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SEPARATION OF THE HUMAN TRANSFERRIN ISOFORMS BY CARRIER-FREE HIGH-PERFORMANCE ZONE ELECTROPHORESIS AND ISOELECTRIC FOCUSING

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SUMMARY

Human transferrin isoforms, *i.e.*, molecules with different carbohydrate contents which differ from each other by only one negative charge, were resolved by high-performance zone electrophoresis in free solution. The di-, tri-, tetra-, penta-, hexa- and heptasialo transferrins could be assigned in the electrophoretic pattern. The pattern changed when iron-free transferrin was treated with neuraminidase, which splits off the sialic acid from the carbohydrate chains. The final digest contained transferrin molecules without sialic acids, as was confirmed by isoelectric focusing.

INTRODUCTION

Human serum transferrin is a mixture of molecules (isoforms) with different carbohydrate contents[1–3] and these isoforms can be separated by isoelectric focusing[4,5]. The di-, tri-, tetra-, penta- and hexasialo transferrins can be assigned in the focusing pattern of transferrin preparations from normal subjects[5,6] and components containing seven or one or zero sialic acids can be seen in some diseases or in pregnancy[6]. The common electrophoretic technique using polyacrylamide gel has failed to resolve the transferrin isoforms. However, in the presence of 6 *M* urea, the ion-free transferrin, two types of mono- and diferric–transferrin complexes (the molecular forms of transferrin) were resolved and identified[7,8]. In previous studies of transferrin we employed high-performance isoelectric focusing[5]. In this investigation we complemented the latter method with high-performance zone electrophoresis in carrier-free solution.

EXPERIMENTAL

Transferrin

Human serum transferrin was purchased from Behring Werke (Marburg, F.R.G.) and was used without further purification. A solution containing 50 μg/μl of

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protein [prepared in 6 m*M* Tris–6 m*M* boric acid–0.1 m*M* EDTA (pH 8.4)] was used for electrophoretic analysis. For the neuraminidase treatment, a 5-mg amount of transferrin was dissolved in 20 μl of sodium acetate buffer (50 m*M*, pH 5).

Neuraminidase treatment

Neuraminidase (Type X, from *Clostridium perfringens*, Sigma, St. Louis, MO, U.S.A.), dissolved in 50 m*M* sodium acetate buffer (pH 5.0) (7 μl, 0.028 units), was added to the transferrin solution and incubated at 37°C[9]. Aliquots (1 μl) were taken at various times and diluted with the electrophoresis buffer (7 μl) for electrophoretic analysis or with 2% Bio-Lyte 5/7 solution (Bio-Rad Labs., Richmond, CA, U.S.A.) (200 μl) for isoelectric focusing.

High-performance electrophoresis and isoelectric focusing

Glass capillaries of 0.1 mm I.D. were used for the electrophoretic analysis of the samples. The tubes were pretreated as described previously[5] to eliminate electro-

