Human Salivary Endo-β-N-acetylglucosaminidase HS Specific for Complex Type Sugar Chains of Glycoproteins*

(Received for publication, December 31, 1992, and in revised form, March 15, 1993)

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The enzyme that catalyzed the conversion of human salivary α-amylase family A (HSA-A) to family B (HSA-B) was identified. It was partially purified from the precipitate obtained by centrifugation of human saliva at 105,000 × g for 60 min by solubilization with S[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate and column chromatographies with Sepharose S-300-HR and hydroxylapatite. The enzyme preparation was practically free from contaminating exoglycosidases and proteases. The enzyme cleaved the N,N′-diacetylchitobiose moiety of the sugar chain of saliva at 106,000 g. The enzyme activity was repressed by N-acetylglucosaminidase and endo-β-N-acetylglucosaminidase. This enzyme was named endo-β-N-acetylglucosaminidase. The enzyme activity was reduced to 13 and 5% using HSA-A with the sugar chains whose outer chain moieties lacked Gal and GlcNAc, respectively, from the nonreducing end. The enzyme also acted on human transferrin and fetuin, and asparagine oligosaccharides of transferrin and fetuin. On the other hand, the enzyme did not act on ovalbumin, RNase B, Taka-amylase, yeast invertase, and ovalbumin asparagine oligosaccharides. These results indicate that human salivary endo-β-N-acetylglucosaminidase is specific for complex type sugar chains and can release the sugar chains from native glycoproteins and glycopeptides regardless of the existence of a Fuc residue on the proximal GlcNAc of the N,N′-diacetylchitobiose core of their sugar chains. The enzyme was named endo-β-N-acetylglucosaminidase. Thus, these studies indicate that the properties of the enzyme are distinct from those of known endo-β-N-acetylglucosaminidase and endo-β-N-acetylglucosaminidase. Human salivary α-amylase (HSA) is separated into multiple forms according to the difference of their isoelectric points (1, 2). These multiple forms are divided into two groups, family A (HSA-A) and family B (HSA-B) (1, 2). HSA-A has asparagine-linked biantennary complex-type sugar chains containing the X-antigen determinant and an α-Fuc on the proximal GlcNAc residue of the sugar chain (3). HSA-B does not have the sugar chain (2, 3). Recently we found the conversion of HSA-A to HSA-B by an enzyme action in human saliva. This enzyme seems to be an intrinsic membrane protein and an endoglycosidase-like enzyme (4).

This paper deals with the identification of the enzyme by analyzing the chemical composition of the reaction products using HSA-A and human transferrin glycopeptide as substrates. Also the specificity of the enzyme for sugar chains was studied by examining the susceptibility of glycoproteins and asparagine oligosaccharides with various types of asparagine-linked sugar chains to the enzyme.

MATERIALS AND METHODS

Enzyme Preparation

Step 1: Solubilization—Human saliva was collected from healthy donors (23–53 years of age) in our laboratory in the presence of NaN3, stored at −20 °C. The saliva (2750 ml) was thawed and centrifuged at 105,000 × g for 60 min. The precipitate (4 g, wet weight) was dissolved with 1% CHAPS in 10 mM sodium-potassium phosphate, pH 7.2. The supernatant was pooled. The precipitate was dissolved with 1% CHAPS in 10 mM sodium-potassium phosphate, pH 7.2. The supernatant was pooled. The procedure was performed until about 80% of the enzyme activity were solubilized.

Step 2: Sephacryl S-300-HR—The solubilized enzyme solution was concentrated to about 14 ml by an Advantec Toyoh ultrafiltration apparatus (Tokyo, Japan) using a UK-10 membrane. The concentrated enzyme solution was applied on a column of Sephacryl S-300-HR (2.2 (inner diameter) × 95.0 cm) that had been equilibrated with 1% CHAPS in 10 mM Tris-HCl, pH 7.2, 50 mM NaCl. The flow rate was 10 ml/h and 10-ml fractions were collected. The enzyme-containing fractions were pooled.

Step 3: Hydroxylapatite—The enzyme from step 2 was applied on a column of hydroxylapatite (1.2 (inner diameter) × 21.5 cm) which had been equilibrated with 1% CHAPS in 10 mM sodium-potassium phosphate, pH 6.0. The column was washed with this buffer until the absorbance at 280 nm of the effluent reached zero. The enzyme was eluted with a linear gradient of 10 mM to 0.2 M sodium-potassium phosphate.

