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Characterization of isoelectric subspecies of asialo-β_2-glycoprotein I

Anni GRIES,* Johannes NIMPF, Helmut WURM, Gerhard M. KOSTNER and Thomas KENNER
Institute of Physiology, University of Graz, Harrachgasse 21/V, A-8010 Graz, Austria

Isoelectric focusing of purified β_2-glycoprotein I (β_2-G-I) revealed five major bands with isoelectric points (pI) between 5.1 and 6.1. Neuraminidase treatment decreased the number of bands to two (pI 8.0 and 8.2). The two asialo subfractions of β_2-G-I were purified by cation-exchange column chromatography. The more basic isoform II was found to have a higher content of lysine. Western-blot analysis of different plasma samples confirmed the heterogeneity of β_2-G-I in plasma. Plasma treated with neuraminidase showed two bands irrespective of the number of isoforms as well as of the concentration in native plasma. This led us to the conclusion that human plasma β_2-G-I consists of two isoproteins that are sialylated to different extents.

INTRODUCTION

β_2-Glycoprotein I (β_2-G-I) was first described in 1961 as an $HClO_4$-soluble human plasma protein [1]. Its normal concentration is in the range 15–30 mg/dl. The M_r is about 54000. It has a carbohydrate content of about 17%.

Although the physiological function of this serum component is so far unknown, recent findings that β_2-G-I binds to platelets [2,3] and to negatively charged phospholipids [4,5], thereby decreasing the prothrombinase sites for binding of Factor Xa, Factor Va, Ca^{2+} or prothrombin to platelets [6], point to an involvement of β_2-G-I in the pathway of blood coagulation.

Previously it was reported [7,8] that the amount of β_2-G-I in plasma is genetically determined such that concentrations are controlled by a pair of autosomal codominant alleles, Bg^N and Bg^D. Individuals homozygous for Bg^N were found to have β_2-G-I concentrations between 16 and 30 mg/dl of plasma, heterozygous ones ($Bg^N Bg^D$) between 6 and 14 mg/dl. Analytical isoelectric focusing of purified β_2-G-I on polyacrylamide gels yielded several bands, which were variously thought to be independent of the amount of sialic acid [9] or to be due to different contents of sialic acid in the different subfractions [10].

In order to make a more detailed investigation of these problems, two subfractions resulting from neuraminidase treatment of β_2-G-I were isolated and characterized, particularly with regard to the amino acid composition.

Furthermore we attempted to demonstrate the isoelectric heterogeneity of β_2-G-I in whole plasma before and after treatment with neuraminidase by immunoblotting of different plasma samples.

MATERIALS AND METHODS

Isolation of β_2-G-I and its subfractions

β_2-G-I was purified from citrated plasma by treatment with 1.4% (v/v) $HClO_4$ followed by affinity chromatography on heparin–Sepharose as reported previously [5].

For separating single isoforms of native β_2-G-I the f.p.l.c.-chromatofocusing technique was utilized. Glycoprotein samples were applied to a Mono P HR 5/20 column (Pharmacia, Uppsala, Sweden) that had been equilibrated previously with 25 mM-imidazole/HCl buffer, pH 7.4. Elution was carried out with Polybuffer 74 (Pharmacia); 1 vol. of Polybuffer was diluted with 7 vol. of distilled water, and the pH was adjusted to 5.0 by addition of 1 M-HCl.

Polybuffer was separated from β_2-G-I by hydrophobic interaction chromatography on phenyl-Sepharose [11].

Neuraminidase treatment of β-G-I and serum samples

Portions (0.5 mg) of β_2-G-I or isolated subfractions dissolved in 1 ml of 0.05 M-ammonium acetate buffer, pH 5.0, were mixed with 0.01 unit of *Clostridium perfringens* neuraminidase (Sigma, Deisenhofen, Germany) and incubated for 12 h at 37 °C. In order to remove free sialic acid the samples were dialysed overnight against distilled water.

Samples (1 ml) of plasma of apparently healthy volunteers (β_2-G-I ranged between 6 and 35 mg/dl) were incubated with 0.1 unit of neuraminidase and treated as described above.

