Carbohydrate Research, 182 (1988) 135–147
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION AND PARTIAL CHARACTERIZATION OF A MALIGNANCY-ASSOCIATED GLYCOPROTEIN*

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(Received April 21st, 1987; accepted for publication in revised form, November 6th, 1987)

ABSTRACT

The isolation from cancer patient serum of a glycoprotein (Cc) associated with the presence of a variety of malignancies was previously reported. Although preliminary chemical and physical data indicated that Cc was different from identified circulating glycoproteins, subsequent immunological studies suggested that it was closely related to α_1-acid glycoprotein. Further analysis revealed the presence of two components in some Cc preparations and prompted a re-examination of the isolation and characterization data. In the present study, Cc was purified by a modified protocol which involved the use of pleural fluid obtained from individuals with cancer, and an α_1-acid glycoprotein antibody column to remove contaminating α_1-acid glycoprotein. Typically, the material not retained by the antibody column gave a single band with M_r 53 000 when subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis. Amino terminal analysis revealed that the protein contained a blocked amino terminus, and carbohydrate analysis indicated that complex, asparagine-linked saccharide units were present. The product could be distinguished from α_1-acid glycoprotein and other previously described circulating glycoproteins by several criteria, including molecular weight, isoelectric point, and amino acid and carbohydrate composition. One of three preparations isolated had an apparent M_r of 59 000. Carbohydrate analysis as well as deglycosylation studies showed that the change in molecular weight was due to increased glycosylation.

INTRODUCTION

Changes in the levels of normal serum glycoproteins have been reported for

*This research was supported by grant CA-15 483 from the National Cancer Institute. National Institutes of Health.

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0008-6215/88/$ 03.50

a variety of diseases, and an increase in serum glycoprotein levels has been correlated with the occurrence of neoplasia[1]. This increase is due primarily to an increase in the acute phase reactants, a family of glycoproteins which are produced by the liver and secreted into the circulation. They include α_1-acid glycoprotein (α_1-AGP), α_1-antitrypsin, ceruloplasmin, and others[2].

Another source of this increase is the tumor itself which may contribute additional circulating glycoproteins, including some of apparently fetal or embryonic type (*e.g.*, carcinoembryonic antigen[3] and α-fetoprotein[4]). Many of these "tumor markers" have been described in terms of a blood test for the detection of cancer. They can be categorized as follows: the oncofetal antigens (aforementioned), ectopic hormones (adrenocorticotropic hormone, parathormone, calcitonin, etc.), the placental proteins (human chorionic gonadotropin and human placental lactogen), enzymes (*e.g.*, prostatic acid phosphatase and lactic dehydrogenase), serum proteins (immunoglobulins), and others (polyamines, ferritin, catecholamine metabolites, etc.)[5]. The ideal biological marker is specific (no false-positive results), sensitive (no false-negative results), and has an absolute correlation wih the extent of disease[6]. To date, no tumor-associated markers which meet these criteria have been found. Although new markers such as the mucin-type glycoproteins CA 12-5 and CA 19-9, and prostate antigen (PA) have not been completely assessed, they are neither organ- nor tumor-specific[7,8].

The isolation from human plasma of a glycoprotein associated with the presence of a variety of malignancies has been reported[9]. Despite the fact that preliminary chemical and physical data indicated that this glycoprotein (Cc) was different from previously characterized circulating glycoproteins, subsequent immunological studies[10–12] suggested that it was closely related to α_1-AGP. However, further chemical analysis revealed that some of the preparations contained contaminating α_1-AGP, which prompted a re-examination of the purification scheme and characterization data.

EXPERIMENTAL

Amino acid analysis. — Amino acid analysis was carried out on a Dionex amino acid analyzer after hydrolysis of the sample in 6M HCl for 24 h *in vacuo* at 110°. Amino terminal analysis was carried out as follows: The protein or glycoprotein (7 nmol) was treated with 1:5 88% formic acid–30% H_2O_2 for 30 min at room temperature and lyophilized. The residue was dissolved in 1% sodium dodecyl sulfate and heated to 100° for 5 min. *N*-Ethylmorpholine (50 μL) and dansyl chloride (75 μL of a 25 mg/mL solution in *N*,*N*-dimethylformamide) were added, and the mixture was incubated for 2 h at 37° in the dark. Protein was precipitated from the reaction mixture with ice-cold acetone (3 vols.). The pellet was washed thoroughly with acetone, subjected to acid hydrolysis (6M HCl, 110° *in vacuo*) for either 4 or 12 h, and the dansylated amino acids detected by t.l.c. on double-sided polyamide sheets (Schleicher & Schuell, Inc., Keene, NH) according

to the method described by Gray[13]. The dansylated amino acids were detected by u.v. illumination.

