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Rapid purification of native group-specific component (vitamin D-binding protein) by differential affinity for immobilized triazine dyes

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The use of pseudo-ligand affinity chromatography on immobilized triazine dyes for purification of proteins has become increasingly popular in recent years[1–7]. However, the widest attention has been concentrated on the use of Cibacron Blue 3-GA[8–15], and it is only recently that studies have emerged showing that other dyes can be used either individually or in combination for the purification of enzymes and proteins. This has led to the concept of tandem chromatography which retains the advantages of dye separation together with selective adsorption–desorption of the protein of interest[16–20].

Group-specific component (Gc) is a plasma protein present at concentrations between 20 and 55 mg/dl[21]. This protein is highly polymorphic and more than 80 alleles have been reported at the Gc locus[22]. Following the discovery of its role as the major carrier protein of vitamin D metabolites in plasma[23], recent interest came from the observation that Gc binds actin[24,25] with high affinity at a specific binding site[26]. This actin-binding property is likely to be the explanation for its presence on the membranes of B[27,28] and T[29] lymphocytes, where it appears to play an important role in immunoglobulin binding[30]. Moreover, Gc was recently recognized as a substrate for phospholipid/Ca^{2+}-dependent protein kinase in isolated pancreatic acini[30]. The purification of this protein has been reported by several groups[31–35] and the techniques employed are usually characterized by their low yield, occasional denaturation[36], and/or are relatively time consuming although improved methods based on its affinity for immobilized Cibacron Blue 3-GA have been recently proposed[37,38].

This paper describes a simple, rapid and high-yield technique providing purification of Gc, based on the differential affinity between the protein and its usual contaminants for three immobilized triazine dyes: Cibacron Blue 3-GA, DEAE Affigel Blue, and Fractogel TSK-AF Green. It also illustrates the usefulness of tandem chromatography using immobilized triazine dyes for the separation of proteins based upon their affinities.

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MATERIALS AND METHODS

Human samples

Blood samples were obtained from healthy blood donors with informed consent, and collected in citrate–soybean trypsin inhibitor (SBTI) as described by Harpel[39]. After centrifugation at 1500 *g* at 4°C for 15 min, the plasma was collected and stored at −20°C until used.

Chromatographic procedures

Chromatography on Cibacron Blue 3-GA. Preparative isolation of Gc was performed at room temperature. Plasma (7 ml) was dialyzed overnight at 4°C against 0.03 *M* sodium phosphate buffer, pH 7.0. Immobilized Cibacron Blue 3-GA (60 ml; a gift from Bio-Rad Labs., Richmond, CA, U.S.A.) was packed in a column (60 × 1.6 cm I.D.) and equilibrated with the above-mentioned buffer. Following the application of the plasma sample, the column was eluted at a flow-rate of 15 ml/h and fractions of 2.5 ml were collected at 4°C. Fractions containing Gc (as analyzed by fused rocket immunoelectrophoresis, see below) were pooled, concentrated under nitrogen pressure to a volume of 4.5 ml, and dialyzed against the equilibration buffer of the DEAE Affigel Blue column (see below).

Chromatography on DEAE Affigel Blue. DEAE Affigel Blue (40 ml; a gift from Bio-Rad Labs.) was packed in a column (20 × 1.6 cm I.D., Bio-Rad Labs.) and equilibrated with a 0.02 *M* potassium phosphate buffer, pH 8.0. After sample application, the column was first washed at a flow-rate of 12 ml/h with 130 ml of the equilibration buffer. Then a linear salt gradient (from 0.0 to 0.5 *M* sodium chloride in the equilibration buffer, total volume 150 ml) was applied to the column followed by additional washes of 0.5 *M* sodium chloride (70 ml) and 0.5 *M* ammonium thiocyanate (80 ml). Fractions of 2.4 ml were collected at 4°C. The fractions containing Gc were pooled, concentrated to 2.5 ml, and dialyzed against the equilibration buffer of the Fractogel TSK-AF Green column (see below).

Chromatography on Fractogel TSK-AF Green. The Fractogel dye (25 ml; a gift from Dr. G. Gunzer, E. Merck, Darmstadt, F.R.G.) was packed in a column of dimensions 20 × 1.6 cm I.D., equipped with plungers and equilibrated with a 0.03 *M* sodium phosphate buffer, pH 7.0 (the same buffer used for the first step). After loading the sample, the column was washed with the equilibration buffer (volume 80 ml) at a flow-rate of 12 ml/h, then with a linear salt gradient (0–1.0 *M* sodium chloride in the above buffer, total volume 90 ml) followed by a wash of 120 ml of 1 *M* sodium chloride. Fractions (2 ml) were collected at 4°C. The tightly bound proteins were removed by elution with 0.5 *M* ammonium thiocyanate.

