Biochimica et Biophysica Acta, 661 (1981) 274–279
Elsevier/North-Holland Biomedical Press

BBA 69367

THE RELEASE OF CARBOHYDRATE MOIETIES FROM HUMAN FIBRINOGEN BY ALMOND GLYCOPEPTIDASE WITHOUT ALTERATION IN FIBRINOGEN CLOTTABILITY

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(Received March 17th, 1981)

Key words: Carbohydrate release; Fibrinogen; Glycopeptidase; Clottability

The possible noninvolvement of the carbohydrate moiety of human fibrinogen in the clotting mechanism was examined by eliminating the neutral sugar chains from desialylated fibrinogen by almond glycopeptidase digestion. 40% of the total neutral sugars was removed from the desialylated fibrinogen. The neutral sugars from both the β- and γ-polypeptide chains were released equally. The protein moiety of the glycopeptidase-digested fibrinogen was found to be intact. No significant change was observed in the thrombin time (fibrinogen clottability) of the resultant fibrinogen. The results suggest that the carbohydrate moiety of fibrinogen is not involved in the clotting mechanism. Oligosaccharide was detected in the glycopeptidase digest of desialylated fibrinogen by thin-layer chromatography (TLC), and was found to be identical with those released quantitatively from the peptic digests of β- and γ-polypeptide chains. The structure of the sugar chain was identified tentatively as Gal_2-$GlcNAc_2$-Man_3-$GlcNAc_2$, by sequential exoglycosidase digestion and quantitative analysis of carbohydrate components.

Introduction

Human fibrinogen, a glycoprotein with a molecular weight of 340 000, consists of two identical halves with each half molecule consisting of α-, β- and γ-polypeptide chains. Each β- and γ-polypeptide chain contains a single asparagine-linked oligosaccharide unit located at Asn-364 in β [1], and at Asn-52 in γ [2]. The oligosaccharide units consist of sialic acid, mannose, galactose and glucosamine [3]; however their complete structure is not yet known.

We intended to examine the role of the carbohydrate moiety of fibrinogen in fibrin clot formation. Laki and Mester [4] reported that there was a 50% loss of clottability by removing the carbohydrate moiety of fibrinogen by oxidizing with periodate. The result was not acceptable, however, because it was not proven that no oxidation occurred on the amino acid residues of fibrinogen [5]. Even a very minor alteration of the fibrinogen polypeptide moiety causes prolongation of thrombin time [6]. To overcome this problem we wanted to cleave off the carbohydrate moiety without changing the protein moiety by digestion using almond glycopeptidase, which cleaves specifically β-aspartylglycosylamine linkages in glycopeptides [7–9].

This paper together with our previous papers on the applications of almond glycopeptidase to structural studies of glycopeptides [10,11], illustrates the usefulness of this enzyme in glycoprotein research.

Materials and Methods

Fibrinogen and its derivatives. Human fibrinogen (Grade L) from AB Kabi, Sweden, was used without further purification. β- and γ-polypeptide chains of human fibrinogen were purified by the slightly modified method of Murano et al. [12]. The reduced and

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carboxymethylated fibrinogen derivatives were applied on a column of CM-cellulose, equilibrated with 25 mM sodium acetate/8 M urea (pH 4.9). Each β- and γ-chain was eluted separately by a linear gradient formed by 25 mM sodium acetate/8 M urea (pH 4.9) and 175 mM sodium acetate/8 M urea (pH 5.15). Glycopeptidase-digested β- and γ-polypeptide chains were prepared from glycopeptidase-digested fibrinogen using the same procedure. Each fraction obtained gave a single band by a polyacrylamide gel electrophoresis in the presence of SDS.

Enzymes. Human thrombin from the Midori-Juji Co. was purified by column chromatography on SP-Sephadex as described previously [13]. The purified thrombin gave a single band using SDS-polyacrylamide gel electrophoresis. Almond glycopeptidase was prepared from almond emulsin (β-glucosidase) from Sigma Chemical Co., as described previously [9]. The final enzyme preparation was free from activities of α-mannosidase, β-*N*-acetylglucosaminidase, β-galactosidase and proteases. Neuraminidase (*Clostridium perfringens*) and pepsin were purchased from Sigma Chemical Co.; β-*N*-acetylhexosaminidase (*Turbo cornutus*) and β-galactosidase (*Charonia lampas*) from Seikagaku Kogyo Co.