1 The abbreviations used are: HSA, human salivary α-amylase; HSA-A, human salivary α-amylase family A; HSA-B, human salivary α-amylase family B; CHAPS, (3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonate; RCA, Ricinus communis agglutinin; BSL II, Bandeiraea simplicifolia lectin II; ConA, concanavalin A; Endo, endo-β-N-acetylglucosaminidase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline.

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phosphate, pH 6.1, with each reservoir containing 100 ml of each buffer solution. The flow rate was 20 ml/h, and 3-ml fractions were collected. The enzyme-containing fractions were pooled.

Recovery of the activity was 41.8% and the specific activity increased about 37-fold relative to the solubilized enzyme solution (Table I). The enzyme preparation was practically free from proteases and exoglycosidases because of the isolation of intact protein moiety and sugar chain from glycoproteins as described under "Results." The enzyme preparation of this step was used for all subsequent studies.

Preparation of Human Salivary α-Amylases

HSA-A and HSA-B were purified from human saliva as described previously (4).

Preparation of Human Transferrin Tetraacetylglucosaminidase

Human transferrin was reduced with dithiothreitol and carboxymethylated with monodiactide acid, then digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. Glycopeptide fraction was obtained by chromatographies with the columns of Sephadex G-25 (1.5 (inner diameter) x 118 cm) and Sephadex G-50 (2.0 (inner diameter) x 143 cm) equilibrated with 0.1 N acetic acid. The glycopeptides were further purified by HPLC with a column of Daisogel-SP-300-5-ODS (0.45 (inner diameter) x 25 cm). The corepeptid fractions were monitored by phenol-CH₃SO₄ method (12). The main glycopeptide was further digested with Staphylococcus aureus V₃ protease, then purified by HPLC with the column. The amino acid sequence and sugar composition of the glycopeptide were deduced to be Asn-Tyr-Asn(GlcNAc)₂(Man)₃(GlcNAc)%(Gal)-Lys by the analysis of amino acid and carbohydrate compositions and the proposed amino acid sequence (6) and sugar chain structure (7) of human transferrin.

Preparation of Asparagine Oligosaccharides

Human transferrin and calf fetuin asparagine oligosaccharides were prepared by exhaustive digestion with pronase. The protein solution (55 mg/ml) was added 20 mg of pronase, and the solution was incubated at 37 °C for 24 h with a few drops of toluene. The reaction mixture was centrifuged at 10,000 rpm for 20 min, and the supernatant was chromatographed with a column of Sephadex G-25 (4.5 (inner diameter) x 83.0 cm) equilibrated with water. Oligosaccharide fractions were monitored by phenol-CH₃SO₄ method (12). The oligosaccharide fractions were pooled and lyophilized. The lyophilized oligosaccharide was dissolved in 7 ml of 40 mM Tris-HCl, pH 7.5, containing 1 mM CaCl₂. Then 2.5 mg of pronase was added, and the solution was incubated at 37 °C for 48 h with addition of 5 mg of pronase after 24 h. The reaction mixture was centrifuged at 10,000 rpm for 20 min, and the supernatant was chromatographed with a column of Sephadex G-25 (1.5 (inner diameter) x 118 cm) equilibrated with 0.1 N acetic acid. The glycopeptide fractions were monitored by phenol-CH₃SO₄ method (12) and pooled. Only aspartic acid was detected as amino acid by amino acid analysis of the final oligosaccharide preparation. The ratio of asparagine and N-acetylglucosammin was deduced to be 1:4 for transferrin asparagine oligosaccharide and 1:5 for fetuin asparagine oligosaccharide.

Ovalbumin asparagine oligosaccharides, GP-I-V were prepared by the method of Huang et al. (48).