Isolation of subfractions of neuraminidase-treated β_2-G-I

Freeze-dried neuraminidase-treated β_2-G-I was dissolved in 0.05 M-sodium acetate buffer, pH 5.6 (2.5 mg/ml), and 200 μl of this mixture was applied to a Mono S cation-exchanger (Pharmacia) that had been equilibrated with the same buffer. Elution of the material was performed with a linear gradient of 0–0.8 M-NaCl in 0.05 M-sodium acetate buffer, pH 5.6. The separated fractions were dialysed overnight against distilled water and freeze-dried.

Amino acid analyses

Samples were hydrolysed in 6 M-HCl in sealed ampoules at 110 °C and analysed on a Biotronic LC 7000

Abbreviation used: β_2-G-I, β_2-glycoprotein I.

* To whom correspondence should be addressed.

amino acid analyser. Amino acid losses due to hydrolysis were corrected by extrapolation from 24 h, 48 h and 72 h hydrolysates. The cysteine + cystine and methionine contents were determined as cysteic acid and methionine sulphone respectively after performic acid oxidation [12].

Sialic acid analyses

Sialic acid was determined by the thiobarbituric acid assay method of Warren [13]. Samples (100–500 μg) of protein were hydrolysed in 0.05 M-HCl at 80 °C for 1.5 h before assay, with *N*-acetylneuraminic acid (type IV; Sigma) as standard.

Electrophoretic methods

The amount of β_2-G-I in serum samples was determined by Laurell electrophoresis [14].

Isoelectric-focusing experiments [15] were carried out on polyacrylamide slab gels with Ampholines, pH 3.5–10, 5–7 and 7–9 (LKB, Bromma, Sweden). Samples (50–80 μg) of purified protein or 15 μl of plasma (containing 5 μg of β_2-G-I/ml) were applied to the gel; 0.01 M-ethanolamine (cathodic solution) and 0.01 M-glutamic acid (anodic solution) were used, and gels were run at 400 V for 5 h. After electrophoresis proteins were either stained with 0.015% Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) in 3.5% (v/v) $HClO_4$ or transferred to nitrocellulose. The pH gradient in the gel was measured by cutting an unfixed part of the gel into $\frac{1}{2}$ cm pieces after focusing. Each piece was eluted with 2 ml of distilled water and the pH of these extracts was measured 5 h later.

Immunoblotting

Proteins separated by isoelectric focusing were transferred to nitrocellulose films (TM-NC 4 ROLL; Bio-Trade, Vienna, Austria) according to the method of Towbin *et al.* [16] with the LKB transblot cell. Transfer was done at 150 V and 0.4 A for 1.5 h with cooling at 4 °C. Afterwards antigens were identified with a double-antibody technique involving monospecific antiserum against β_2-G-I prepared in our own laboratory as described previously [17] and IgG coupled to horseradish peroxidase (Bio-Trade) with 4-chloro-1-naphthol (Sigma) as substrate [18].

Tryptic peptide mapping

Tryptic peptide mapping was performed by the method of Cleveland *et al.* [19]. Briefly, isolated isoforms of asialo-β_2-G-I were dissolved in 0.1 M-Tris/HCl buffer, pH 8.2. Proteolytic digestions were carried out with trypsin from bovine pancreas attached to cross-linked beaded agarose (Sigma) for 2 h at 37 °C (1 unit/50 μg of protein).

Then 2-mercaptoethanol and SDS were added to final concentrations of 10% (v/v) and 2% (w/v) respectively and proteolysis was stopped by boiling the samples for 2 min. Tryptic peptides were separated on a SDS/15–18% polyacrylamide gradient gel and stained with $AgNO_3$.

RESULTS AND DISCUSSION

Total β_2-G-I, purified from pooled plasma samples, yielded five major isoforms when separated by isoelectric focusing. Their isoelectric points are 5.1, 5.4, 5.6, 5.8 and 6.1. Neuraminidase treatment of this fraction decreased the number of isoelectric species to two. Thereby the

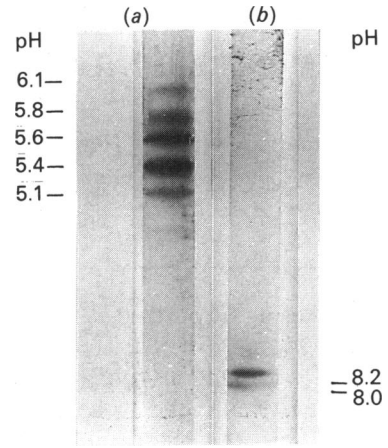

Fig. 1. Analytical isoelectric focusing of isolated β_2-G-I in 7.5% polyacrylamide gels containing 6 M-urea

Staining was performed with Coomassie Brilliant Blue G-250. The gels contained (*a*) 40 μg of native β_2-G-I focused within a pH range of 5–7 and (*b*) 40 μg of neuraminidase-treated β_2-G-I focused within a pH range of 7–9.

