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Generation of Carbohydrate-Deficient Transferrin by Enzymatic Deglycosylation of Human Transferrin

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ABSTRACT

Carbohydrate-deficient transferrin (CDT) molecules are transferrin isoforms that lack one or both of the carbohydrate groups attached to a normal human transferrin molecule. CDT has been reported to be a sensitive and specific marker for diagnosing alcoholism. This report demonstrates the in vitro generation of CDT molecules that can potentially be used as the standard in measuring CDT concentrations. This was achieved by deglycosylation of human transferrin with the enzyme Endo-β-*N*-acetylglucosaminidase F_2 (Endo-F_2). The enzyme was immobilized on sepharose beads, which were packed into a column. The immobilization of the enzyme not only eliminated the Endo-F_2 contamination of CDT, but also rendered the enzyme suitable for repetitive use. In this manner, it was possible to obtain at least 200 mg of CDT over a period of more than 3 mo, without any noticeable decrease of enzyme activity, using only 3.0 μg of enzyme. This proved to be an efficient method for generating CDT.

Index Entries: Carbohydrate-deficient transferrin (CDT); alcoholism; Endo-β-*N*-acetylglucosaminidase F_2; deglycosylation; immobilized enzyme.

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INTRODUCTION

Transferrin is the major iron transport glycoprotein of the blood with a mol wt of 79.5 kD. The protein portion consists of a single polypeptide chain with two homologous halves, each having an iron-binding site. The carbohydrate chains of the molecule are attached to asparagine (Asn) residues at the 413 and 611 positions in the C-terminal domain of the glycoprotein *(1)*. The carbohydrate moiety on transferrin belongs to glycans of the *N*-acetyllactosaminic type. The majority of the oligosaccharide chains on transferrin have the high-mannose-type biantennary structure *(2)*.

When saturated with iron, five diferric transferrin isoforms have been isolated from human serum, based on isoelectric focusing (pI 5.4, 5.6, 5.7, 5.8, and 5.9) *(3,4)*. The main isoform has a pI of 5.4, and has the complete carbohydrate moieties attached at amino-acid residues 413 (Asn) and 611 (Asn) on the transferrin peptide chain. This pI 5.4 isoform is called the normal transferrin (or intact transferrin). Isoforms of pI 5.7 (and higher) are found to lack one (or both) of the carbohydrate moieties *(5)*, and these isoforms are called carbohydrate-deficient transferrin (CDT). Evidence has shown that elevation of CDT in blood is directly related to alcoholism *(5–8)*. As a potential alcoholic marker, CDT has proven its superiority over all of the other currently known markers. It is widely believed that CDT is the most sensitive and specific marker for alcoholism *(5,9–13)*. CDT-related research has become a major area in alcoholic study. Easiness of access to a CDT standard is very beneficial to the CDT research community.

The most obvious method of obtaining a CDT standard is the purification of total alcoholic transferrin by immunochromatography (using antibodies directed against epitopes available on all transferrin isoforms), followed by isoelectric focusing (or ion-exchange chromatography), to separate the different isoforms of transferrin. Isoforms of pI 5.7 and/or higher are collected and used as CDT. This presumably straightforward approach is actually a very difficult and expensive route. It is by no means an easy task to obtain a large quantity of alcoholic blood sample(s). The further separation and purification procedures may not produce a high yield of CDT. Alternatively, CDT can also be cloned using recombinant DNA technology, or it can be generated enzymatically by using deglycosylation enzymes. In this report, an efficient method for generating CDT is described. This was carried out via deglycosylation of normal human transferrin, using enzyme Endo-β-*N*-acetylglucosaminidase F_2 (Endo-F_2). The enzyme was immobilized on sepharose beads, which were packed into a column. The immobilization of the enzyme not only prevented the Endo-F_2 contamination of CDT, but also rendered the enzyme suitable for repetitive use. In this manner, it was possible to obtain at least 200 mg of

CDT over a period of more than 3 mo, without any noticeable decrease of enzyme activity, using only 3.0 μg of enzyme. This method for generating CDT standard is especially helpful in developing an in vitro diagnostic method for alcoholic screening, using CDT as the biological marker.