### Chemicals

Human transferrin, calf fetuin, and bovine pancreatic RNase B were purchased from Sigma. Asialo human transferrin and asialo calf fetuin were prepared by incubation with sialidase. Taga-amylase was from Sankyo Co., Ltd. (Tokyo, Japan). Ovalbumin was from Wako Pure Chemical Industries (Osaka, Japan). Sephararyl S-300-HR was from Pharmacia (Uppsala, Sweden). Toyopearl HW-50 (superfine) was obtained from Tosoh Co., Ltd. (Tokyo, Japan). Hydroxyapatite HP 40-50 was from Asahi Optical Co., Ltd. (Tokyo, Japan). Daisogel-SP-300-5-OBS was gifted from Dai-ichi Co., Ltd. (Osaka, Japan). Silica Gel 60 TLC plate was purchased from Merck. Nitrocellulose membrane was purchased from Advantec Toyoy Co., Ltd. (Tokyo, Japan). CHAPS was from Dojin Laboratories (Kumamoto, Japan). Sialidase from Arthrobacter ureafaciens (8) was purchased from Nakarai Tesque Co., Ltd. (Kyoto, Japan). α-L-Fucosidase from Charonia lampas (9), β-galactosidase from Streptococcus 8846K (10), β-N-acetylglucosaminidase H from Streptomyces pluto (23) were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Horseradish peroxidase-conjugated Ricinus communis agglutinin (RCAl) and concanavalin A (ConA) were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Biotinylated Bandeiraea simplicifolia lectin II (BSL II) and Vectastain ABC kit-4000 were purchased from Vector Laboratories, Inc. (Burlingame, CA). Anti-mouse IgG conjugated with horseradish peroxidase was purchased from Cappel Laboratories. Other chemicals were of reagent grade.

### Enzyme Assay

The enzyme was assayed by the same procedure as described previously (4). Purified HSA-A was used as a substrate. HSA-A (10 μl; 0.02%, w/v) dissolved in 0.2 M sodium phosphate buffer, pH 6.0, was mixed with 10 μl of the enzyme solution. The reaction mixture was incubated at 37 °C. The enzyme reaction was stopped by addition of 20 μl of a mixture of 2% SDS, 40% glycerol, 4% 2-mercaptoethanol, 0.004% bromphenol blue, and 10 mM Tris-HCl, pH 6.0. The reaction mixture (20 μl) was subjected to SDS-PAGE with 7.5% polyacrylamide gel according to the method of Laemmli (13). The amounts of the proteins in the gel were measured with a densitometer (Advantec Toyoy DMU-33C, Tokyo) after staining with 0.1% Coomassie Brilliant Blue R-250 (Fig. 1). One unit of the enzyme activity was defined as the enzyme amount that converted 1 μg of HSA-A per min under the conditions described above.

### Analytical Methods

Amino acid compositions of glycoproteins and peptides were analyzed with Hitachi 835 amino acid analyzer after hydrolysis at 110 °C for 24, 48, and 72 h with 6 N HCl in evacuated tubes. Amino sugars of glycoproteins or glycopeptides were determined with Hitachi 835 amino acid analyzer after hydrolysis at 100 °C for 6 h with 4 N HCl in evacuated tubes. Sugar compositions of glycoproteins or glycopeptides were determined with Dionex DX-300 sugar analysis system after hydrolysis at 100 °C for 6 h with 2.5 M trifluoroacetic acid in evacuated tubes. Neutral sugar was determined by phenol-CH₃SO₄ method (12).

### HPLC of Peptides

HPLC of peptides was performed with Hitachi L-6200 HPLC system with a column of Daisogel-SP-300-5-OBS (0.45 inner diameter) x 25 cm) equilibrated with 0.1% trifluoroacetic acid, 0.1% acetonitrile, and 0.1% acetic acid (v/v). The flow rate was 0.5 ml/min, and the eluted fraction was collected at 2.5 ml/min. The peptide fractions were monitored by phenol-CH₃SO₄ method (12). The main glycopeptide fraction was obtained by chromatographies with the columns of Sephadex G-50 (2.0 (inner diameter) x 25 cm), Sephadex G-50 (2.0 (inner diameter) x 118 cm), and Sephadex G-50 (2.0 (inner diameter) x 143 cm) equilibrated with 0.1 N acetic acid.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td>protein*</td>
<td>units/mg</td>
<td></td>
<td>-fold</td>
</tr>
<tr>
<td>105000 x 8 ppt.</td>
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<td>3800</td>
<td>ND</td>
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<td>1010</td>
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<td>88.4</td>
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<td>111</td>
<td>15.5</td>
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<td>4.65</td>
</tr>
<tr>
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<td>1590</td>
<td>12.8</td>
<td>124</td>
<td>41.8</td>
<td>37.2</td>
</tr>
</tbody>
</table>

* Protein determined by measuring the absorbance at 280 nm of protein solution assuming that absorbance at 280 nm of 1% of protein solutions was 10.