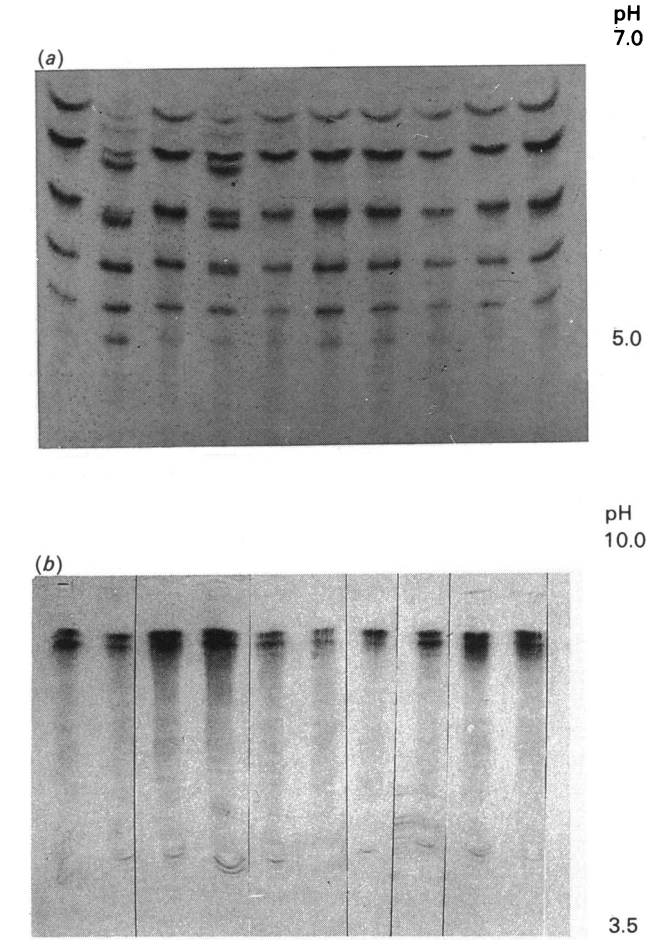

Fig. 2. Isoelectric focusing of ten different plasma samples (*a*) in a pH gradient of 5–7 and (*b*) in a pH gradient of 3.5–10 after neuraminidase treatment

A 15 μl sample of plasma containing 5 μg of β_2-G-I/ml was applied to each slot. Subsequently proteins were transferred to nitrocellulose and incubated with monospecific antibodies against β_2-G-I.

isoelectric points shifted to 8.0 and 8.2 respectively (Fig. 1). That the demonstrated heterogeneity of purified β_2-G-I was not an artifact caused by $HClO_4$ treatment, the first step of the isolation procedure, was shown by isoelectric focusing followed by Western-blot analysis of freshly drawn plasma with specific antibodies against β_2-G-I (Fig. 2).

Recently genetic variants of human serum β_2-G-I were demonstrated by isoelectric focusing and Western-blot analysis [20,21]. In these studies it was concluded that three autosomal alleles (*B2G*1*, *B2G*2* and *B2G*3*) determine six different phenotypes but the frequency for the common gene *B2G*2* is nearly 90%. No correlation was found between a certain genetic isoform and the plasma concentrations of β_2-G-I.

In view of these findings we analysed the β_2-G-I patterns of 50 different donors with plasma concentrations ranging from 6 to 35 mg/dl by isoelectric focusing followed by Western-blot analysis before and after treatment with neuraminidase. Focusing of total plasma was performed in a pH gradient of 5–7 and that of neuraminidase-treated plasma in a pH gradient of 3.5–10. The blots of ten donors are shown in Fig. 2.