MATERIALS AND METHODS

Endo-F_2 enzyme and Lectin Link carbohydrate assay kits were from Genzyme (Cambridge, MA). CNBr-activated sepharose 4B, human serum transferrin, and protease-free BSA (fraction V) were from Sigma (St. Louis, MO). Precast SDS-PAGE Ready Gels and protein standards were from Bio-Rad (Hercules, CA). SDS-PAGE was performed using a Bio-Rad Mini-Gel System with a Model 1000/500 power supply, according to the standard procedure. Western blotting was carried out using a Semi-Phor™ system from Hoefer Scientific Instruments (San Francisco, CA), according to procedures suggested by manufacturers.

Immobilization of Endo-F_2 Enzyme on CNBr-Activated Sepharose 4B Beads

Twenty mg of BSA was dissolved in 20.0 mL coupling buffer (0.1 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.3). 3.0 μg Endo-F_2 (6 milli-units) was added into the BSA solution and mixed well.

Three g freeze-dried CNBr-activated sepharose 4B beads were swelled in 30.0 mL of 1.0 m*M* HCl for 15 min. The gel was then filtered through a 0.8-μm filter. The beads in the filter cup were washed 4× with 1.0 m*M* HCl (200 mL each time), once with 100 mL DI water, and once with 100 mL coupling buffer. The washed beads were immediately transferred into 20.0 mL Endo-F_2/BSA/coupling buffer. The resulting mixture was incubated 2 h at room temperature and mixed on an end-over-end rotator. Ten mL ethanolamine (1.0 *M*, pH 8.3) was then added into the coupling mixture and incubated for 2 h at room temperature, to block any unreacted—OCN groups on the sepharose beads. The beads were then washed through a 0.8-μm filter, once with 200 mL coupling buffer, then twice with 200 mL NaAc buffer (0.1 *M* NaAc, pH 4.0, containing 0.5 *M* NaCl), followed by 200 mL coupling buffer. The washed beads were then resuspended in 20 mL coupling buffer and loaded into a 1.5 cm × 20 cm low-pressure column for future use.

Transferrin Deglycosylation by Endo-F_2 Enzyme

The Endo-F_2 column was washed extensively (5×, 15 mL each) with 10.0 m*M* NaAc buffer (pH 4.5, containing 100 m*M* NaCl), followed by an extensive wash with Endo-F_2 reaction buffer (0.2 *M* NaAc, pH 4.75), deliv-

ered by a peristaltic pump (200 mL buffer, at a rate of 2.0 mL/min). Then, 10.0 mL transferrin solution (1.0 mg transferrin/mL reaction buffer) was loaded on the column and circulated through the column overnight at room temperature, to saturate any nonspecific adsorption of transferrin onto the column. The solution in the column was eluted, and a fresh aliquot of 10.0 mL transferrin solution (1.0 mg/mL reaction buffer) was added to the column. The column was incubated at 37°C for 3 d with mixing on an end-over-end rotator. The Endo-F_2-treated transferrin was harvested. A fresh aliquot of normal transferrin solution was then added to the column, incubated as before, and harvested. This process was repeated continuously (no less than 30 ×). Each harvest was analyzed by SDS-PAGE for the completion of deglycosylation. Some of the harvests were analyzed by the lectin link blot to confirm the extent of deglycosylation.

RESULTS AND DISCUSSION

Endo-F_2 is a rare enzyme. Using a general enzymatic reaction condition of incubating free enzyme and transferrin together wastes the enzyme and contaminates the CDT with enzyme. The immobilization of the enzyme eliminates its potential contamination of CDT, allowing repetitive deglycosylation of multiple lots of transferrin.

