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Purification of Vitamin D Binding Proteins (Group-Specific Components) in Human Plasma Using a Chromatofocusing Method[1)]

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Human plasma vitamin D binding proteins (DBP, also denoted as group-specific components; Gc) were highly purified from commercial human α-globulin (also denoted as Cohn Fraction IV; HαG). Chromatofocusing separated the partially purified HαG into three DBPs which had the same molecular size in gel permeation high-performance liquid chromatography. They each exhibited binding affinity with 25-hydroxyvitamin D_3 [25(OH)D_3] and cross-immunoreactivity against anti-human plasma group-specific components (Gc) antiserum. However, the three DBPs were completely different in charge on chromatofocusing, and gave distinct peaks having apparent isoelectric points of 4.64, 4.59 and 4.54. It is well known that the Gc has three phenotypes separable only by isoelectric focusing. The relationship between the three purified DBPs and the three phenotypes of Gc has not been clarified. However, we suggest that the chromatofocusing method may provide a convenient procedure for the purification of DBP from human plasma and commercial HαG.

Keywords——vitamin D binding protein; human alpha globulin; Cohn Fraction IV; gel filtration chromatography; affinity chromatography; ion exchange chromatography; gel permeation high-performance liquid chromatography; chromatofocusing

It has been shown that vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 [25(OH)D_3] in the liver and subsequently to 1α,25-dihydroxyvitamin D_3 [1α,25-(OH)$_2D_3$] or 24*R*,25-dihydroxyvitamin D_3 [24*R*,25-(OH)$_2D_3$] in the kidneys at lower or higher calcium concentrations than normal, respectively.[2,3)] Vitamin D_3 and its metabolites are transported to the target tissues by circulating as forms bound with a specific binding protein denoted as vitamin D binding protein (DBP) in blood,[3b)] as is the case with most steroid hormones. Recently, the DBPs were isolated and purified from the plasma of humans,[4)] rats[5)] and chicks[6)] and their physicochemical properties were reported. The human DBP is synthesized in the liver[4f)] and circulates in the blood at concentrations (6—8 μM) higher than those of other steroid hormone binding proteins.[4d,e)] The reported physicochemical properties of human DBP are as follows: (a) it is a glycoprotein with a molecular weight of 52000—58000 daltons[4a-c)]; (b) it has a binding site per 1 mol of protein[4a-c)]; (c) it has high affinity for 25(OH)D_3 and 24*R*,25-(OH)$_2D_3$ whereas the affinity for vitamin D_3 and 1α,25-(OH)$_2D_3$ is comparatively low.[3b)] However, detailed information on the higher structure of DBP, the structure of the glyco-moiety and the binding site and mode is not yet available. For further investigations, a convenient method to obtain a large quantity of purified DBP is essential. In this paper, we describe a suitable procedure, including chromatofocusing, for the purification of DBP from commercial human α-globulin, Cohn Fraction IV (HαG).

Experimental

Chemicals——Commercial grade (Duphar Co., The Netherlands) of 25(OH)D_3 was used as a standard. HαG

used as a source of DBP was purchased from Miles Co. (U.S.A.), while human serum albumin (Fraction V), human γ-globulin, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome c were obtained from Sigma Co. (U.S.A.). Anti-human plasma group-specific components (Gc) antiserum was purchased from Dako Co. (Denmark), while Sephadex products, Blue Sepharose CL-6B, DEAE-Sepharose CL-6B and Polybuffer 74 were purchased from Pharmacia Fine Chemicals Co. (Sweden). Organic solvents were purified by the usual methods and distilled before use. Other chemicals were of analytical grade.

Incubation of HαG with 25(OH)D_3——A solution of 40 μg of 25(OH)D_3 in 0.5 ml of ethanol was added to 800 mg of powdered HαG in 40 ml of 1/15 M phosphate buffer solution (pH 7.4) containing 0.15 M NaCl and 0.03% of sodium azide, and the mixture was incubated for 48 h at 4 °C. The incubated solution was directly subjected to the following isolation and purification steps.