Other chemicals. *p*-Nitrophenyl derivatives of α-mannose, β-galactose and β-*N*-acetylglucosamine were purchased from Nakarai Chemicals, and sialic acid from Seikagaku Kogyo Co. Glucose oligomers used as a standard for TLC were prepared by acid hydrolysis of Dextran [14].

Desialylation of fibrinogen. Intact fibrinogen was desialylated by digesting fibrinogen (50 mg) with 50 μl neuraminidase (50 munit) in 450 μl 10 mM citrate buffer/10 mM $CaCl_2$ (pH 6.0) at 37°C for 1 h. More than 98% of the total sialic acid present were released by the neuraminidase digestion.

Almond glycopeptidase digestion of desialylated fibrinogen. The above mixture of desialylated fibrinogen was digested with 150 μl almond glycopeptidase (12 munit) at 37°C for 4 h (reaction mixture G). The reaction mixture G was used for the following: Sephadex G-25 column chromatography, analysis of carbohydrate content of β- and γ-polypeptide chains, and measurement of thrombin time.

Measurement of thrombin time. Thrombin time was determined at 37°C in triplicate as follows: 100 μl thrombin were added to 200 μl fibrinogen containing 28 mM barbital buffer (pH 7.35) [15]. The clotting time was measured on a Fibrometer (Baltimore Biological Lab.).

Gel permeation chromatography. Bio-Gel P-4 (100–200 mesh) column chromatography was performed with a column (0.5 × 90 cm) equipped with a water jacket. The column was kept at 55°C by circulating warm water in the jacket during this procedure.

Thin-layer chromatography. Thin-layer chromatography was performed on silica gel 60 plates (Merck Art. 5553). The following solvent systems were used: *n*-propanol/acetic acid/water (3 : 3 : 2, v/v) for oligosaccharides [16], and isopropanol/acetone/0.1 M lactic acid (2 : 2 : 1, v/v) for monosaccharides [17]. The color-producing reagents on silica gel plates were orcinol-H_2SO_4 reagent for oligosaccharides [16], and diphenylamine reagent for monosaccharides [17]. The intensity of each color developed was determined on a Shimazu TLC Scanner CS-900.

Analytical procedures. For desalting, the oligosaccharide mixture was successively passed through column 1 ml each of Amberlite IR-120 (H^+) and Amberlite IRA-400 ($CO_3^=$). Activities of the exoglycosidases were determined by using the corresponding *p*-nitrophenyl glycosides as described by Muramatsu and Egami [18]. After elimination of protein by heating at 80°C for 10 min, protease activity was assayed by measuring digested protein by the method of Lowry et al. [19]. In addition, protease activity was detected by polyacrylamide gel electrophoresis in the presence of SDS according to the method of Weber and Osborn [20]. Sialic acid was determined by the thiobarbituric acid method [21]. Total neutral sugar was determined by orcinol-H_2SO_4 reaction [22]. Analyses of sugar components were performed after hydrolysis of oligosaccharides with 2.5 M trifluoroacetic acid at 100°C for 6 h [23]. Total protein was determined by the method of Lowry et al. [19].

Results

Hydrolysis of desialylated fibrinogen with Almond glycopeptidase

The oligosaccharides in reaction mixture G (see Materials and Methods), which is glycopeptidase digest of desialylated fibrinogen, were separated by chromatography on Sephadex G-25 (Fig. 1). The first peak reflects the amount of carbohydrate left