Endo-F_2 enzyme (mol wt 39.5 kDa) hydrolyzes the di-N-acetylchitobiose linkage of asparagine-linked glycans, with a preference for the complex biantennary oligosaccharide. It catalyzes the deglycosylation of biantennary carbohydrate moieties of transferrin through the chemistry shown in Fig. 1.

The effectiveness of transferrin deglycosylation by Endo-F_2 enzyme is shown in Fig. 2A. Before deglycosylation, transferrin migrates as two bands (lanes 6 and 7), a major, higher-mol-wt band a, and a minor, lower-mol-wt band b. These two bands probably represent two transferrin isoforms. Lanes 1 and 2 contain a typical migration pattern of transferrin after deglycosylation with Endo-F_2 enzyme (on column) for 3 d at 37°C. The four bands are most likely transferrin proteins in various states of deglycosylation: band a represents intact transferrin (same as band a in lanes 6 and 7). Bands c and d are from the deglycosylation of transferrin in band a, and most likely contain transferrin molecules missing one and both of the two carbohydrate moieties, respectively. This is further supported by the apparently equal migration distance between bands a, c, and d, which appear to correspond to transferrin proteins having the mol wt of 79,570, 77,562, and 75,554 Da, respectively *(14)*. This becomes more evident in lane 5, which contains the migration pattern of transferrin after deglycosylation with Endo-F_2 enzyme for 7 d at 37°C. In lane 5, band a almost completely disappeared; the intensity of band c became fainter and band d became

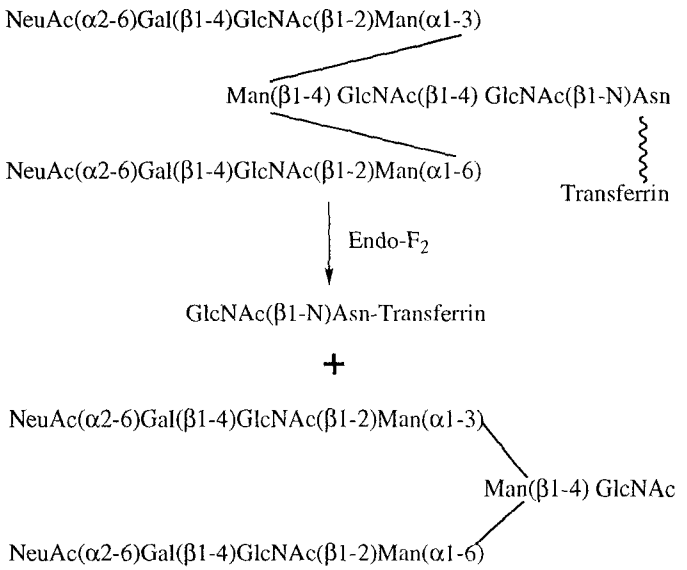

Fig. 1. Enzymatic deglycosylation of transferrin by Endo-F_2 enzyme.

stronger, compared to the corresponding bands in lanes 1 and 2. Band e is probably the deglycosylated form of transferrin in band b of lanes 6 and 7. The very faint appearance of band a in lanes 1 and 2 reveals that the majority of transferrin has been successfully deglycosylated (*see* discussion below on Fig. 3).

In order to demonstrate that bands a–d are indeed unique bands corresponding to different forms of transferrin, a mixture of deglycosylated and intact transferrin was electrophoresed in the same lane. Lanes 3 and 4 of Fig. 2B show the migration pattern of mixed sample. Bands a–c are well distinguished on this gel. Bands b and d are not well separated here. However, the lectin blotting described below distinguishes these two bands.

The degree of deglycosylation of transferrin by Endo-F_2 was further analyzed by a Western-type lectin blotting. After SDS-PAGE, the proteins in the gel were transferred to a sheet of nitrocellulose membrane. After fixing and blocking, a biotinylated lectin, which binds to the sialic acid residues of the carbohydrate moieties of transferrin, was added to the nitrocellulose membrane. After washing, alkaline-phosphatase-labeled avidin was added to the nitrocellulose paper. The alkaline phosphatase–avidin, specifically linked to the carbohydrate groups via the biotinylated lectin, was visualized by the development of substrates NBT/BCIP (NBT: nitro-blue tetrazolium; BCIT: 5-bromo-4-chloro-3′-Indolyphosphate *p*-toluidine) and the appearance of dark brown bands. The band intensity should be proportional to the amount of carbohydrate-containing transferrin.