Purification of Human Plasma DBP from HαG——The incubated solution obtained above (40 ml) was successively subjected to gel filtration on Sephadex G-200 (5 × 65 cm) and G-100 (5 × 65 cm) columns, affinity chromatography on a Blue Sepharose CL-6B column (2 × 89 cm), ion exchange chromatography on a DEAE-Sepharose CL-6B column (2.5 × 18 cm) and again the same affinity chromatography as mentioned above, yielding partially purified human DBP. The experimental conditions were described in our previous reports.[7] The DBP fraction thus obtained was further purified by chromatofocusing using a Mono P HR 5/20 column (Pharmacia Fine Chemicals Co., Sweden) according to the previous report.[7c]

Procedures for Analysis——Assay of 25(OH)D_3 in the eluates was performed according to the previous reports.[7] Protein was determined by the method of Lowry *et al.*[8] using human serum albumin as a standard protein. Polyacrylamide disc gel electrophoresis was performed by the method of Davis[9] using a separating gel containing 7.5% (w/v) polyacrylamide with a running buffer of Tris–glycine (pH 8.9). The molecular weight of the purified human DBP was determined by the method described in the previous paper.[7a] Immunodiffusion in gel was performed by the method of Ouchterlony[10] using gel prepared from 1% agarose in Tris barbiturate buffer (pH 8.6, ionic strength = 0.02). The procedure was described in the previous paper.[7c]

Results

Identification of DBP in HαG

In order to investigate the presence of DBP in HαG, the following studies were performed. After dissolving 10 mg of powdered HαG in 20 ml of the phosphate buffer solution (pH 7.4), 25 μg of 25(OH)D_3 in 0.3 ml of ethanol was added, and the mixture was incubated as described in Experimental. When the difference spectrum of the incubated solution (in phosphate buffer) was estimated by using the HαG solution without addition of 25(OH)D_3 as a reference solution, a characteristic spectrum of 25(OH)D_3 was observed, as shown in Fig. 1. Then, the incubated solution was subjected to gel filtration on a Sephadex G-75 column (2.4 × 75 cm) and the protein fractions bound to 25(OH)D_3 (fraction no. 14—23) were collected. About 54% of the incubated 25(OH)D_3 was found in the fractions as a bound form.

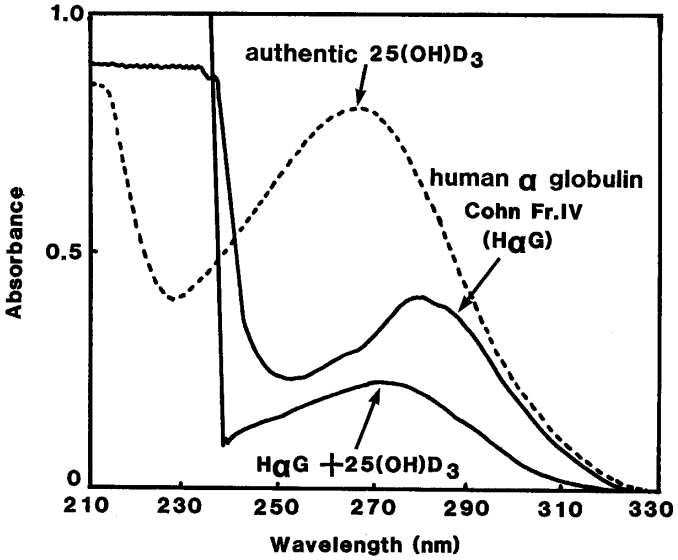

Fig. 1. Ultraviolet Spectra of HαG and 25(OH)D_3

In immunodiffusion, the HαG solution gave a clear single line of immunoprecipitation against anti-human Gc antiserum. Since the existence of DBP in commercial HαG was confirmed by these preliminary experiments, the HαG was used as a source of human DBP in the following investigations.