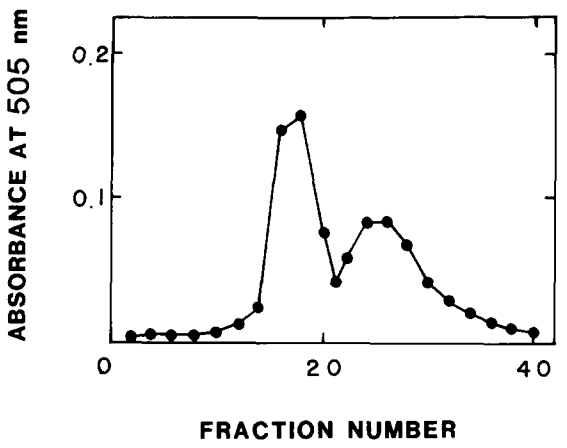


Fig. 1. Sephadex G-25 column chromatography of the glycopeptidase digest of desialylated fibrinogen. Reaction mixture G, described in Materials and Methods, was applied on a column (0.5 X 96 cm) of Sephadex G-25, fine, equilibrated with saline and eluted with the same solution at a flow rate of 4 ml/h. 0.5-ml fractions were collected. Carbohydrate concentrations were determined by the orcinol-H_2SO_4 reaction.

in desialylated fibrinogen, and the second reflects the amount of oligosaccharides (40% of the parent carbohydrate) released from desialylated fibrinogen.

When glycopeptidase-digested β- and γ-polypeptide chains were prepared from the reaction mixture G, the amount of neutral sugar released was found to be 38 and 39%, respectively.

Oligosaccharide profile

The oligosaccharides released from desialylated fibrinogen were analyzed according to the following procedure. The fraction (second peak in Fig. 1) was collected, heated at 60°C for 10 min and centrifuged. The supernatant was subjected to a plate of TLC. Fig. 2-1 shows a typical oligosaccharide profile. A single oligosaccharide detected had a molecular size compatible with that of isomaltododecaose. When fibrinogen, previously digested by pepsin and desialylated, was digested with almond glycopeptidase, 98% of the oligosaccharide was released. Fig. 3 shows the chromatogram on Bio-Gel P-4. The oligosaccharide fraction (main peak in Fig. 3) was further desalted and analyzed by TLC. The oligosaccharide from pepsin-digested and desialylated fibrinogen is shown in Fig. 2-2. The oligosaccharide of each β- and γ-polypeptide chain was also quantitatively released by digestion with almond glycopeptidase. when each polypeptide had previously been digested by pepsin and desialylated. The oligosaccharides from β- and γ-polypeptides were identical to that in reaction mixture G of desialylated fibrinogen (Fig. 2-3 and 2-4).

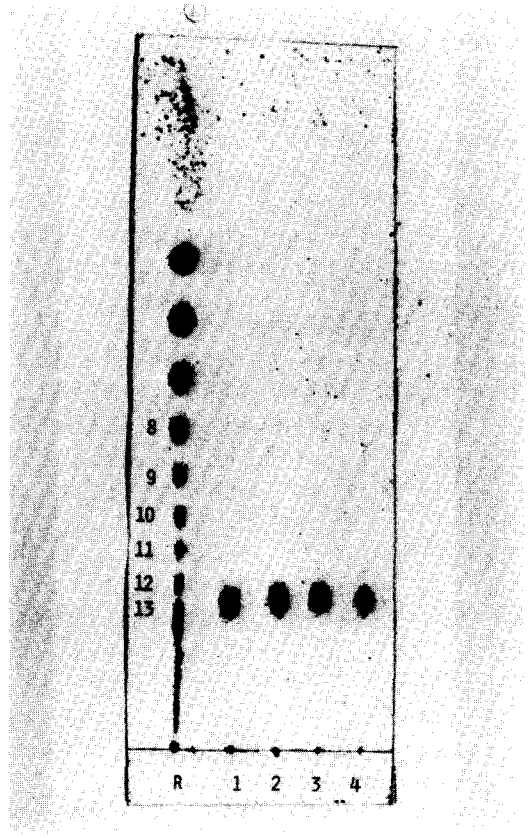


Fig. 2. Thin-layer chromatogram of the oligosaccharides released by almond glycopeptidase digestion. Each 2-μl sample was applied to a silica gel plate. The plate was developed at 25°C for 17 h. Oligosaccharides were visualized with orcinol-H_2SO_4 reagent. R, mixture of glucose oligomers as a reference; 1, from desialylated fibrinogen; 2, from pepsin-digested and desialylated fibrinogen (main peak in Fig. 3); 3, from pepsin-digested and desialylated β-polypeptide chain; 4, from pepsin-digested and desialylated γ-polypeptide chain; vertical numbers indicate glucose units.