† Not determined.

### Human Salivary Endo-β-N-acetylglucosaminidase

Anti-HSA antiserum was obtained from mice as described previously (5).

### Enzyme Activity

The enzyme was assayed by the same procedure as described previously (4). Purified HSA-A was used as a substrate. HSA-A (10 μl; 0.02%, w/v) dissolved in 0.2 M sodium phosphate buffer, pH 6.0, was mixed with 10 μl of the enzyme solution. The reaction mixture was incubated at 37 °C. The enzyme reaction was stopped by addition of 20 μl of a mixture of 2% SDS, 40% glycerol, 4% 2-mercaptoethanol, 0.004% bromphenol blue, and 10 mM Tris-HCl, pH 6.0. The reaction mixture (20 μl) was subjected to SDS-PAGE with 7.5% polyacrylamide gel according to the method of Laemmli (13). The amounts of the proteins in the gel were measured with a densitometer (Advantec Toyoy DMU-33C, Tokyo) after staining with 0.1% Coomassie Brilliant Blue R-250 (Fig. 1). One unit of the enzyme activity was defined as the enzyme amount that converted 1 μg of HSA-A per min under the conditions described above.
Human Salivary Endo-β-N-acetylglucosaminidase

**RESULTS**

**Amino Acid and Carbohydrate Composition of HSA-A after the Enzyme Reaction—**Amino acid and carbohydrate composition of the enzyme reaction products was analyzed with HSA-A as a substrate. HSA-A was incubated with or without the enzyme. Fig. 2A shows that HSA-A was completely converted to HSA after 30 s of incubation at 37°C. The reaction mixture was subjected to SDS-PAGE as described under "Materials and Methods." Lane 1, HSA-A without the enzyme; 2, HSA-A with the enzyme; M, molecular weight markers. The reaction mixture was chromatographed with a column of Toyopearl HW-550 (superfine, 2.0 inner diameter) × 65.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.2, containing 5 mM NaCl at a flow rate of 0.5 ml/h at 4°C. The effluent from the sieve was centrifuged at 200 × g for 5 min, and the resultant pellet was resuspended in 9 ml of PBS.

**Homodetection of Epithelial Cells**

Epithelial cells obtained from human saliva with 500-mesh stainless sieve as described above were suspended in PBS (1 × 10⁶ cells/ml). The epithelial cells were homogenized with Teflon pestle in a Potter homogenizer at 1500 rpm at 4°C until most epithelial cells were disrupted.

**Fig. 1. Standard assay method of the enzyme.** The enzyme activity was measured with HSA-A as a substrate as described under "Materials and Methods." Inset shows the SDS-PAGE of the reaction product.

<table>
<thead>
<tr>
<th>Reaction time, h</th>
<th>Converted HSA-A, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>

**Fig. 2. Chromatography of HSA-A after the enzyme reaction.** HSA-A (10 mg) was incubated with or without the enzyme (5.2 units) in 5.0 ml of 0.1 M sodium-potassium phosphate, pH 6.0, containing 0.05% Tween 20 and 0.15 M NaCl at room temperature for 1 h and washed three times with the solution for 10 min. The membrane was incubated in a peroxidase-conjugated lectin or a biotinylated lectin (2 μg/ml in the Tween solution) at room temperature for 1 h with continuous shaking, then washed three times with 10 ml of the Tween solution. The membrane incubated with a biotinylated lectin was further stained with a Vectastain ABC kit-4000. Peroxidase reaction was performed by dipping the membrane in 0.0025% o-dianisidine, 0.1% H₂O₂, 10 mM Tris-HCl, pH 7.2, at room temperature until spots emerged.

**Immunodetection of HSA on Nitrocellulose Membrane**

HSA on nitrocellulose membrane was detected with anti-HSA antiserum as described previously (5).