Purification of Human Plasma DBP from HαG

Human plasma DBP was purified as described in Experimental. Figure 2 shows the profile of the affinity chromatography on Blue Sepharose CL-6B performed before chromatofocusing. The protein fractions bound to $25(OH)D_3$ (fraction no. 29—33) were collected. At this stage, the yield of protein was 2.8 mg and the DBP was purified about 50.4-fold from the original HαG.

The partially purified DBP obtained above was chromatofocused as described in Experimental for further purification. As shown in Fig. 3, it was separated into three peaks having different isoelectric points. The peaks were denoted as P-1, P-2 and P-3 in order of elution. When each separated peak was independently subjected to the same chromatofocusing, a single peak was obtained, showing that the purification was essentially complete. All three peaks had binding ability with $25(OH)D_3$. However, the degrees of binding were different: about 85—90% of $25(OH)D_3$ was bound with P-2 while the remainder was bound with the other two.

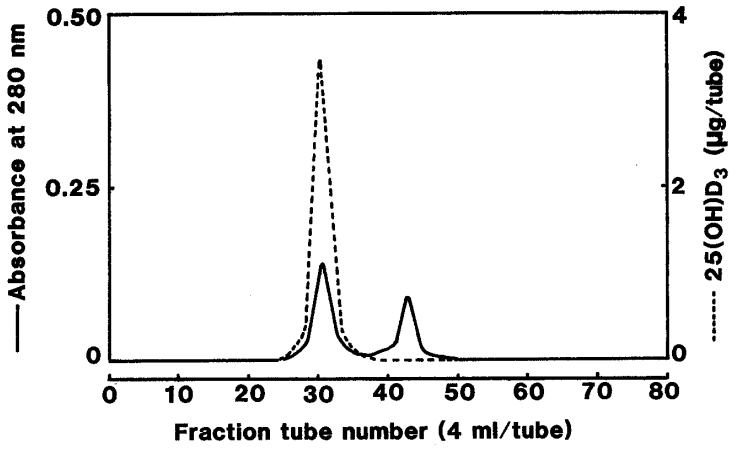

Fig. 2. Profile on Final Affinity Chromatography of Human DBP on a Blue Sepharose CL-6B Column

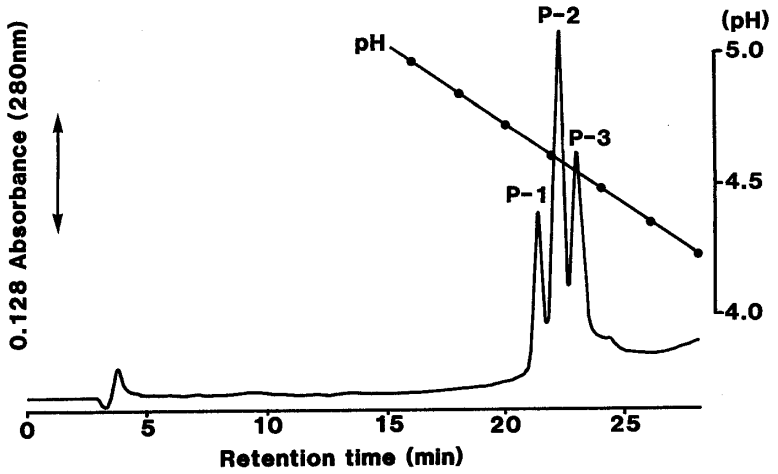

Fig. 3. Profile on Chromatofocusing of Human DBP on a Mono P Column

Physicochemical and Immunological Characteristics of Human Plasma DBP

1) Polyacrylamide Disc Gel Electrophoresis——The purified P-1, P-2 and P-3 were subjected to polyacrylamide disc gel electrophoresis. As shown in Fig. 4, P-1, P-2 and P-3 each gave a sharp, single band of protein. A mixture of the three peaks gave two bands (Fig. 4D); the upper band (near the cathode) contained P-1 while the lower one (near the anode) contained P-2 and P-3.