Other methods

Gc quantitation was performed by single radial immunodiffusion[40] using a monospecific antiserum from Dako (distributed by Accurate Chemicals and Scientific Co., Westbury, NY, U.S.A.) and a serum standard from Calbiochem-Behring (La Jolla, CA, U.S.A.). Protein detection in the eluates of the columns was carried out by fused rocket immunoelectrophoresis[41]. Protein measurement, immunoelectrophoresis, and sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis were performed according to standard procedures[42–44]. The biological activity of Gc was

assessed by its ability to bind its known ligands, *i.e.* actin and 25-hydroxycholecalciferol.

Actin was obtained from leg muscles of New Zealand white rabbits and purified as described[45]. Measurement of the affinity (K_a) between Gc with actin was performed according to Goldschmidt-Clermont *et al.*[46] using polyacrylamide gel electrophoresis with Gc radiolabeled with iodine-125 (specific activity: 1–2 μCi/μg), and analyzed as described by Scatchard[47]. 25-Hydroxycholecalciferol was obtained from Roussel Laboratories (Paris, France). Thin-layer analytical isoelectric focusing performed as previously described[48] was used to assess the Gc phenotype[49] and verify that the purified protein has retained its native properties.

RESULTS AND DISCUSSION

During the process of chromatography of whole human plasma on Cibacron Blue 3-GA, Gc was found to be included in the trailing part of the unbound peak

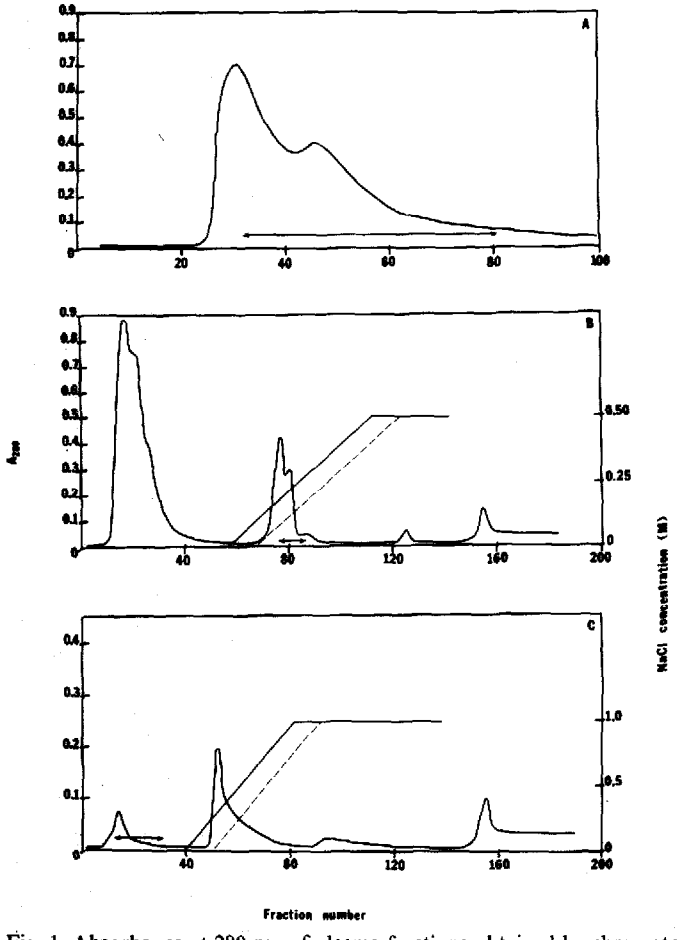

Fig. 1. Absorbance at 280 nm of plasma fractions obtained by chromatography on Cibacron Blue 3-GA (A), DEAE-Affigel Blue (B) and Fractogel TSK-AF Green (C). The bar with two arrows indicates the position where Gc was eluted. In B and C, the broken line indicates the development of the sodium chloride gradient, taking into account the void volume of the column.

(Fig. 1A). A small fraction of the protein (approximately 3–5%) was eluted earlier in the major peak, corresponding to Gc–25-hydroxycholecalciferol complexes[37]. Analysis of the Gc fractions by immunoelectrophoresis (Fig. 2) and by SDS–polyacrylamide gel electrophoresis (Fig. 3) indicated that they were contaminated mostly with immunoglobulin G (IgG), transferrin, ceruloplasmin and traces of albumin. This first step provided a Gc protein enriched by a factor of 10.7 with a yield of 95% (Table I). It is also important to note that, using this first step, the genetic variants of Gc could, as noted previously[38], be separated from one another in heterozygous individuals.