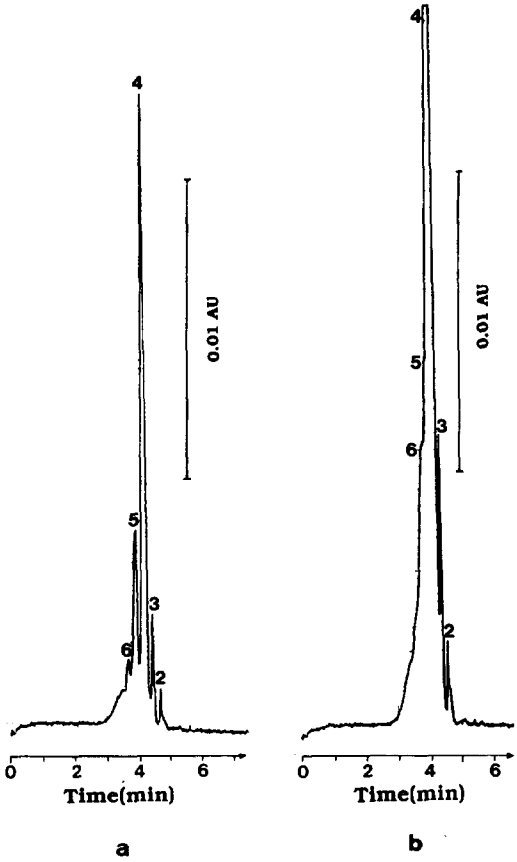

Fig. 1. High-performance electrophoresis of human serum iron-free transferrin in free solution. Experimental conditions: electrophoresis buffer, 18 m*M* Tris–18 m*M* boric acid–0.3 m*M* EDTA (pH 8.4); tube dimensions, 200 mm × 0.1 mm I.D. × 0.3 mm O.D.; voltage, 8000 V; current, 10 μA; on-tube detection at 280 nm. The transferrin isoforms (di-, tri-, tetra-, penta- and hexasialo, marked 2, 3, 4, 5 and 6, respectively) are better resolved when the starting zone of the sample is short: (a) 0.5 mm; (b) 1.5 mm.

endosmosis. The length of the tubes was varied between 185 and 200 mm. A modified Spectroflow 783 instrument (Kratos, Ramsey, NJ, U.S.A.) was used for on-tube detection of the migrating protein zones. The detection was performed at a distance of 25–30 mm from the opposite end of the capillary to that where the sample was applied.

The samples to be subjected to electrophoresis were applied to the tube in the following way. The capillary was dipped into the electrophoresis buffer [18 m*M* Tris–18 m*M* boric acid–0.3 m*M* EDTA (pH 8.4)], which rose in the tube by capillary force. When the buffer had risen to within 0.5–1.5 mm of the opposite end of the tube, the capillary was transferred to the sample solution. The length of the starting zone was thus 0.5–1.5 mm. The isoelectric focusing experiments were performed as described earlier[5]. All experiments were repeated 2–5 times to control the reproducibility.

RESULTS

Electrophoretic studies of transferrin in capillaries in the presence of urea[10] were preceded by experiments using the same buffer as above but without urea. The electrophoresis of iron-free transferrin in this buffer provided, surprisingly, several well resolved components (Fig. 1a). At least five components could be identified in the pattern. When this experiment was repeated with a longer starting zone (1.5 mm), the components were not resolved so clearly (Fig. 1b).

We assumed that this separation of various components of iron-free transferrin was due to charge differences in the carbohydrate moiety (sialic acid content) of the transferrin molecules[3,5]. To test this hypothesis, we used neuraminidase to split off the sialic acid(s) from the molecule as described under Experimental and analysed the final products by electrophoresis and isoelectric focusing. The electrophoretic analyses of samples taken from the enzymatic digestion mixture showed that the removal of the sialic acids changes the mobilities and the proportions of the different components in the sample with time (Fig. 2). After 20 h the sample contained one main component and some very minor components (Fig. 2).

Isoelectric focusing of the original iron-free transferrin sample and the digestion product is shown in Fig. 3. The prefocusing pattern[5] of the undigested transferrin (Fig. 3a) shows the components (di-, tri-, tetra-, penta- and hexasialo transferrins) that are normally present[1,5] together with some impurities (see ref. 5) with lower isoelectric points than the tetrasialo transferrin (the order of migration of the components in the prefocusing and mobilization steps is opposite). After digestion for 20 h the product contained one main component and some minor components with lower isoelectric points (Fig. 3b). The isoelectric focusing pattern of a mixture of the undigested and digested transferrin samples showed that the main component of the digested sample had the highest isoelectric point (Fig. 3c).

DISCUSSION

Conventional polyacrylamide gel electrophoretic methods (without or with urea) were not able to resolve the transferrin isoforms[11]. In this work we studied the possibility of resolving these isoforms in iron-free transferrin preparations by high-performance electrophoresis in free solution.