Carbohydrate analysis. —Hexosamine analysis was carried out with a Dionex amino acid analyzer after hydrolysis of the sample in 4M HCl for 8 h *in vacuo* at 100°.

For hexoses, the glycoprotein (5 nmol) and an internal standard (D-xylose) were hydrolyzed in 2M trifluoroacetic acid *in vacuo* for 4 h at 110°. The acid was removed in a vacuum desiccator over P_2O_5. The sugars were reduced by dissolving the sample in a 0.2% $NaBH_4$–M NH_4OH solution and incubating for 4 h at room temperature. In order to destroy the excess of $NaBH_4$ and to neutralize NH_4OH, AG 50 (H^+) cation-exchange resin (Bio-Rad) was added slowly. The mixture was poured into a Pasteur pipette plugged with glass wool, and washed with M NH_4OH (10 bed vols.). The eluate was evaporated to dryness on a rotary evaporator. Borate ions were removed by addition of 1000:1 methanol–HCl and evaporation to dryness; this was repeated 5 times. The residue dissolved in water was quantitatively transferred to a screw-capped test tube, lyophilized, and dried in a desiccator over P_2O_5. The alditols were *O*-acetylated by adding equal volumes of anhydrous acetic anhydride and pyridine to the dried residue, mixing vigorously, and heating the mixture for 30 min at 100°. The sample was evaporated to dryness using a stream of N_2 and then dissolved in pyridine. The alditol acetates were analyzed by gas chromatography (Hewlett–Packard Gas Chromatograph, model 5890A, Avondale, CA) according to the method of Clamp *et al.*[14]. The analysis was carried out in a DB225-30N J and W Durabond fused-silica capillary column (0.25 mm × 0.25 μm; Alltech Associates, Inc., Deerfield, IL) under the following conditions: the carrier gas used was He (99.995%), the linear velocity was 50 cm/second, and the temperature program ranged from 200 to 240° increasing at 4°/min.

In order to determine the sialic acid content, the sample glycoprotein (2 nmol) was hydrolyzed in 0.05M H_2SO_4 for 1 h at 80°, neutralized with M NaOH, and analyzed by h.p.l.c. (Waters liquid chromatograph, Model 6UK, Milford, MA). The analysis was carried out in an Aminex Ion Exclusion HPX-87H column (300 mm × 7.8 mm, Bio-Rad), equilibrated in 6mM H_2SO_4. The flow rate was maintained at 0.1 mL/min, and fractions were monitored by u.v. absorbance at 206 nm.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. — SDS–polyacrylamide gel electrophorsis was performed according to the methods described by Laemmli[15], and Hass and Kennett[16] using 1.5-mm thick slab gels. The running gel used was 10% polyacrylamide with a stacking gel of 5%, cross-linked with bis(acrylamide). The molecular weight markers (Bio-Rad Laboratories, Richmond, CA) were phosphorylase B (92 000), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 000), and lysozyme (14 000). After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 50:50:1 (v/v) methanol–water–acetic acid and destained in the same solution without the dye. The gels were also stained with silver according to the method of Wray *et al.*[13] if bands could not be detected with

the Coomassie stain. The gels containing ^{125}I-labeled samples were dried on Whatman 3MM filter paper directly after staining with the Coomassie stain and were detected by autoradiography at −80° with Kodak X-O-MAT AR film.

Adsorption on lectins. — The immobilized lectins, *Lens culinaris* agglutinin-, concanavalin A-, *Ricinus communis* agglutinin I (mol. wt. 120 000)-, peanut agglutinin-, and soybean agglutinin-agarose, were purchased from Vector Laboratories (Burlingame, CA). Wheat germ agglutinin-agarose was prepared in this laboratory. The Con A- and LCA-agarose columns wer equilibrated in TBS (10mM Tris·HCl, pH 8.0, containing 0.15M NaCl, mM $CaCl_2$, and mM $MgCl_2$). Bound material was specifically eluted, first with 10mM methyl α-D-glucopyranoside and then with 0.5M methyl α-D-mannopyranoside in TBS according to the method of Kornfeld *et al.*[18]. RCA I- and PNA-agarose columns were equilibrated in phosphate-buffered saline (PBS), and bound material eluted with 0.1M lactose in PBS. The SBA-agarose column was equilibrated in modified TBS (mM $MnCl_2$ instead of $MgCl_2$), and bound material was eluted with 0.2M D-galactose in the same buffer. The WGA-agarose column was equilibrated with 50mM Tris·HCl, pH 8, and the bound material was eluted with 0.1M 2-acetamido-2-deoxy-D-glucose in the same buffer[9].