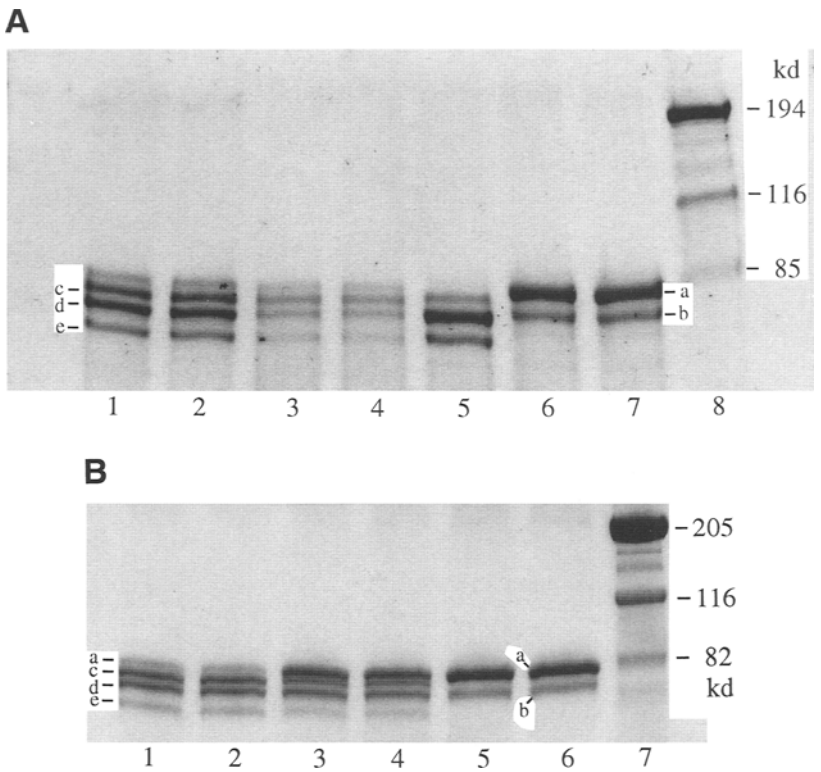

Fig. 2. **(A)** SDS-PAGE gel of different forms of transferrin. Lanes 1 and 2: transferrin after Endo-F_2 (immobilized) deglycosylation for 3 d at 37°C, each lane contained 15 μL of 0.2 mg/mL protein. Lanes 3 and 4: similar to lanes 1 and 2, but loaded with 15 μL of 0.07 mg/mL of deglycosylated transferrin in each lane. Lane 5: transferrin after Endo-F_2 (immobilized) deglycosylation for 7 d at 37°C, loaded with 15 μL of 0.2 mg/mL protein. Lanes 6 and 7: normal transferrin, 15 μL of 0.2mg/mL protein loaded in each lane. Lane 8: protein standards. All proteins were reduced with DTT. **(B)** SDS-PAGE of mixed sample of intact and deglycosylated transferrin. Lanes 1 and 2: transferrin after Endo-F_2 (immobilized) deglycosylation for 3 d at 37°C, each lane contained 10 μL of 0.2 mg/mL protein. Lanes 3 and 4: mixed sample, each lane was loaded with 10 μL of 0.2 mg/mL of a mixture of intact and deglycosylated transferrin (0.1 mg/mL each). Lanes 5 and 6, intact (normal) transferrin, 10 μL of 0.2mg/mL protein in each lane. Lane 7: protein standards. All proteins were reduced with DTT.