A low ionic strength (low conductivity) Tris–borate–EDTA buffer was used for

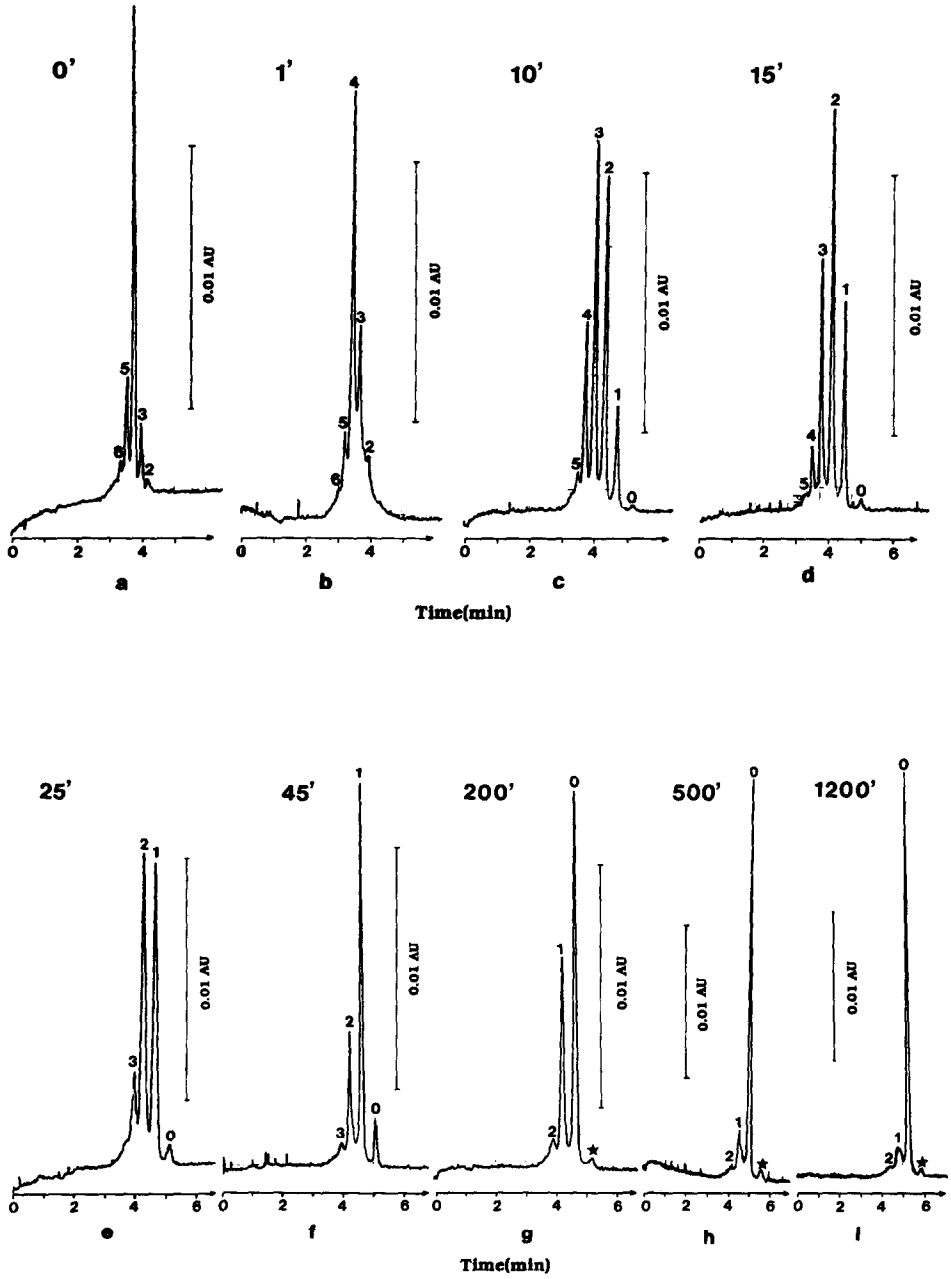

Fig. 2. High-performance electrophoresis of iron-free transferrin following incubation with neuraminidase (see Experimental). Experimental conditions as in Fig. 1, except for tube length (185 mm) and current (12 μA). The length of the starting zone was 0.5 mm. The samples for electrophoresis were taken after various incubation times: (a) 0; (b) 1; (c) 10; (d) 15; (e) 25; (f) 45; (g) 200; (h) 500; (i) 1200 min. The proportions of the transferrin isoforms (asialo, mono-, di-, trisialo, etc., marked 0, 1, 2, 3, etc.) changed with time. The sample taken after 20 h still contained transferrin molecules having one and two sialic acids. The small peak (labelled with an asterisk) appeared after 50–80 min but did not increase in size on prolonged incubation time (g–i).