Purification of perchloric acid fraction of pleural fluid. — Perchloric acid precipitation of pleural fluid, obtained from a variety of cancer patients, was performed as described for plasma by Bolmer and Davidson[9]. Pleural fluid, which had been stored at −20° for no longer than 6 months after collection, was kept on ice during the preparation. After precipitation, the neutralized supernatant was dialyzed against several changes of de-ionized water at 4°, lyophilized, and stored at −20° for no longer than 3 months. Perchloric acid precipitation of 1 L of pleural fluid generally yielded 1 g of protein (Lowry *et al.*[19]) and 50 mg of sialic acid (Jourdian *et al.*[20]).

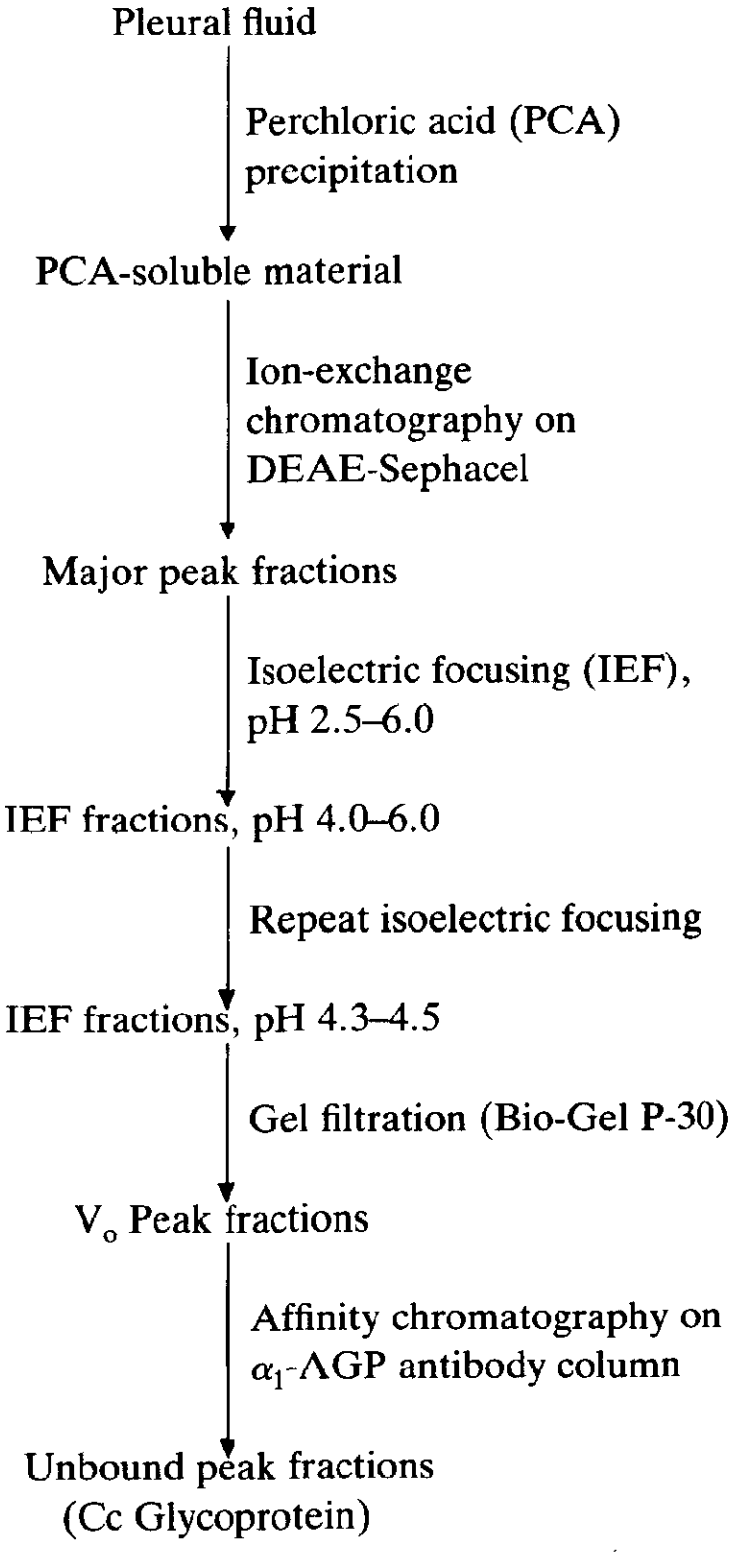
The perchloric acid-soluble fraction from 500 mL of pleural fluid was reconstituted in 0.1M pyridinium acetate, pH 5.2 (20 mL) and applied to a 2.5 × 57 cm column of DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO), equilibrated in 0.1M pyridinium acetate, pH 5.2, at room temperature. The column was washed with the same buffer (at least 2 bed vols.), and then eluted with a linear gradient (0.1–1.0M) of pyridinium acetate (1 L). Fractions (10 mL) were collected and assayed for protein[13] and sialic acid[14]. The major peak fractions were combined, dialyzed against de-ionized water at 4°, lyophilized, and stored at −20° for no longer than 3 months.