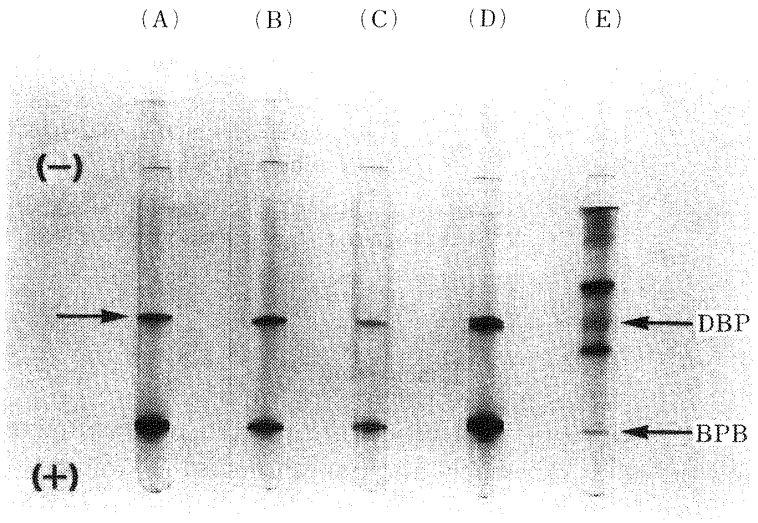

Fig. 4. Polyacrylamide Disc Gel Electrophoreses of Purified P-1, P-2 and P-3 Fractions of Human DBP

(A) P-1; (B) P-2; (C) P-3; (D) A+B+C; (E) human α globulin, Cohn Fr. IV. BPB: bromphenol blue.

2) Determination of Molecular Weight——Figure 5 shows the high performance liquid chromatographic (HPLC) profiles of P-1, P-2 and P-3 on a gel permeation column (see Experimental). The fractions each gave a single peak showing the same retention time (95.7 min), which suggested that the three peaks have the same molecular weight. When the retention time was compared with a calibration curve prepared with five standard proteins (human serum γ-globulin, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome c), the apparent molecular weight was calculated to be 57000.

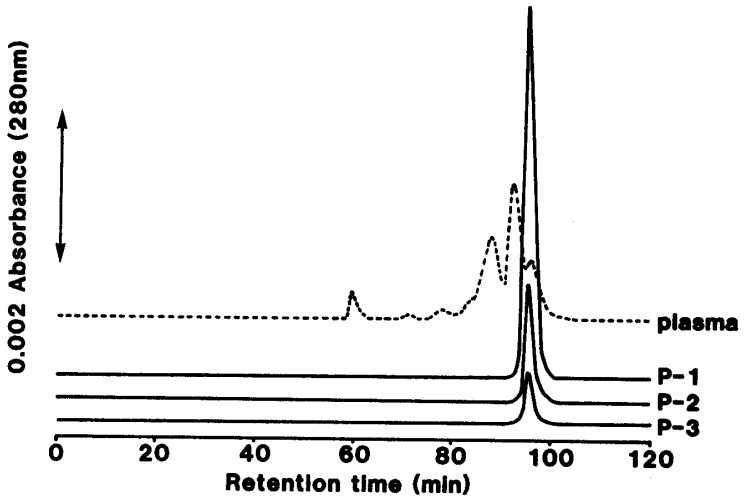

Fig. 5. HPLC Profiles of Purified P-1, P-2 and P-3 Fractions of Human DBP on a Gel Permeation Column

3) Immunodiffusion of P-1, P-2 and P-3 against Anti-human Gc Antiserum——When the anti-human Gc antiserum was allowed to diffuse in gel against P-1, P-2 and P-3, a clear single fused line of immunoprecipitation was formed as shown in Fig. 6.