In our experiments approx. 95% of the investigated plasma samples showed the common isoelectric-focusing pattern: five major isoforms with isoelectric points between 5.1 and 6.1. In some cases the two bands, with pI values of 6.1 and 5.8, were split in two, implying the presence of genetic variants (Fig. 2*a*, lanes 2 and 4). When these plasma samples were treated with neuraminidase, which cleaves off the terminal residues of *N*-acetylneuraminic acid, each of the samples showed a pattern of two isoelectric subspecies shifted towards the cathode, irrespective of the original number of isoforms in native plasma (Fig. 2*b*). The presence of genetic variants was also independent of the concentration of β_2-G-I in plasma [lanes 3 and 8 in Figs. 2(*a*) and 2(*b*) show plasmas with concentrations less than 10 mg/dl; the others ranged between 15 and 35 mg/dl].

To investigate the identity of the isoforms in more detail, five different isoelectric subspecies of β_2-G-I representing the most common genetic variants were separated by preparative chromatofocusing as described in the Materials and methods section and treated individually with neuraminidase.

Isoelectric focusing of these five asialo subfractions of β_2-G-I in a pH gradient of 7–9 gave rise to the formation of two bands with pI values of 8.0 and 8.2, exactly the same two bands as observed with total asialo-β_2-G-I (Fig. 3).

Subsequently we separated these two isoforms obtained by treatment of total β_2-G-I with neuraminidase by cation-exchange column chromatography at pH 5.6.

Despite the fact that the two isoforms differed in their isoelectric points only by 0.2 pH unit, both fractions could be obtained in virtually pure form in one step. Fig. 4 displays the elution pattern of the incubation mixture containing total β_2-G-I and neuraminidase. The material was eluted in three peaks. Neuraminidase with a pI of approx. 5.5 was eluted in our system in the void volume of the column (peak 1). The other two peaks consisted of β_2-G-I subfractions I and II. The material belonging to each of peak 2 and peak 3 was pooled and checked for purity by isoelectric focusing. Each fraction was found to be over 96% pure. The sialic acid content of both subfractions, determined by the thiobarbituric acid method [13], was found to be less than 0.1 mol/mol of protein.

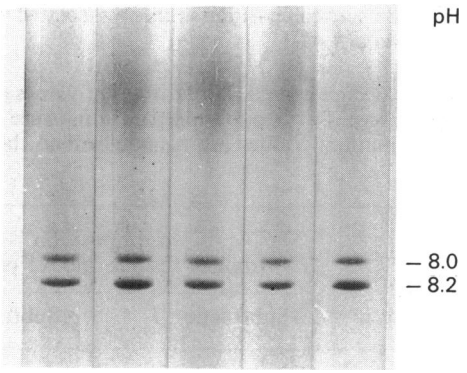

Fig. 3. Isoelectric focusing of purified neuraminidase-treated subfractions 1–5 of β-G-I

A 20 μg portion of protein was applied to each gel, and focusing was performed in a pH gradient of 7–9. From left to right the gels contained fractions 1–5 respectively, and staining was performed with Coomassie Brilliant Blue G-250.

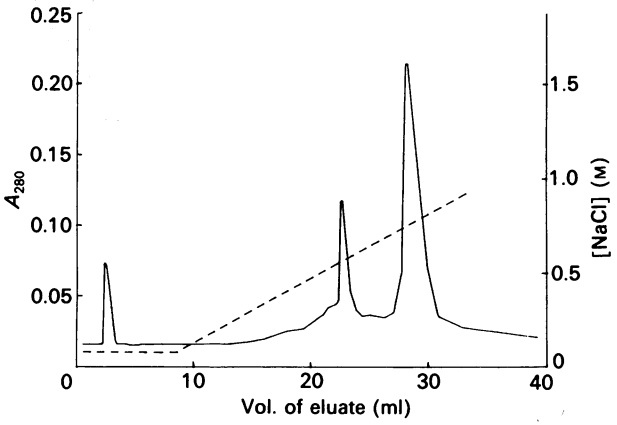

Fig. 4. Chromtography of neuraminidase-treated β_2-G-I on a Mono S cation-exchanger

A 500 μg portion of neuraminidase-treated protein dissolved in 0.05 M-sodium acetate buffer, pH 5.6, was applied to the column. Elution was carried out with the same buffer containing a linear gradient of 0–0.8 M-NaCl (----). The first peak eluted is neuraminidase, and the second and third are the two isoforms of asialo-β_2-G-I.