Apoprotein properties

Although the fibrinogen clottability could possibly be reduced by contamination of proteases, if any, in

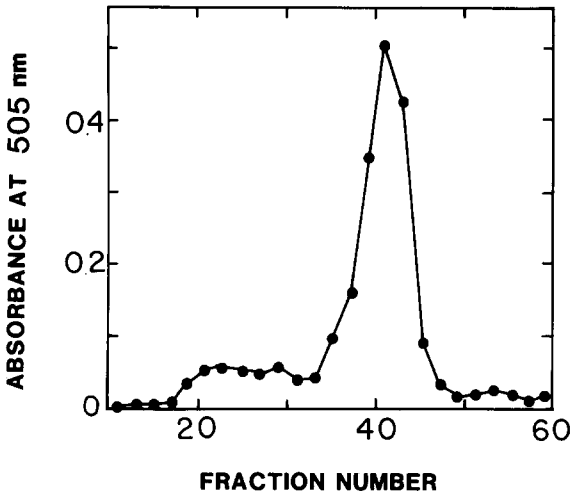


Fig. 3. Bio-Gel P-4 column chromatography of the glycopeptidase digest of pepsin-digested and desialylated fibrinogen. Fibrinogen (10 mg) was dissolved in 1 ml 10 mM HCl and incubated with pepsin (0.1 mg) at 37°C for 20 h. The peptic digest was then desialylated by mild acid hydrolysis, pH 2, at 90°C for 1 h. The incubation mixture was evaporated to dryness. The residue was dissolved in 25 μl 0.1 M citrate-phosphate buffer (pH 5.0), and incubated with 10 μl glycopeptidase (approx. 0.8 munit) at 37°C for 20 h. The mixture was heated to terminate reaction and applied on a column of Bio-Gel. The column was eluted with water. Chromatographic conditions are described in Materials and Methods. Oligosaccharides were determined by the orcinol-H_2SO_4 reaction.

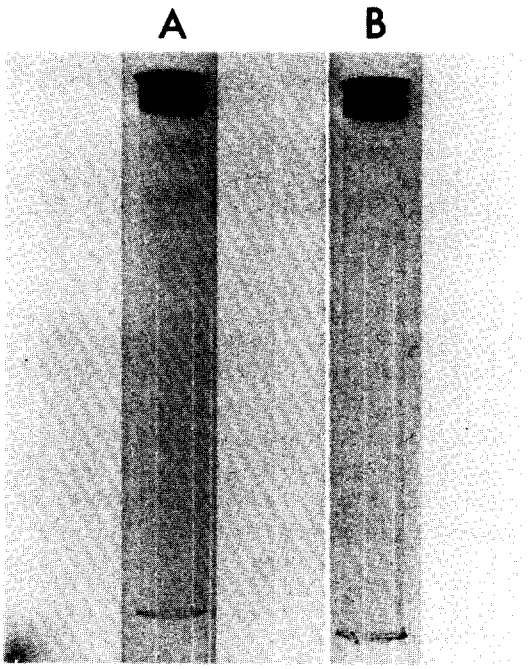


Fig. 4. SDS-polyacrylamide gel electrophoresis of desialylated fibrinogen before and after glycopeptidase digestion. The conditions are described in Materials and Methods. Approx. 10 μg protein was subjected to 10% SDS polyacrylamide gels. Migration is from top to bottom. A, desialylated fibrinogen before glycopeptidase digestion; B, desialylated fibrinogen after glycopeptidase digestion.

the glycopeptidase preparation, no protease activity was detected at all in our enzyme preparation. Furthermore, no fragmentation of glycopeptidase-digested fibrinogen was noted by a SDS-polyacrylamide gel electrophoresis (Fig. 4).