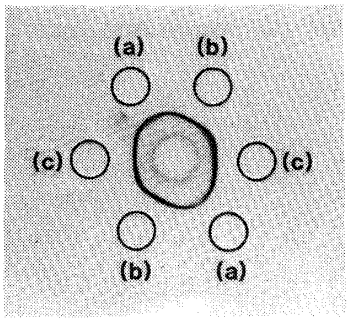

Fig. 6. Ouchterlony Immunodiffusion of Purified P-1, P-2 and P-3 Fractions of Human DBP against Anti-human Plasma Gc Antiserum

(a) human plasma P-1 fraction; (b) human plasma P-2 fraction; (c) human plasma P-3 fraction.

Discussion

In previous papers,[7] we reported a procedure for the purification of DBP from rat plasma or lymph, and the physicochemical and immunological characteristics were clarified. The procedure included gel filtration, affinity chromatography, ion exchange chromatography and chromatofocusing. In this study, a similar procedure was successfully applied for the purification of human plasma DBP from commercial HαG.

Several liters of human plasma were formerly required to obtain purified DBP,[4c] and such a large volume of human plasma is not readily available. Moreover, to purify DBP directly from human plasma, time-consuming dialysis is essential for desalting. On the other hand, Haddad and Walgate[4a] proposed HαG as a source for purifying DBP instead of human plasma, because the product was confirmed to contain a 2.1 times higher concentration of DBP than human plasma. This simplified the purification, but the preparative gel electrophoresis used as the final purification step still required 12—16 h for separation. Therefore, we used chromatofocusing for the final purification step, since it could be performed in only 25 min.

Chromatofocusing is a new method for purifying proteins by focusing in a pH gradient developed in the column without using an electrophoretic system. This method gives very good reproducibility and about 25 mg of protein can be loaded on a column for an analysis. By this method, we separated one major peak (P-2) and two minor peaks (P-1 and P-3) as shown in Fig. 3. P-2 showed a higher binding affinity to $25(OH)D_3$ than the others. The three proteins (P-1, P-2 and P-3) showed the same immunological characteristics and similar molecular weight (about 57000), though their isoelectric points differed from one another.

In 1959, Hirschfeld[11] reported the presence of Gc in the α-globulin fraction of human sera by an immuno-electrophoretic technique. The Gc-globulin was classified into three phenotypes (Gc 1-1, Gc 2-1 and Gc 2-2) on the basis of immuno-electrophoresis.[12] On the other hand, Daiger *et al.*[13] observed that radioactivity of plasma samples containing bound [^{14}C]vitamin D_3 or [^{3}H]$25(OH)D_3$ was associated with the Gc-globulin on polyacrylamide gel electrophoresis and immuno-electrophoresis. They concluded from these and other results that Gc-globulin and DBP were the same protein. Recently, Constants and Viau[14] investigated isoelectric focusing of the Gc-globulin and found that the Gc 1 phenotype could be classified into two Gc subtypes denoted as Gc 1F and Gc 1S (F and S mean fast and slow migration of bands on isoelectric focusing polyacrylamide plates). They concluded that the Gc-globulin included three alleles (Gc-1F, Gc-1S and Gc-2S) which provided the six

phenotypes denoted as Gc 1F-1F, Gc 1S-1S, Gc 1F-1S, Gc 2S-1F, Gc 2S-1S and Gc 2S-2S.[14] The decision of blood types using the six phenotypes has become available as a new method, like the ABO and MN systems.[15]

Since the three proteins (P-1, P-2 and P-3) purified by us showed binding ability with $25(OH)D_3$ and cross-immunoreactivity against anti-human plasma Gc antiserum, it was strongly suggested that they were identical with Gc-globulin. Therefore, the utilization of chromatofocusing for the determination of blood types based on these phenotypes might be possible. In this study, we could not establish the relationship between the three proteins and the phenotypes, but further studies on this problem are in progress.

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