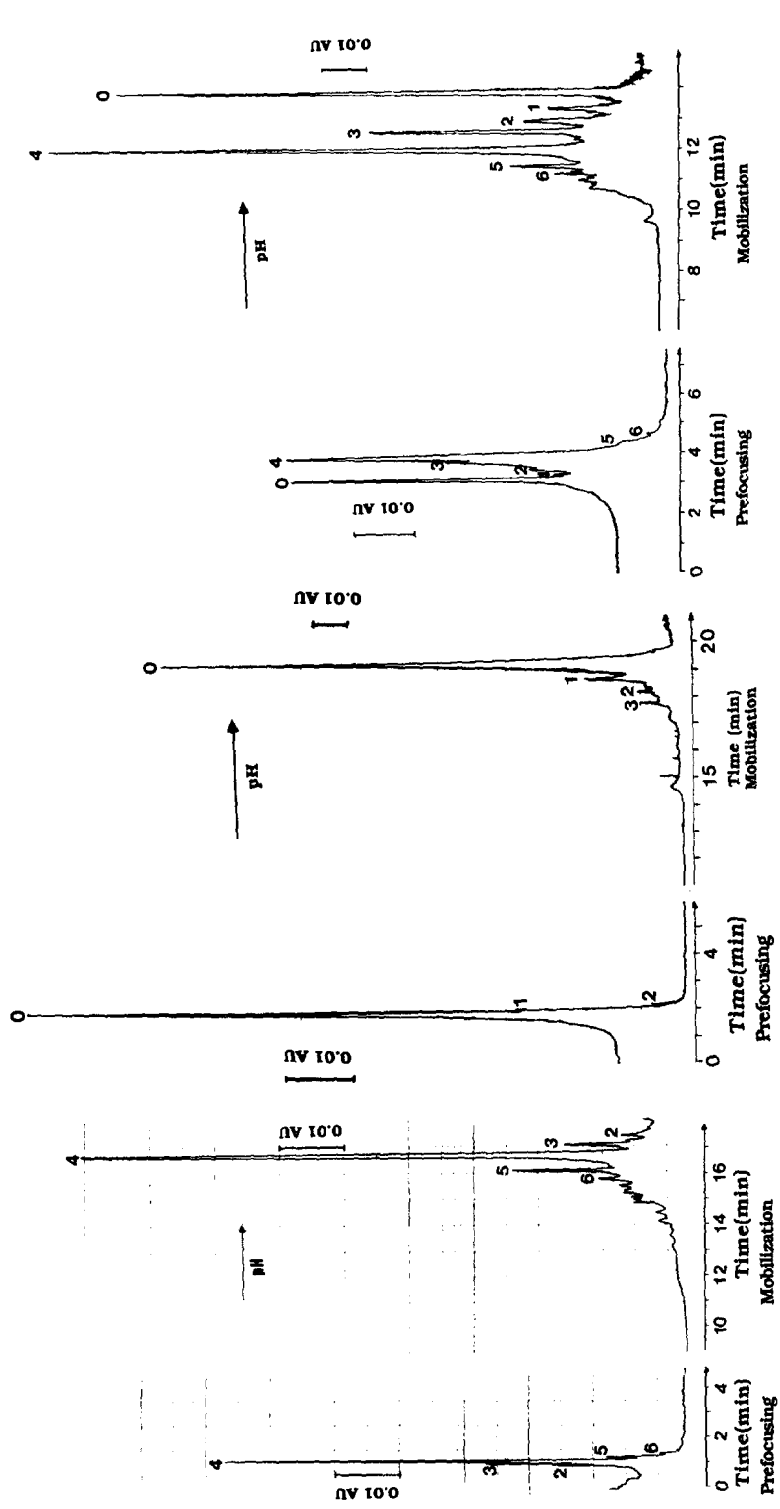

Fig. 3. High-performance isoelectric focusing of iron-free transferrin (a) before and (b) after incubation (20 h) with neuraminidase. Isoelectric focusing of a mixture of these two samples (1:1) shows that neuraminidase treatment of the transferrin results in asialo-transferrin (c). Experimental conditions: tube dimensions, 185 mm × 0.1 mm I.D. × 0.3 mm O.D.; protein concentration, 1 μg/μl; carrier ampholytes, 2% Bio-lyte 5/7; voltage, (a) 5000 V, (b, c) 6000 V; on-tube detection at 280 nm; anolyte, 20 m*M* phosphoric acid (prefocusing) or 20 m*M* sodium hydroxide (anodic mobilization); catholyte, 20 m*M* sodium hydroxide. The peaks assigned to the asialo and mono-, di-, tri-, tetra-, penta- and hexa-sialo transferrin components are marked 0, 1, 2, 3, 4, 5 and 6, respectively.

zone electrophoresis in order to shorten the time of analysis (conventional polyacrylamide gel electrophoresis in the presence of urea takes about 15–20 h). The observation that iron-free transferrin could be resolved into several peaks in free-solution (Fig. 1) led us to investigate the nature of these components. It appeared that they corresponded to isoforms of transferrin.

It has been shown previously that the main component of a transferrin sample (tetrasialo transferrin) undergoes a four-step decrease in mobility on treatment with neuraminidase[1,2]. As the sialic acids were split from the transferrin molecules we could follow the stepwise decrease in the mobilities of the products (see Fig. 2). Samples taken from the digestion mixture at different times contained transferrin isoforms in altered proportions. The electrophoretic pattern of the final product (Fig. 2i) differed completely from that of the starting material and showed only one main component, the asialo-transferrin. It is known that the desialation is not complete even if the enzyme concentration or the incubation time is increased[3], which explains the presence of mono- and disialo transferrins in the digest (Fig. 2h and i). The asialo-transferrin form appeared after digestion for only a few minutes, but its amount increased dramatically after 50–80 min. The proportions