Preparative isoelectric focusing[9] was performed in a column (440 mL) from LKB (Gaithersburg, MD) at 500 mV of constant voltage for at least 30 h at 4° in the pH range of 2.5–6. Ampholytes (LKB) of pH 2.5–4, 4–6, and 5–7 in a ratio of 2:2:1 (resulting in a final concentration of 1%) were used to form the pH gradient. The electrode solutions were 0.16M H_3PO_4(anode) and 0.5% ampholyte, pH 5–7(cathode). After focusing, fractions (5 mL) were collected and monitored for pH, and for protein. Peak fractions in the pH range of 4–6 (first focusing) or at pH 4.4

(second focusing) were combined, dialyzed against de-ionized water at 4°, lyophilized, and stored at −20° for no longer than 3 months.

Radio-iodination of glycoproteins. — Radioiodination ($Na^{125}I$, Amersham, Arlington Heights, IL) of glycoproteins was performed with Chloramine-T (Sigma, St. Louis, MO); specific activities were in the range of 3.5–3.7 MBq/mg.

Bio-Gel P-30 (Bio-Rad, Richmond, CA) was equilibrated in 10mM Tris·HCl, pH 8.0, and packed in a 1.5 × 45 cm column at room temperature. Peak fractions from the second isoelectric focusing containing a radiolabeled marker were dissolved in 10mM Tris·HCl (1 mL), applied to the column, and eluted with the



Scheme 1. Modified scheme for the isolation and purification of Cc.

same buffer at room temperature. Fractions (1 mL) were collected and monitored for radioactivity. Void-peak fractions were combined, concentrated, desalted on a 1 × 15 cm column of Bio-Gel P-10 (Bio-Rad), and lyophilized.

Deglycosylation of glycoproteins. — Glycoproteins were deglycosylated with *N*-glycanase (Genzyme, Boston, MA). The glycoprotein sample (10 μL, 10 mg/mL) was heated for 3 min at 100° in the presence of 0.5% sodium dodecyl sulfate and 0.1M 2-mercaptoethanol. The sample was diluted with sodium phosphate buffer, pH 8.6 (0.2M final concentration) containing 10mM 1,10-phenanthroline and 1.25% NP-40. *N*-Glycanase was added to a final concentration of 10 munits/mL, and the mixture was incubated overnight at 37°. The reaction was stopped by adding 1% sodium dodecyl sulfate and heating for 3 min at 100° or by freezing in a dry ice–acetone bath. The carbohydrate was separated, and removed from the protein by gel filtration chromatography in Bio-Gel P-30 as described above. The void-peak fractions containing a radiolabeled marker were combined and applied to a 1-mL Extract-Gel D column (Pierre Chemical Co., Rockford, IL), equilibrated in distilled water, to remove detergents used during the deglycosylation reaction. The samples were eluted and monitored for radioactivity. Unbound-peak fractions were combined, lyophilized, and stored at −20°. Deglycosylation was essentially quantitative based on hexosamine analysis of the product.

Coupling of α_1-AGP antibodies. — The antibodies (Accurate Chemicals, Westbury, NY) were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) as described by March *et al.*[21]. The conjugated gel (0.6 mg protein/mL of Sepharose) was equilibrated in 50mM Tris·HCl, pH 8.0, and packed in a 1 × 6 cm column at 4°. Once the column had been calibrated with authentic α_1-AGP, the void-peak fractions containing a radioiodinated marker from the gel-filtration step were dissolved in 50mM Tris·HCl, pH 8.0 (1 mL), and applied to the column; bound material was eluted with 3M potassium thiocyanate. Fractions (1 mL) were collected and monitored for radioactivity. Unbound-peak fractions were combined, desalted on a Bio-Gel P-10 column (1 × 15 cm) at room temperature, lyophilized, and stored at −20°.

RESULTS

Several modifications of the original procedure are illustrated in Scheme 1. Firstly, pleural fluid was used instead of plasma because large volumes were easier to obtain and we found that the glycoprotein constituents were essentially the same as those in plasma; secondly was the substitution of Bio-Gel P-30 for Sephadex G-25 in order to prevent glucose (dextran) contamination; and third was chromatography on an α_1-AGP antibody column. Lastly, the WGA affinity chromatography step was omitted.