Thrombin time of fibrinogen before and after glycopeptidase digestion

More than 98% of the fibrinogen was desialylated. 40% of the neutral sugar was released from desialylated fibrinogen by digestion with glycopeptidase. Thrombin clotting times were determined on three different preparations; intact fibrinogen, desialylated fibrinogen, and glycopeptidase-digested fibrinogen (reaction mixture G), with a constant concentration of each fibrinogen at different thrombin concentrations and at varied concentrations of each fibrinogen (the thrombin concentration remaining constant) (Fig. 5). The thrombin time was inversely propor-

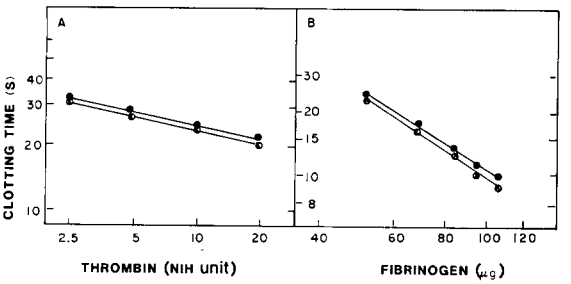


Fig. 5. Thrombin time of fibrinogen before and after glycopeptidase digestion. desialylated fibrinogen and glycopeptidase-digested fibrinogen (reaction mixture G) were prepared as described in Materials and Methods. Control fibrinogen was treated in the same manner. Thrombin time of each diluent was measured as described in Materials and Methods. (A) fibrinogen, 50 μg; thrombin, 2.5–20 NIH units, (B) fibrinogen, 50–100 μg; thrombin, 10 NIH units. •———•, intact fibrinogen; o———o, desialylated fibrinogen; X———X, glycopeptidase-digested fibrinogen.

tional to the fibrinogen concentration in these conditions. No significant difference was observed between desialylated fibrinogen and glycopeptidase-digested fibrinogen. The removal of 98% sialic acid from fibrinogen caused a slight shortening in clotting time, however, the carbohydrate moiety of fibrinogen did not significantly contribute to formation of fibrin clot (see Discussion).

Structure of oligosaccharides

The oligosaccharides prepared from pepsin-digested and desialylated fibrinogen were isolated by TLC, extracted with water, and hydrolyzed by trifluoroacetic acid. The resulting monosaccharide mixture was analyzed by another TLC process. The oligosaccharide that derived from pepsin-digested and desialylated fibrinogen (oligosaccharide I in Table I) and corresponded to isomaltododecaose, was composed of the following sugars: Gal/Man/GlcNAc. Its average molar ratio was 2 : 3 : 4. The structure was determined by sequential exoglycosidase digestion. Galactose was removed from oligosaccharide I by the reaction of β-galactosidase (oligosaccharide II in Table I). No sugar was released by incubation with β-*N*-acetylglucosaminidase. 2 mol *N*-acetylglucosamine were liberated from oligosaccharide II by incubation with β-*N*-acetylglucosaminidase (oligosaccharide III in Table I). The structure of the oligosaccharide of desialylated fibrinogen was tentatively identified as $Gal_2GlcNAc_2Man_3GlcNAc_2$.

TABLE I

MOLAR RATIO OF COMPONENTS OF FIBRINOGEN OLIGOSACCHARIDE AND ITS STEPWISE DEGRADATION PRODUCTS OBTAINED BY EXOGLYCOSIDASE DIGESTION

Oligosaccharide I (Fig. 2--2) was isolated from the glycopptidase digest of pepsin-digested and desialyated fibrinogen by the combination of Bio-Gel P-4 gel filtration, desalting, and TLC as descirbed in Materials and Methods. Oligosaccharide II was obtained by digestion of oligosaccharide I with 0.5 unit β-galactosidase in 30 μl 0.1 M citrate-phosphate buffer (pH 4.0) at 37°C for 20 h. The resulting oligosaccharide II was purified again by TLC. Oligosaccharide III was obtained by digestion of oligosaccharide II with 0.5 unit β-*N*-acetylhexosaminidase, in 30 μl 0.1 M citrate-phosphate buffer (pH 4.0) at 37°C for 20 h. The resulting oligosaccharide III was also purified by TLC. Monosaccharide compositions of each oligosaccharide, extracted from the plate with water, were analyzed as described in Materials and Methods. The values represent the average of five separate experiments.