**Fractionation of Cells in Human Saliva**

Human saliva was obtained from healthy donors (23–53 years of age). The oral cavity of each donor was thoroughly washed with 50 ml of phosphate-buffered saline (PBS) for 30 s. Collected saliva (40 ml) was centrifuged at 200 × g for 5 min. The precipitate was resuspended in 9 ml of PBS and the suspension was passed in sequence through 500-mesh stainless sieve. The sieve-retained cells were collected and suspended in 9 ml of PBS. The effluent from the sieve was centrifuged at 200 × g for 5 min, and the resultant pellet was resuspended in 9 ml of PBS.

**TLC of Oligosaccharides**

TLC of oligosaccharides was performed on Silica Gel 60 plates with the solvent system: propanol-1/acetic acid/water (3:3:2, v/v) followed by visualization of oligosaccharides by spraying orcinol-H₂SO₄ solution (14).

**Trimming of Sugar Chain of HSA-A**

Two hundred micrograms each of purified HSA-A were incubated with the following glycosidase(s) in 250 μl of 0.1 M sodium acetate, pH 5.5, containing 0.02% leupeptin, 0.02% antipain, 0.01% pepstatin at 37°C for 30 h; preparation I, no glycosidase; preparation II, a mixture of sialidase (0.02 unit) and α-fucosidase (0.02 unit); preparation III, a mixture of sialidase (0.02 unit), α-fucosidase (0.02 unit), and β-galactosidase (0.02 unit); preparation IV, a mixture of sialidase (0.02 unit), α-fucosidase, β-galactosidase (0.02 unit) and β-N-acetylhexosaminidase (0.2 unit).

**Dot-Lectin Analysis**

Dot-lectin analysis was performed as follows. Glycosidase-trimmed HSA-A (0.15 μg) prepared by the method described above was dotted on nitrocellulose membrane using microcapillary. The membrane was incubated in 10 mM sodium-potassium phosphate, pH 6.0, containing 0.05% Tween 20 and 0.15 M NaCl at room temperature for 1 h and washed three times with the solution for 10 min. The membrane was incubated in a peroxidase-conjugated lectin or a biotinylated lectin (2 μg/ml in the Tween solution) at room temperature for 1 h with continuous shaking, then washed three times with 10 ml of the Tween solution. The membrane incubated with a biotinylated lectin was further stained with a Vectastain ABC kit-4000. Peroxidase reaction was performed by dipping the membrane in 0.0025% o-dianisidine, 0.1% H₂O₂, 10 mM Tris-HCl, pH 7.2, at room temperature until spots emerged.

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**RESULTS**

**Amino Acid and Carbohydrate Composition of HSA-A after the Enzyme Reaction—**Amino acid and carbohydrate composition of the enzyme reaction products was analyzed with HSA-A as a substrate. HSA-A was incubated with or without the enzyme. Fig. 2A shows that HSA-A was completely con-
verted. The reaction mixture was chromatographed on a column of Toyopearl HW-50. Fig. 2C shows that two sugar chain fractions were liberated from the protein moiety of HSA-A after the enzyme reaction. It has been reported that the sugar chains of HSA-A were separated to sialo and asialo type (3). Sialo sugar chains are known to be eluted faster than asialo sugar chains on gel permeation chromatography. Fractions 32–37 in Fig. 2C were pooled and further analyzed as a sugar chain fraction because they were completely separated from the protein moiety. Fractions 20–24 were pooled as a protein fraction and further analyzed because they were completely separated from the liberated sugar chain fractions.

The amino acid composition of the protein fraction was the same as that of native HSA-A. Carbohydrate analysis of native HSA-A showed 4.05 mol of GlcN, 3.08 mol of Man, 2.34 mol of Gal, and 2.99 mol of Fuc per mole of HSA-A. Neuraminic acid was not determined. Thus, the carbohydrate composition of HSA-A was approximated to be GlcN:Man:Gal:Fuc = 4.32:3.18:2.72:1.41. On the other hand, the carbohydrate analysis of the protein fraction after the enzyme reaction showed 0.94 mol of GlcN and 0.84 mol of Fuc per mole of the protein moiety. Thus, the carbohydrate composition of HSA-A after the enzyme reaction was approximated to be 1 GlcNAc and 1 Fuc. The released sugar chain fraction showed the carbohydrate composition of GlcNAc:Man:Gal: Fuc = 3.00:2.60:2.00:1.30. These results indicate that the enzyme cleaved the sugar chain of HSA-A at the N,N'-diacetylchitobiose core to release the protein moiety having the proximal GlcNAc with its linked Fuc and the residual sugar chains.