The perchloric acid-soluble material was applied to a DEAE-Sephacel column (Fig. 1). The major protein and sialic acid peak was eluted with ~0.5M pyridinium acetate. The total recovery based on protein ranged from 85 to 90%,

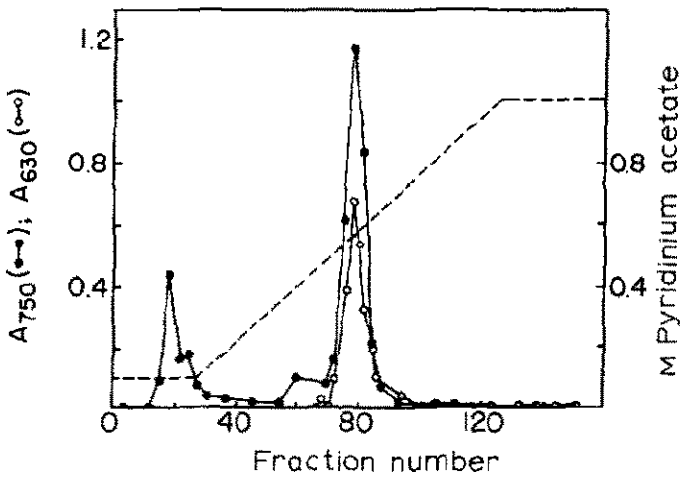


Fig. 1. Ion-exchange chromatography of perchloric acid-soluble material on a DEAE-Sephacel column (2.5 × 57 cm). A solution containing 300–400 mg of protein was applied to the column; see Experimental section for details. Protein (A_{750}, —●—●—), sialic acid (A_{630}, —○—○—), and M pyridinium acetate (----).

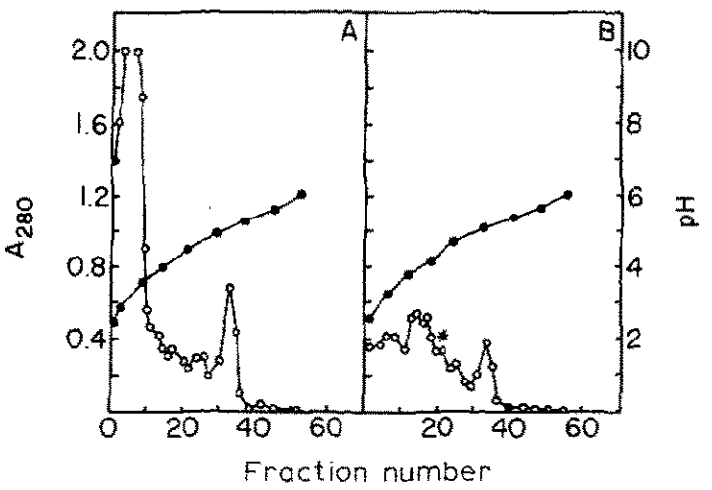


Fig. 2. Preparative isoelectric focusing, pH range 2.5–6.0, of: (A) The major sialic acid-containing peak from ion-exchange chromatography, and (B) the material above pH 4.0 from isoelectric focusing. The protein (100–200 mg) was dissolved in the heavy-density gradient solution and introduced into the column during the formation of the sucrose density gradient; see Experimental section for details. The star indicates the shoulder peak at pH 4.4. Protein (○), pH (●).

with 75–81% in the peak fractions. These fractions were combined and subjected to preparative isoelectric focusing (Fig. 2); the major peak in panel (A) corresponds to α_1-AGP. Fractions in the pH range 4.0–6.0 were combined and recovered. The material from several experiments was pooled and subjected to a second preparative isoelectric focusing under the same conditions. The starred shoulder (Fig. 2B), corresponding to a pH of 4.4, was combined and recovered by dialysis and lyophilization. The amount of protein was generally insufficient to be monitored *via* absorbance at 280 nm; consequently, an aliquot of the isoelectric focusing material (generally 10 μg) was radioiodinated and mixed with the unlabeled material.

