radioactivity and the phenol-sulfuric acid method (14).

Activity. The carbohydrate moiety is covalently linked with the protein, and although heterogeneous in size, has an average molecular weight of 1800. The finding of carbohydrate-containing membrane proteins is not surprising, but the carbohydrate content of 21 to 51% was unexpected. Moreover, the lack of sialic acid and the high fucose content were not typical for membrane proteins (21), especially since sialic acid has been reported in intestinal brush border saccharides (22). The carbohydrate composition of intestinal disaccharidases (fucose, mannose, galactose, and hexosamines) did not suggest contamination with mucus, since mucus contains no mannose, but has uronic acids (23) not found in the disaccharidases. In his extensive review of glycoproteins, Eylar found that the only proteins to have no sialic acid and high carbohydrate content (30%) were blood group substances (23). Intestinal disaccharidases now provide another such example.

Purified disaccharidases clearly seem to have blood group antigens associated with them, since the enzymes react with type-specific antibody as demonstrated by the Sepharose affinity chromatography, polyacrylamide gel electrophoresis, and sucrase density centrifugation experiments. However, their composition differs from that of purified blood group substances. Soluble blood group substances obtained from ovarian cyst fluid contain 74 to 93% carbohydrate, and threonine, serine, proline, and alanine comprise 37% of the amino acids (20). Disaccharidases contain 20 to 50% carbohydrate, and threonine, serine, proline, and alanine comprise only 25% of the total amino acid content (4). Purified blood group substances also contain very little mannose (11), consistently found in the disaccharidases. The finding of a glycoprotein with blood group antigenicity but somewhat different carbohydrate composition is not unique. Springer has found in **Escherichia coli** a blood group active glycoprotein that contains glucose and another in **sassafras albidum** that contains xylose, rhamnose, arabinose, galacturonic acid, and glucose (24).

The alkaline borohydride experiment suggests that there are multiple carbohydrate chains on each molecule, as in blood group substances (25). This conclusion is suggested by the size heterogeneity of the carbohydrate released by alkaline borohydride, and by the low average molecular weight of the oligosaccharides. Assuming a molecular weight of the purified maltase of 205,000 (4), a 25% carbohydrate content (Table I), and an average oligosaccharide molecular weight of 1800, there would be approximately 28 carbohydrate residues per enzyme molecule. With
approximately 170 serine and threonine residues per molecule (4), this chain number seems reasonable.

The role of the large amount of carbohydrate and especially the blood group reactivity is not certain. First, the carbohydrate may be needed for enzyme activity. Increased enzymatic instability has been reported after extensive periodate treatment of glycoamylase from Aspergillus niger (26). The configuration of the blood group antigen itself is probably not essential to the enzyme activity, since rat enzymes which lack blood group reactivity have the same enzymatic properties as the human enzymes (27). Another possible explanation for the role of the carbohydrate may be related to the position of the enzymes within the cell. Disaccharidases seem to be functionally placed in the membrane so that they face the external surface of the cell (2). Resistance to pancreatic proteases might possibly be conferred by the high carbohydrate content. This explanation would be consistent with the recent suggestion that sugars in protein structures code for the topographical location within a cell (28). Further experiments are needed to prove the merit of these hypotheses.

Acknowledgments—We would like to thank Mrs. Carol Goodwin for excellent technical assistance and Dr. Stuart Kornfeld for valuable advice.

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