Oligosaccharides	Man	Gal	GlcNAc
Oligosaccharide I	3.0	2.0	4.0
Oligosaccharide II	3.0	0	4.0
Oligosaccharide III	3.0	0	2.0

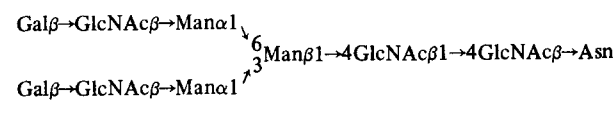
Discussion

We provide evidence that carbohydrate moieties of fibrinogen, both sialic acids and neutral sugars, may not be directly involved in the clotting mechanism. We set the experimental conditions to determine thrombin time such that there was a linear relationship between thrombin time and the amount of functioning fibrinogen (Fig. 5). With regard to neutral sugar in fibrinogen, equal amounts of the oligosaccharides were released from β- and γ-polypeptide chains by glycopeptidase digestion and both sugar chains had the same constituents. If the carbohydrate moiety of fibrinogen played a role in the formation of fibrin clot, 40% removal of the carbohydrate moiety should be proportional to the decrease of functioning fibrinogen, since the structures of the sugar chains were identical, and should cause 40% delay in thrombin time. Our results, however, indicate that thrombin time is not affected by the presence or absence of the carbohydrate moieties of fibrinogen (both sialic acids and neutral sugars). Chandrasekhar et al. [24] reported accelerated clotting time in desialylated fibrinogen obtained by using a crude neuraminidase preparation. Others reported no difference in clotting time after desialylation with different neuraminidase preparations [25]. The removal of 98% sialic acid from fibrinogen caused a slight shortening in clotting time (Fig. 5), which was probably caused by contaminants in the neuraminidase preparation. The carbohydrate moieties of fibrinogen may be crucial in its function, but certainly not in blood clotting.

Desialylated fibrinogen was used as a substrate of almond glycopeptidase because the enzyme does not act on sialylated glycoprotein [9]. The enzyme hydrolyzes glycoprotein more slowly than glycopeptide digested by pepsin, as shown previously in stem bromelain and ovalbumin [9]. The situation in

analogous in the case of fibrinogen. A maximum of 40% of carbohydrate was released from desialylated fibrinogen when incubated with a sufficient quantity of glycopeptidase. This amount is small in comparison to the 98% release of carbohydrates from pepsin-digested and desialylated fibrinogen. An incubation time of 4 h was used because the rates of hydrolysis of the desialylated fibrinogen by the glycopeptidase were slow especially beyond 4–5 h [9].

We decided the structure of the sugar chain to be $Gal_2GlcNAc_2Man_3GlcNAc_2$. It was reported in the previous papers that complex-type sugar chain of a glycoprotein had a common core structure, Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAcβ→Asn, and two to four outer chains composed of sialic acid, galactose and *N*-acetylglucosamine which are attached to two nonreducing terminals of α-mannosyl residues [26,27]. The outer chain is most often Sia-Gal-GlcNAc as reported in bovine IgG [28] and human α_1-acid glycoprotein [29]. Human fibrinogen oligosaccharide, therefore, might also have the general structure as follows:



We have demonstrated that almond glycopeptidase is useful for the analyses of asparagine-linked oligosaccharides; high-mannose-type and hybrid-type oligosaccharides [11] and an unique oligosaccharide of plant origin [10]. This report expands the application of this enzyme in the study of complex-type oligosaccharides. Furthermore, glycopeptidase has been shown to be useful, not only in structural studies, but also in functional studies of glycoproteins.

Acknowledgments

This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan, and by the Naito Foundation Research Grant for 1980. We are greatly indebted to Dr. Ryo Tanaka, Nagoya City University, for generous support and helpful discussions. We also wish to express our gratitude to Dr. Takashi Muramatsu, Kagoshima University, and Dr. James M. Watanabe for encouraging discussions and valuable suggestions regarding the manuscript. We wish to thank Mrs. K. Yamamoto and E. Takeuchi for their technical assistance.

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