The structure of the released sugar chain was estimated. The structure of the sugar chain has been determined by Yamashita et al. (3) and is a biantennary complex type. Microheterogeneity at the side chain increases the variety of the sugar chain (3). They have a common core structure, Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)-GlcNAc-Asn. The enzyme cleaved the citobiose as described above. Thus, the released sugar chain has the core structure of Manα1→6(Manα1→3)Manβ1→4GlcNAc. The side chain structure of it was estimated by sequential exoglycosidase digestion. Fig. 3 shows that the sugar chain fraction showed single spot on TLC and had a molecular size compatible with that of isomaltotrihexaose. The size of the sugar chain was not changed by sialidase digestion, indicating that no sialic acid was attached at the nonreducing end of the sugar chain.

The size of the sugar chain reduced by α-fucosidase digestion indicated the presence of α-fucose at the side chain. The size of the sugar chain became smaller by β-galactosidase digestion. Also the size of the sugar chain reduced by further β-N-acetylatedehexosaminidase digestion after β-galactosidase digestion indicated the order of the side chain of Gal-GlcNAc.

The data of the carbohydrate composition of the released sugar chain and the proposed structure of the sugar chain of HSA-A determined by Yamashita et al. (3) suggest the side chain structure of (Fuc)(Gal-GlcNAc)2. The linkage of the sugar chain structure and the position of the fucose residue on the side chain have been determined by Yamashita et al. (3). Thus, the structure of the released sugar chain was estimated as follows: Galβ1→4(Fucα1→3)GlcNAcβ1→2Manα1→6 or 3(Galβ1→4GlcNAcβ1→2Manα1→3 or 6)Manβ1→4GlcNAc.

**Amino Acid and Carbohydrate Composition of Human Transferrin Tetracyloecptide after the Enzyme Reaction**

The enzyme reaction products using transferrin tetracyloecptide as a substrate were also analyzed. The glycopeptide was incubated with or without the enzyme, then subjected to HPLC. Fig. 4 shows that the peptide was eluted faster from the column after the enzyme reaction. The fractions indicated as II and IV in Fig. 4 were pooled and further analyzed as the peptide fraction. The fractions indicated as I and III in Fig. 4 were pooled and further analyzed as the sugar chain fraction because sugar chains released from glycopeptides are not retained on an ODS column (15). The amino acid composition of the peptide fractions (II and IV) did not change after the enzyme reaction. The carbohydrate composition of the peptide fraction (GlcNAc:Man:Gal = 4.10:2.90:2.10) was changed to 0.95 mol of GlcNAc per mol of the peptide. The sugar chain fraction (III) showed carbohydrate composition of GlcNAc:Man:Gal = 3.10:3.00:2.20 and no amino acid. The released sugar chain from the glycopeptide had a molecular size compatible with that of isomaltotetraose (Fig. 4C). These results indicate that the enzyme cleaved the sugar chain of the tetracyloecptide at the N,N'-diacetylchitobiose core to release an equimolar amount of the tetrapeptide containing 1 GlcNAc and the residual sugar chain.

**Activity of the Enzyme for Sugar Chain-trimmed HSA-A**

We examined the specificity of the enzyme for the structure of the sugar chains using HSA-A with trimmed sugar chains as substrates. The sugar chain of HSA-A was sequentially trimmed from the nonreducing end by sequential exoglycosidase digestion as shown in Fig. 5A. Fig. 5B shows that molecular weight of HSA-As (I–IV), treated with different combinations of exoglycosidases, gradually decreased by depletion of their sugar moieties. The deletion of carbohydrate was further confirmed by detection of the exposure of certain sugar residues with specific lectins, as shown in Fig. 5C.