The isoform I/isoform II ratio of asialo-β_2-G-I obtained from pooled plasma was approx. 1:5, as determined from the peak areas of the elution patterns in Fig. 4. By scanning the isoelectric-focusing gels stained with Coomassie Brilliant Blue we found the proportions 1:3:3:2:1 for isoforms 1–5 of native β_2-G-I and the ratio 1:5 for asialo isoforms 1 and 2. In further experiments we determined the amino acid content of the two asialo-β_2-G-I isoforms (Table 1). It was found that they differ from each other in the content of lysine and glycine. Subfraction I had 28 mol and subfraction II 29 mol of lysine residues/mol of protein, whereas glycine was found to be 26 mol/mol in subfraction I and 25 mol/mol in subfraction II.

Table 1. Amino acid composition of the two isolated subfractions of asialo-β_2-G-I

The values are expressed as the average values ± S.D. for five hydrolyses. Two sample preparations were used for the determination. Values are rounded off to the nearest integer.

Amino acid	Composition (mol of residue/mol of protein)	
	Subfraction I	Subfraction II
Asp	29±0.5	29±0.3
Thr	25±0.3	25±0.3
Ser	20±0.4	20±0.3
Glu	28±0.4	28±0.4
Pro	37±0.2	37±0.3
Gly	26±0.3	25±0.4
Ala	18±0.3	18±0.3
Val	19±0.2	19±0.1
Cys	22±0.1	22±0.1
Met	3±0.1	3±0.1
Ile	13±0.4	13±0.6
Leu	19±0.6	19±0.6
Tyr	13±0.6	13±0.5
Phe	17±0.4	17±0.3
Lys	28±0.3	29±0.3
His	5±0.2	5±0.1
Arg	10±0.4	10±0.5

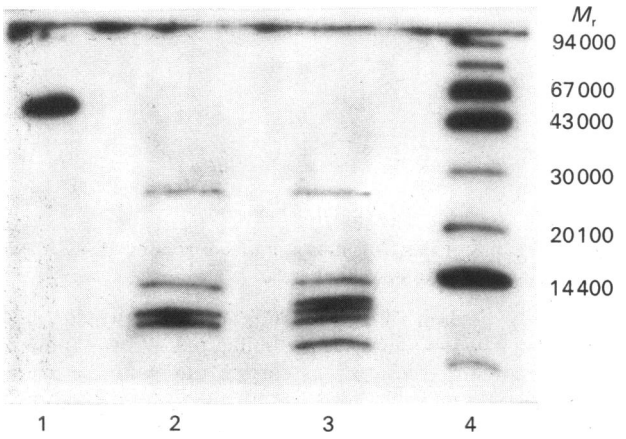

Fig. 5. SDS/polyacrylamide-gel electrophoresis of tryptic fragments of asialo-β_2-G-I subfractions I and II on a 15–18% polyacrylamide gradient gel

Digestions were carried out with 1 unit of trypsin/50 μg of protein for 2 h at 37 °C. A 50 μg portion of protein was applied to each gel. Lane 1, native β_2-G-I; lane 2, tryptic fragments of subfraction I; lane 3, tryptic fragments of subfraction II; lane 4, low-M_r standard.

To substantiate these results we digested the purified asialo subfractions with trypsin. This proteinase is known to cleave specifically at the *C*-terminal side of lysine and arginine residues. Fig. 5 shows the SDS/polyacrylamide-gel electrophoresis patterns of digested asialo subfractions I and II at enzyme concentrations of 1 unit/50 μg of protein incubated for 2 h at 37 °C. The enzyme was bound to agarose and therefore no trypsin bands were detectable on the gel. Subfraction II yielded five tryptic fragments, whereas with subfraction I only three tryptic fragments were found. As both subfractions have the same amount of arginine residues, the greater number of peptide fragments in isoforms II could be explained by a higher content of lysine.

Summarizing our results, we have demonstrated that human plasma β_2-G-I consists of five major subfractions in native form. Each of these subfractions yields two isoforms with pI values of 8.0 and 8.2 when desialylated by treatment with neuraminidase. These two isoforms differ in their content of the basic amino acid lysine.

We conclude that these two isoproteins are sialylated to different extents, giving rise to the formation of various isoelectric-focusing patterns in plasma.

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