Figure 3 shows the result of the Western-type lectin blotting of various transferrin forms. The gel used for the Western blotting was electrophoresed, together with the gel shown in Fig. 2A. The experimental conditions for the two gels were exactly the same, including the lane allocations. As with the staining method, two bands appeared in lanes 6 and 7, a major upper band and a minor lower band. Both of these transferrin bands have either one or both carbohydrate moieties, and, therefore, they can be visualized in lectin blotting. Lanes 1 and 2 contain enzyme-digested

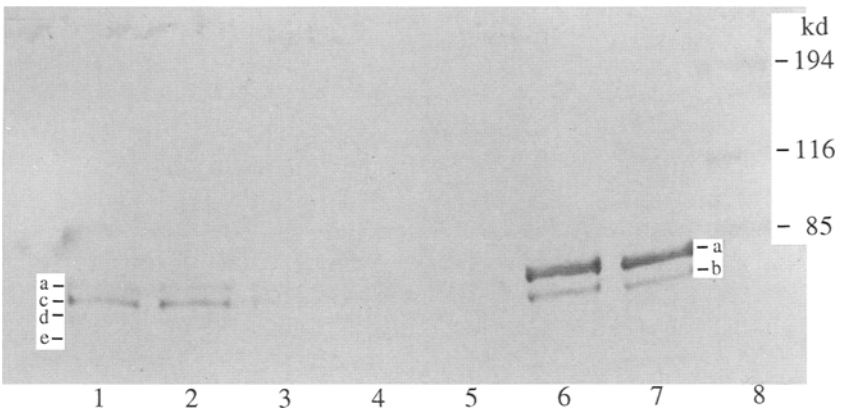

Fig. 3. Western-type lectin blotting from SDS-PAGE gel of different forms of transferrin. Lane allocations, protein loading, and all other experimental conditions are the same as that of Fig. 2A.

transferrin. In the lectin blot, only two of the corresponding four bands seen in the stained gel are visualized a and c. The very faint upper band (a) in lanes 1 and 2 probably corresponds to intact transferrin possessing two carbohydrate moieties. The lower band c in lanes 1 and 2, which is also faint (but much stronger than band a), is most likely a result of transferrin missing one of the two carbohydrate moieties. The fact that bands d and e, which are strong in Fig. 2A, failed to develop in lectin blotting indicates that transferrin molecules in these two bands have both carbohydrate moieties removed. This also suggests that bands b and d are different, since band b appeared both in the regular staining and in the lectin blotting assay, but the much stronger band d, obtained via regular staining (especially in the case of lane 5, where longer deglycosylation was conducted), failed to develop in the lectin-blotting assay. The almost complete disappearance of band a in lanes 1 and 2 of both Figures 2A and 3 indicates that the deglycosylation is close to completion.

It is noteworthy that the Endo-F_2 catalyzed deglycosylation is temperature-dependent. At 4°C, the enzyme is essentially inactive, and no deglycosylation was evidenced by SDS-PAGE and lectin blotting (data not shown). Endo-F_2 enzyme immobilized on sepharose beads retained its enzymatic activity for at least 3 mo at 37°C. Transferrin harvested from Endo-F_2–sepharose beads, which had been stored at 37°C for 3 mo, demonstrated the same staining pattern as that in Figures 2A and 3 (data not shown).

In conclusion, a method for the efficient removal of carbohydrate groups from glycoproteins has been developed. The method is demonstrated by the deglycosylation of transferrin with the enzyme Endo-F_2 immobilized on sepharose beads. As a result, the immobilized enzyme can

be used repeatedly at 37°C, and the generated CDT is free of Endo-F_2 contamination. This method may also provide a means to perform efficient sugar-mapping studies of glycoproteins. Enzymes that possess known specificity toward certain carbohydrate sequences can be immobilized and packed into different columns to establish an enzyme library for multiple uses. The sequential incubation of a carbohydrate protein with selected columns from this library would theoretically yield saccharides and glycoproteins with different identifiable terminal carbohydrate units.

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