After chromatography on a Bio-Gel P-30 column (recovery between 92 and 96%), the void-peak fractions were applied to an α_1-acid glycoprotein–antibody column. The bulk of the labeled material (*i.e.*, 90% of the total radioactivity

TABLE I

AMINO ACID COMPOSITIONS OF WGA-BOUND AND WGA-UNBOUND Cc_1[a]

Amino acid	*WGA-bound Cc_1*	*WGA-unbound Cc_1*
$CySO_3H$		
Asx	46	47
Thr	30	35
Ser	91	124
Glx	99	113
Gly	100	100
Ala	64	52
Pro	[b]	[b]
Val	25	18
Met		
Ile	9	12
Leu	22	21
Tyr	10	13
Phe	18	346[c]
His	16	24
Lys	39	64
Arg	13	7
Trp	[b]	[b]

[a]Values were normalized to glycine which is arbitrarily given a value of 100. [b]Not determined. [c]Abnormal result likely due to a contaminant or analyzer noise.

recovered) was in the nonadsorbed fraction; the 10% which did bind could be eluted with potassium thiocyanate but was not characterized further. Aliquots of the radiolabeled material in the unbound peak were examined by SDS–polyacrylamide gel electrophoresis. The unbound material showed one major band corresponding to an apparent mol. wt. of 53 000. As another measure of purity, amino-terminal analysis of malignancy-related serum glycoprotein (Cc) indicated that the NH_2-terminus was blocked, suggesting the presence of one protein.

The last step in the original purification scheme was a WGA-affinity-chromatography step. Because both WGA-bound and -unbound "Cc" have the same pI and the same apparent mol. wt., the amino acid compositions of both were determined and are presented in Table I. Except for the phenylalanine value in the unbound sample, which was unusually high and likely due to a contaminant, the amino acid compositions were virtually identical. As a result, the WGA affinity step was omitted from the purification scheme. When the amino acid compositions of either WGA-bound or -unbound products from several preparations were compared to those previously reported[9], the values were found to be the same within experimental error and greatly different from that of α_1-AGP[23].

The carbohydrate composition and the sugar-protein linkage(s) of Cc were examined. Preliminary data obtained by lectin affinity chromatography are presented in Table II. Iodinated Cc was applied first to a Con A-agarose column.

TABLE II

LECTIN-AFFINITY CHROMATOGRAPHY OF ^{125}I-LABELED Cc[a]

Lectin	*Eluting sugar*	*Cc_1 and Cc_2*			*Cc_3*		
		Unbound[b]	*Bound[b]*	*Recovery[c]*	*Unbound[b]*	*Bound[b]*	*Recovery[c]*
Con A	D-Mannose	63	27	88	55	28	76
	D-Glucose		10			17	
LCA	D-Mannose	81	12	81	92	7	75
	D-Glucose		6			1	
WGA	D-GlcNAc	70	30	87	19	81	81
RCA I	Lactose	54	46	81	36	64	85

[a]Less than 2% bound to peanut agglutinin (eluting sugar, lactose) and soybean agglutinin (eluting sugar, D-galactose). [b]Expressed as percent of recovery. [c]Expressed as percent of applied.

Thirty-seven percent bound to the column, two-thirds of which was eluted with a D-glucose-containing buffer, and the remainder with a D-mannose-containing buffer. These data indicated that there was heterogeneity in the carbohydrate moiety. A separate aliquot was applied to an LCA-agarose column. Since 18% of the material bound to the column, it was tentatively concluded that some portion of the saccharides contained L-fucose. Analogous experiments with WGA-, RCA I-, PNA-, and SBA-agarose columns suggested the presence of sialic acid and D-galactose, and the absence of 2-acetamido-2-deoxy-D-galactose, indicating that complex Asn-linked saccharide units were present.

Analysis of the carbohydrate composition was also carried out by g.l.c. of the alditol acetates (Table III); sialic acid was separately analyzed by h.p.l.c. As indicated in the table, Cc contains fucose, mannose, galactose, glucosamine, and some sialic acid, again suggesting complex-type saccharide chains. The absence of galactosamine supports the finding that the oligosaccharide chains are all Asn-linked. To confirm these findings, Cc was treated with *N*-glycanase which cleaves

TABLE III

CARBOHYDRATE COMPOSITION OF Cc AND α_1-AGP[b]

Carbohydrate	*Cc_1, Cc_2*	*Cc_3*	*α_1-AGP[b]*
Fucose	1.3	0.8	0.7
Mannose	3.9	4.8	4.9
Galactose	4.1	6.2	9.8
Glucosamine	3.4	6.6	13.9
Galactosamine	0	0	0
Sialic acid	0.5	1.8	12.1
Total	13.2	20.2	41.4

[a]Values expressed as percent (w/w). [b]Ref. 23.