The lectins used were RCA120, a galactose-binding lectin (16), BSL II, an N-acetylglucosamine-binding lectin (17), and ConA, a mannoside-binding lectin (18). The appearance of the strong reactivity of preparation II with RCA120 indicated that the main sugar residue of the nonreducing end of the sugar chain of HSA-A was galactose. The disappearance of the reactivity of preparation III with RCA120 and the appearance of reactivity with BSL II indicated that the sugar residue of the non-
action with the enzyme. The rate of the conversion was to lower molecular weight proteins on SDS-PAGE after reusing glycoproteins with various types of asparagine-linked transferrin, human asialotransferrin, calf fetuin, and calf asialofetuin at a rate similar to that for HSA-A. On the other hand, the enzyme did not act on Taka-amylase, RNase B, invertase, and ovalbumin. The same results were obtained with asparagine oligosaccharides. The enzyme completely released oligosaccharides from asparagine oligosaccharides prepared from human transferrin and calf fetuin. On the other hand, the enzyme did not act on ovalbumin asparagine oligosaccharides (GP-I–V) that were hydrolyzed by endo-β-N-acetylglucosaminidase H from S. plicatus (data not shown).

Cells in Human Saliva and the Enzyme Activity—The enzyme exists in the insoluble fraction of human saliva (4). Epithelial cells, leukocytes, and microorganisms are observed.
Human Salivary Endo-β-N-acetylglucosaminidase

TABLE II
Relative activity of the enzyme for sugar chain-trimmed HSA-A

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sugar chain structure</th>
<th>Relative activity</th>
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<tbody>
<tr>
<td>I</td>
<td>(Fucα1-3Galβ1-4GlcNAcβ1-2Manα1-3)</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>(NeuAc2-6)Galβ1-4GlcNAcβ1-2Manα1-3</td>
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<tr>
<td>III</td>
<td>GlcNAcβ1-2Manα1-3</td>
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<tr>
<td>IV</td>
<td>Manβ1-4GlcNAcβ1-4GlcNAc</td>
<td>5.0</td>
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</table>

*The sugar chains of native HSA-A contain the same core portion, and the diversity is limited to the outer chain moiety (3). The diverse sugar moieties are shown in parentheses.

**Deduced from the proposed structure of the sugar chain of HSA-A by Yamashita et al. (3) and the result of dot-lectin analysis described in the text.

FIG. 6. Action of the enzyme of other glycoproteins. Glycoproteins (2 μg) were incubated with the enzyme (0.0053 unit) under the conditions of a standard assay method, as described under “Materials and Methods,” then subjected to SDS-PAGE. The rate of conversion of glycoproteins was measured as described under “Materials and Methods.” O, HSA-A; □, human transferrin; ■, asialo human transferrin; Δ, calf fetuin; ▲, asialo calf fetuin; ●, bovine RNase B, ovalbumin, Taka-amylase, yeast invertase.

in human saliva (49, 50). Fig. 7 shows that cells in human saliva were separated into two fractions with 500-mesh stainless sieve. The number of epithelial cells and leukocyte-like cells in the sieve-retained fraction and the sieve-passing fraction were counted. More than 95% of epithelial cells were retained on the sieve, and more than 96% of leukocyte-like cells passed through the sieve. Microorganisms were also thought to pass through the sieve because they were smaller than leukocyte-like cells, but the number of microorganisms was not counted. More than 95% of the enzyme activity were recovered in the sieve-retained fraction (epithelial cell fraction). The epithelial cells obtained by scratching the oral cavity epithelium with a spatula also showed the enzyme activity. These results strongly suggest that the origin of the enzyme is the epithelial cells peeling from the oral cavity epithelium into saliva.

The suspension and the homogenate of the epithelial cells showed similar enzyme activity (data not shown), indicating that the enzyme is located on the surface of the epithelial cells.

DISCUSSION

This enzyme was found as the enzyme that converted HSA-A to HSA-B in human saliva by depletion of sugar chains (4). Two types of enzymes, glycopeptidase (19-21) and endo-β-N-acetylglucosaminidases (22-26), are known to deplete sugar chains from glycoproteins or glycopeptides. Since this enzyme cleaved the sugar chains of HSA-A and transferrin tetraglycopeptide at the N,N’-diacetylchitobiose core to release residual sugar chains, this enzyme was identified as an endo-β-N-acetylglucosaminidase.