Further separation was then attempted using DEAE Affigel Blue (Fig. 1B). The use of a buffer containing 0.09–0.20 *M* sodium chloride resulted in elution of Gc together with ceruloplasmin and traces of IgA and haptoglobin (Fig. 2). The rationale for using this matrix instead of a classical anion-exchange gel was the better resolution of Gc on this gel, together with the complete separation achieved from IgG and transferrin, which did not bind to the column under the above conditions[50]. The last step, involving Fractogel TSK-AF Green (Fig. 1C), provided a pure protein fraction (Figs. 2 and 3) in which Gc was enriched by a factor of 256 with a total yield of 67% (Table I). Traces of Gc were found in the salt gradient and ammonium thiocyanate fractions.

This three-step purification of Gc appears to have several advantages over previously reported techniques. First, it provides a high yield and a purification factor superior to those obtained by Bowman[31], Bouillon *et al.*[32] and Svasti and Bow-

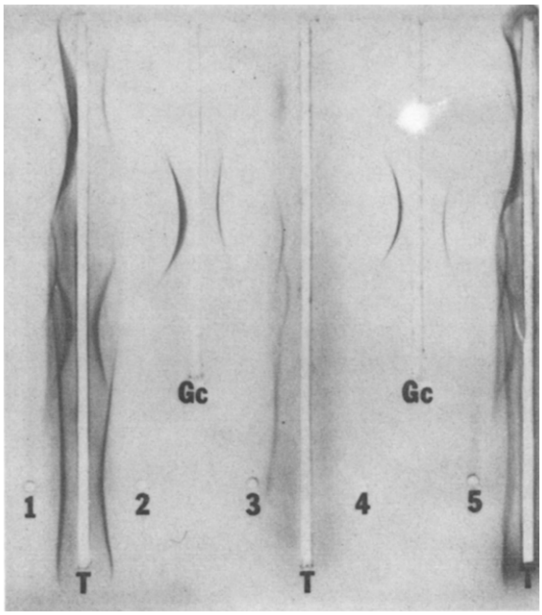

Fig. 2. Immunoelectrophoresis of human control plasma (lanes 1 and 5), of fractions pooled and concentrated from Cibacron Blue 3-GA column (fractions 40–81, lane 2), from DEAE-blue column (fractions 76–86, lane 3) and from Fratogel TSK-AF Green column (fractions 11–32, lane 4). T, antiserum to total human serum; Gc, specific antiserum to Gc protein. The anode is at the top.

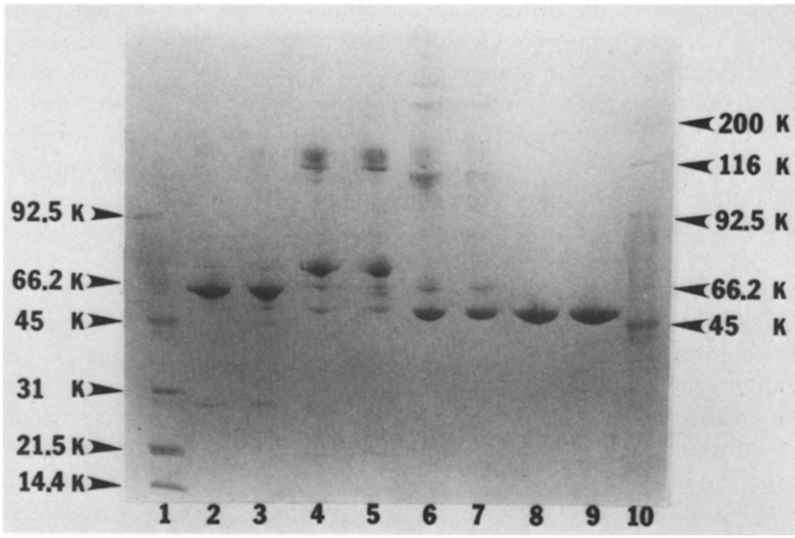

Fig. 3. SDS–gradient-polyacrylamide (5–20%) gel electrophoresis of Gc fractions after each purification step. Lanes 1 and 10: low- and high-molecular-weight markers (from Bio-Rad). Lanes 2 and 3: starting plasma (6.9 μg), non-reduced and reduced. Lanes 4 and 5: concentrated Gc fractions after Cibacron Blue 3-GA (6.4 μg), non-reduced and reduced. Lanes 6 and 7: concentrated Gc fractions after DEAE-blue (6.2 μg), non-reduced and reduced. Lanes 8 and 9: concentrated Gc fractions after Fractogel TSK-AF Green (6.1 μg), non-reduced and reduced. The gel was stained with 0.5% Coomassie Blue R250 in ethanol–water–acetic acid (9:9:2, v/v). The anode is at the bottom. K = Kilodaltons.