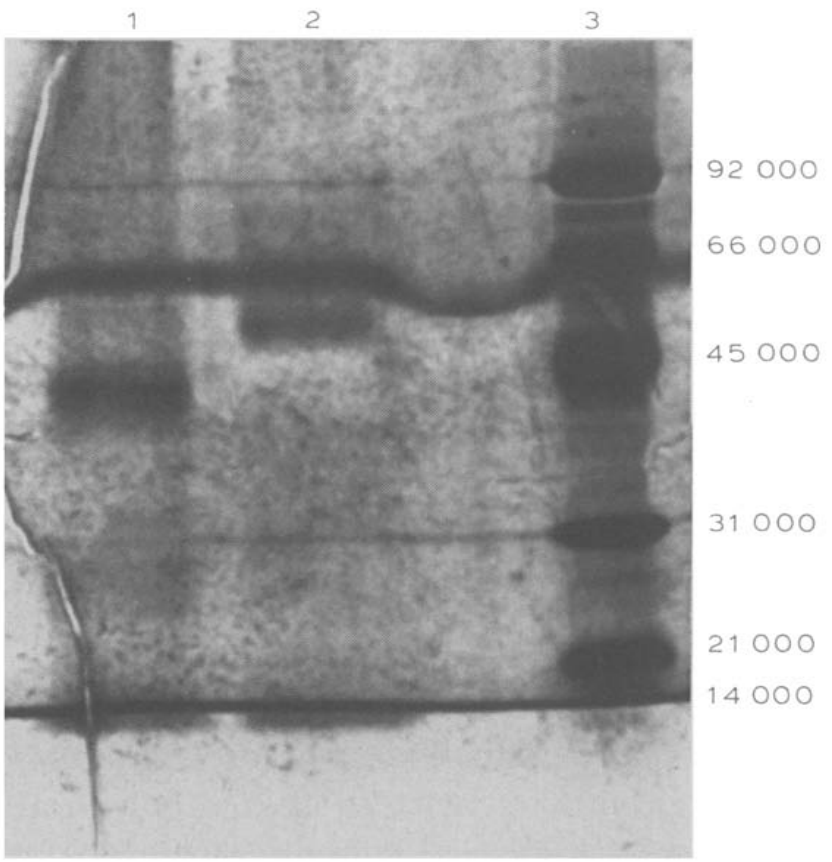


Fig. 3. Poly(acrylamide) gel electrophoresis of deglycosylated Cc, followed by silver staining. Samples (10 μg each) were applied to a slab gel containing a 10% running and 5% stacking gel, respectively. After electrophoresis in the presence of SDS, the gel was stained with silver. The wavy line is a detergent artifact. Lanes are as follows: (1) Deglycosylated Cc, (2) native Cc, and (3) protein standards.

off bi-, tri-, and tetra-antennary complex, hybrid, and high-mannose chains[24]. Complete deglycosylation was confirmed by hexosamine analysis. An aliquot of the treated sample was examined by SDS–PAGE (Fig. 3). Lane 1 contained the deglycosylated Cc, apparent mol. wt. of 44 000; lane 2 the starting material (53 000); and lane 3 the protein standards. Based on molecular weight, the total percentage of carbohydrate of Cc was calculated to be 14.4%.

In this study, three different preparations of Cc were isolated. The first, Cc_1, was isolated from pooled pleural fluid of unknown etiology. The second and third, Cc_2 and Cc_3, respectively, were isolated from the pleural effusions of a patient with mesothelioma (associated with asbestos exposure). The latter was isolated from pleural fluids obtained after the patient had undergone chemotherapy. The apparent mol. wts. of Cc_1 and Cc_2 were 53 000, whereas Cc_3 had an apparent mol. wt. of 59 000 (Fig. 4).

Based on differences in lectin affinity (Table II), it was suspected that the difference in apparent molecular weight might be due to variation in the carbohydrate portion of the molecule. Thus, experiments with LCA-, WGA-, RCA I-, PNA-, and SBA-agarose columns indicated that Cc_3 contained less L-fucose but more sialic acid and D-galactose than Cc_2, and that 2-acetamido-2-deoxy-D-galactose was apparently not present. These data were confirmed by analysis of the monosaccharides by g.l.c. and h.p.l.c. as used before (Table III). Additional