This enzyme can act on complex-type sugar chains regardless of the existence of sialic acid and fucose residues on the
FIG. 7. Cells in human saliva. Cells in human saliva were separated into two fractions with 500-mesh stainless sieve as described under "Materials and Methods." A, cells in human saliva; B, cells retained on 500-mesh stainless sieve; C, cells passed through 500-mesh stainless sieve; D, epithelial cells obtained by scratching human oral cavity epithelium with a spatula.

side chain moieties of the sugar chain as shown by the action on HSA-A. On the other hand, the enzyme was less active on the incomplete complex-type sugar chains because the activity of the enzyme for HSA-A decreased by sequential trimming of the sugar chain. This property was completely opposite to that of endo-\(\beta\)-N-acetylglucosaminidase obtained from rat liver (26) or fibroblast of fucosidosis patients (27). This enzyme also acted on human transferrin having the sugar chains of biantennary complex type (7) and calf fetuin having the sugar chains of triantennary complex type (7). On the other hand, this enzyme did not act on the glycoproteins, such as RNase B (28), Taka-amylose (29), and invertase (30), which had high mannose-type sugar chains, and ovalbumin (7, 31), having high mannose and hybrid-type sugar chains. Those results indicate that this endo-\(\beta\)-N-acetylglucosaminidase is specific for complex-type sugar chains of glycoproteins. This was confirmed by the experiment using glycopeptide as substrate. This enzyme acted on transferrin tetraglycopeptide having the sugar chain of complex type. The enzyme also acted on transferrin and fetuin asparagine oligosaccharides. On the other hand, the enzyme did not act on any ovalbumin asparagine oligosaccharides, GP-I and GP-II having hybrid type sugar chain (40), GP-III being the mixture of asparagine oligosaccharides having hybrid and high mannose-type sugar chains (31), and GP-IV and GP-V having high mannose-type sugar chain (33). These results indicate that this endo-\(\beta\)-N-acetylglucosaminidase is specific for complex type sugar chains and can directly release the sugar chains from glycoproteins as well as glycopeptides. This enzyme can be used for typing the sugar chain structures of glycoproteins. Also it can be used for investigating the functions of sugar chains of the complex type. We named this enzyme endo-\(\beta\)-N-acetylglucosaminidase (Endo) HS.

The specificity of Endo HS mentioned above is distinct from those of the microbial endo-\(\beta\)-N-acetylglucosaminidases, such as Endo D (23, 32-35), Endo H (23, 36-40), CI, CII (24, 31, 39), and Endo M (41, 42), and the animal endo-\(\beta\)-N-acetylglucosaminidases from hen oviduct (43), rat liver (26), and fibroblast of fucosidosis patients (27). Recently an Endo F preparation (21, 25) from Flavobacterium meningosepticum was resolved into three distinct activities designated Endo F1, Endo F2, and Endo F3 (51). They hydrolyze high mannose-type sugar chains, while only Endo F3 can hydrolyze preferentially both bi- and triantennary sugar chains (51, 52). Endo F3 is most similar to Endo HS in substrate specificity. Sequence studies of Endo H and Endo F1 that hydrolyze only high mannose- and hybrid-type sugar chains revealed a 32% structural identity over the entire sequence (53). Sequencing of Endo HS might be expected to reveal structural factors responsible for the specificity for complex type sugar chains. Studies are now in progress in our laboratory to clone and sequence the gene of Endo HS.

Endo HS acted on HSA-A having Fuc residue on GlcNAc residue proximal to asparagine, and the reaction rate was similar to that for human transferrin whose sugar chains did not have the Fuc residue. The Fuc residue on the GlcNAc residue proximal to asparagine is generally a hindrance to the action of endo-\(\beta\)-N-acetylglucosaminidases (21). It is another distinct property of Endo HS concerning specificity for glycans that Endo HS acts on complex-type sugar chains having Fuc residues on the proximal GlcNAc of \(N,N'\)-diacetylchitobiose core.
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The origin of Endo HS was thought to be epithelial cells peeling from epithelium into saliva; the enzyme is solubilized with detergents, not with EDTA or NaCl (4). The suspension of oral cavity epithelium showed similar Endo HS activity. It is likely that Endo HS is an intrinsic membrane protein and integrated on the surface of the cell membrane of the epithelial cells of the oral cavity epithelium. The microbial endo-β-N-acetylglucosaminidases are secreted into culture medium (22–25, 41). Animal enzymes so far reported are thought to be cells of the oral cavity epithelium. The microbial endo-p-N-acetylglucosaminidases are assumed to split the N-acetylglucosamine-asparagine linkage. However, these enzymes can not release GlcNAc linked to asparagine from protein moieties. The amidase that releases GlcNAc from protein moieties is assumed to be amidase-like enzyme in human saliva.

REFERENCES