TABLE I

PROTEIN CONCENTRATION, Gc CONCENTRATION AND YIELD AFTER THE DIFFERENT PURIFICATION STEPS

Total protein concentration was measured by the technique of Bradford[42]. Gc was measured by single radial immunodiffusion[40] by comparison with a protein standard obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.)

Material	*Volume (ml)*	*Total protein concentration (mg/dl)*	*Gc globulin concentration (mg/dl)*	*Yield (%)*	*Purification factor*
Plasma	7	5460	20.4		
Cibacron Blue 3GA, pH 7.0 (fractions 40–81 (concentrated)	4.5	750	30.1	95	10.7
DEAE-Affigel Blue (fractions 76–86 concentrated)	2.5	190	41.5	73	58.5
Fractogel TSK-AF Green (fractions 11–32 concentrated)	1.7	58.8	56.2	67	256.0

man[51]. Others have proposed techniques based upon affinity chromatography using specific ligands, with the drawback of higher cost and often difficulty of specific desorption from their ligand[52,53]. Second, it does not involve time consuming experiments, and the purified protein can be obtained within six working days. Also, the reagents involved are easily available, relatively inexpensive and can be re-used for several experiments.

Special attention has been given to the study of a possible denaturation of the protein. Indeed Gc is fragile and can be easily altered during its purification process[54]. Furthermore, the recent evidence for the role of Gc both as an actin-binding protein and as an important component of the cytoskeleton implies the use in such experiments of a protein whose properties are as close as possible to those of the native protein. We therefore used, in addition to classical immunoelectrophoresis and molecular weight determination, functional techniques to verify the absence of apparent denaturation of Gc. These included the ability to form complexes with its two known ligands, 25-hydroxycholecalciferol and G-actin (Fig. 4). Regarding its interaction with G-actin, measurement of the affinity constant appears to be the most accurate method to detect any alteration of native properties[26,27]. It is therefore important to

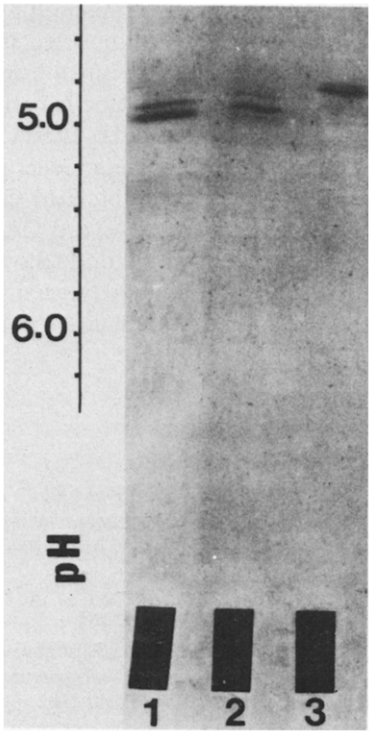

Fig. 4. Thin-layer analytical isoelectric focusing of purified Gc and its complexes with ligands. Lane 1: Gc_1 after three purification steps. Note the typical microheterogeneity with the fast band (p*I*, 4.92) and the slow band (p*I*, 4.98). Lane 2: Gc_1 incubated with saturating amounts of 25-hydroxycholecalciferol. Lane 3: Gc_1 mixed with purified G-actin at a 1:1 molar ratio. Note the characteristic anodal shifts of the Gc bands before complexation with the ligands. Stain: Coomassie Blue R-250 0.5%. The anode is at the top.

stress that the affinity constant obtained ($1.9 \cdot 10^8\ M^{-1}$) was similar to or somewhat better than that reported by others[55]. Similarly, the binding of Gc to 25-hydroxycholecalciferol as analyzed by the characteristic change in p*I*, which accompanies their complex formation, showed that no apparent alteration of the physicochemical and biological properties of Gc could be detected.

Finally, our procedure illustrates the versatility of the "tandem chromatography" system using different dye columns in sequence as a combination for the purification of plasma proteins. This system, involving a "negative" and "positive" column as proposed by Hey and Dean[2], can be especially useful in the separation of certain proteins having different affinities for the dye gels, as in the above example where Gc does not bind to the Fractogel TSK-AF Green, whereas its usual contaminant (ceruloplasmin) binds to this gel and is released by a salt gradient. In addition, apart from this important practical aspect, further studies on the differences in the behavior of Gc towards two closely related triazine dyes could contribute to a better understanding of protein–dye mechanisms of interaction[10].

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