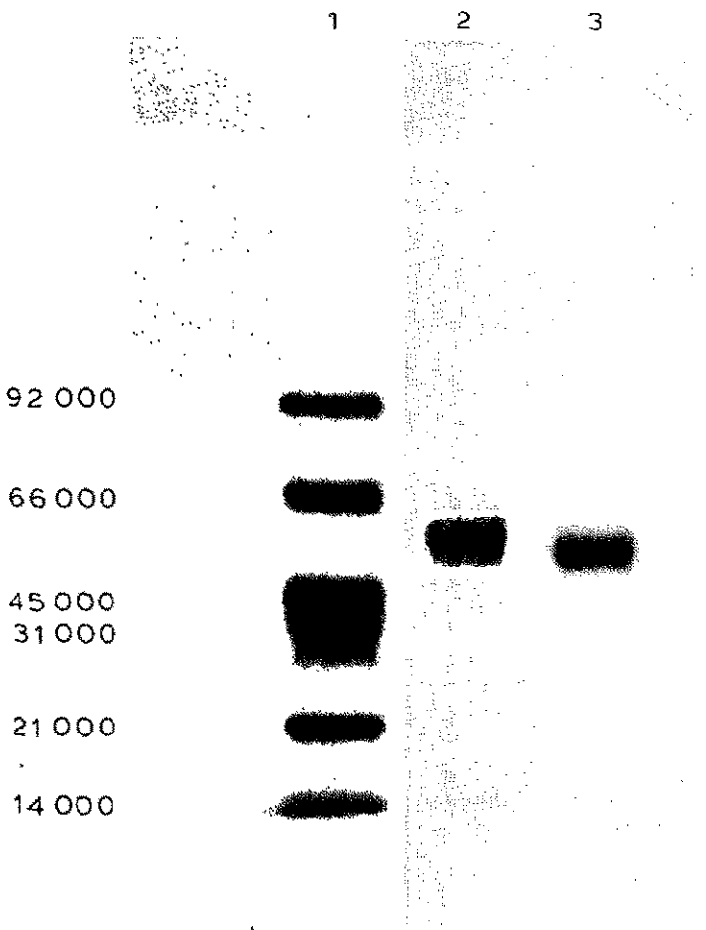


Fig. 4. Poly(acrylamide) gel electrophoresis of Cc_2 and Cc_3, followed by silver staining. Samples (50 μg each) were applied to a slab gel, and electrophoresis and staining were performed as described in Fig. 3. Lanes are as follows: (1) Protein standards, (2) Cc_3, and (3) Cc_2.

evidence as obtained by deglycosylating Cc_3 with *N*-glycanase and examining the sample by SDS–PAGE; the deglycosylated Cc_3 had an apparent mol. wt. of 44 000 (data not shown).

DISCUSSION

The original procedure used to isolate Cc from serum was modified to improve both yield and contaminant separation. By use of the described procedure, the average yield of Cc was 1 mg per liter of pleural fluid, somewhat higher than that originally reported for plasma[9]. An α_1-AGP antibody column was employed to remove α_1-AGP and contaminants immunologically related to it. The WGA affinity-chromatography step was omitted because variation in the carbohydrate component led to poor recoveries in this step, and there were no differences in the amino acid compositions or immunologic properties[25] of unbound and bound material. Amino terminal analysis of the purified material showed that the NH_2-terminal of Cc was blocked, and SDS–PAGE showed one major component with an apparent mol. wt. of 53 000; consequently, the preparation was considered pure.

Because it had been suggested[10–12] that Cc was closely related to α_1-AGP, it was important to determine not only whether the protein isolated was identical to that originally described, but also to determine whether this protein was related to

α_1-AGP. From isoelectric focusing, it was known that the protein isolated has a pI of 4.4 ±0.1, as does the Cc glycoprotein isolated by Bolmer[22]. In contrast, α_1-AGP has a pI of 2.7 (ref. 23). SDS–PAGE indicated that the protein has an apparent mol. wt. of 53 000, which is in the range previously reported; the M_r of α_1-AGP is 44.1 kDa. Comparison of the amino acid compositions of the isolated Cc and α_1-AGP showed that the protein isolated in this study and that described earlier were identical and different from α_1-AGP. The data from lectin affinity and gas–liquid chromatography suggested the presence of Asn-linked complex type saccharide units, and that the approximate percentage of carbohydrate by weight was 14%.

The complete susceptibility of the saccharide chains of Cc to *N*-glycanase supported the conclusion that all saccharide units of Cc are Asn-linked. A higher molecular weight isolate, Cc_3 (M_r 59 kDa) differed in degree of glycosylation, possibly related to chemotherapy[26–28].

In conclusion, we have purified a glycoprotein associated with a variety of malignancies (*e.g.*, breast, lung, and liver carcinoma, melanoma, sarcoma, mesothialioma, and others)[9,25]. Several pilot studies have indicated a statistically significant difference in the levels of circulating Cc between non-cancer groups (normal volunters, cancer patients having no evidence of the disease at the time of the study, patients with diseases other than cancer, and cancer patients free of the disease for 5 years or more) and active cancer groups (localized or metastatic)[12,25].

ACKNOWLEDGMENTS

The authors thank Nancy S. Ringler, Mordecai Moore, and Douglas Rea for their technical assistance, and Dr. Gerald L. Bartlett for his valuable suggestions and discussions.

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