of the tri-, di- and monosialo transferrins changed via maxima, whereas the other components decreased monotonically with time. A minor component appeared after digestion for 50–80 min (labelled with an asterisk in Fig. 2g–i) but did not increase very much in amount on prolonged digestion and may be some impurity or unspecific digestion product. Another possibility is that the transferrin preparation (which was produced commercially from blood) contains two genotypes in different amounts. As the frequencies of the C1 and C2 alleles in a European population are 90% and 10%, respectively[1,3], and they have different isoelectric points[4], this may also explain our pattern.

In zone electrophoresis it is important to keep the starting zone as narrow as possible. This is clearly documented in Fig. 1, where the elctrophoretic pattern obtained with a longer starting zone shows lower resolution. In these experiments (Fig. 1) we also used a zone-sharpening effect, as the samples were dissolved in diluted buffer. However, narrow starting zones were even more important in the experiments shown in Fig. 2 (0.5 mm was used), because in these runs the neuraminidase-treated samples were diluted 1:8 with the electrophoresis buffer, *i.e.*, the conductivity of the starting zone was almost unchanged. The protein concentrations in the samples were lower than in Fig. 1.

The isoelectric focusing experiments show that the enzymatic cleavage resulted in transferrin (asialo-transferrin) with a higher isoelectric point than the common transferrin isoforms in a native preparation. The prefocusing patterns of the native (Fig. 3a) and neuraminidase-treated (Fig. 3b) transferrin provided similar information about the composition of the samples as did the mobilization patterns, and the components were clearly identifiable and their relative positions in the pH gradient were also detectable.

High-performance electrophoresis provided the possibility of detecting components with small differences in charge (as small as one unit) and allowed very accurate monitoring of the enzymatic cleavage of the sialic acid from transferrin.

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