Purification and Characterization of Human Serum C-Reactive Protein

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A simple and rapid purification method for human serum C-reactive protein (CRP) was developed. CRP was strongly adsorbed on a DEAE-cellulose column and was easily separated from other serum proteins. CRP was purified approximately 1,000-fold with a high yield (50%). The final preparation showed a single band as judged by SDS-polyacrylamide gel electrophoresis, polyacrylamide gel disc electrophoresis, and polyacrylamide gel isoelectric focusing.

The fluorescence of the complex of CRP and 8-anilino-1-naphthalene sulfonate (ANS) changed with change of the pH, suggesting that CRP may show pH-dependent conformational change. This finding could account for the peculiar behavior of the protein in isoelectric focusing; it shows an isoelectric point of 7.4 when the starting pH is 7.0, whereas it shows two isoelectric points, 5.3 and 7.4, when the starting pH is 5.5. Ca\(^{2+}\)-dependent change of the fluorescence of the complex of CRP and ANS was also detected. These results suggest a pH- and Ca\(^{2+}\)-dependent conformational change of CRP.

C-Reactive protein (CRP) is one of the characteristic proteins which appear in serum in cases of infection or tissue injury resulting from trauma, burns or a variety of inflammatory diseases. CRP is reported to mediate many reactions; precipitation with C polysaccharide derived from the cell wall of *Streptococcus pneumoniae* (1), agglutination of lipid suspensions containing phosphatidylycholine, cholesterol, and Span 60 (2), binding to liposomes containing positively charged substances (3) and interaction with certain polycations (4). It is believed that CRP may participate in the non-specific resistance to infections.

Several chemical and physicochemical properties of CRP, such as apparent molecular weight (5), subunit structure (6), amino acid composition (6), and primary structure (7), have been reported. There have also been several reports in connection with the isolation of CRP from pooled ascites fluid (8–10), but CRP obtained by these methods often contained other contaminant proteins.

In this report, we describe a simple and rapid method for purification of CRP from pooled sera, and the characterization of some properties of CRP purified by this method. We also report a pH- and Ca\(^{2+}\)-dependent conformational change of CRP.
MATERIALS AND METHODS

Chemicals—Egg yolk phosphatidylcholine was prepared by chromatography on columns of neutral aluminum oxide and Unisil. Cholesterol was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Span 60 was from Wako Pure Chemical Co., Tokyo, Japan.

CRP-Positive Sera—Pooled CRP positive sera were kindly provided by the Section of Serology, Central Clinical Laboratory, University of Tokyo Hospital.

Preparation of Lipid Suspensions—Preparation of lipid suspensions was described earlier (2). In brief, a mixture of 9 mg of cholesterol, 3 mg of egg yolk phosphatidylcholine and 6 mg of Span 60 was dispersed in 5 ml of veronal-buffered saline (pH 7.4) containing 0.15 mM CaCl₂ and 0.45 mM MgCl₂.

Agglutination of Lipid Suspensions—Quantitative assay of CRP was performed by following the agglutination of lipid suspensions as described earlier (2). In brief, the change of intensity of light scattering with time was measured as the change in optical density with time using a spectrophotometer (Shimadzu UV-140) with constant shaking at room temperature. The change of optical density was measured at 340 nm.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (11) with columns of 10% polyacrylamide gel containing 1% SDS. Denatured proteins were prepared by incubating proteins in boiling water for 5 min in a solution containing 1% SDS, 2% mercaptoethanol, and 25% glycerol. Electrophoresis was performed at room temperature for 10 h at 5 mA per tube. The standard proteins used for molecular weight calibration were as follows: bovine serum albumin (67,000), hen ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700).

Isoelectric Focusing Fractionation—Isoelectric focusing fractionation was carried out with an Ampholine column (column size, 110 ml), using an LKB apparatus as developed by Vesterberg and Svensson (12). The pH range of carrier ampholyte was 3–10 or 4–6 and electrophoresis was carried out for 40 h at constant voltage (300 V) with a cooling system (4°C). Fractions of 2 ml were collected and the pH values of these fractions were measured with a Hitachi-Horiba pH meter. Protein fractions were dialyzed overnight against veronal buffer and then CRP activity was determined.

Amino Acid Analysis—Protein samples (30 μg) were hydrolyzed in evacuated tubes for 24, 48, and 72 h at 110°C in 0.15 ml of 6 N HCl. The amino acid analysis was carried out with a Hitachi amino acid analyzer, Model 835-50. Cysteine and cystine were estimated as cysteic acid after performic acid oxidation (13).

Fluorescence Measurement—The fluorescence dye 8-anilino-1-naphthalene sulfonate (ANS) (Tokyo Kasei Co., Tokyo) was used as a probe. A Shimadzu RE-501 spectrophotofluorimeter was used with an excitation wavelength of 413 nm. The emission curve of 10 μM ANS was measured in the presence or absence of 10 ng of purified CRP. A solution was made up to 0.5 ml with 10 mM Tris-maleate buffer. All these fluorescence emission curves were measured at 25°C in quartz cells.

Polyacrylamide Gel Disc Electrophoresis—Disc electrophoresis was carried out according to Davis (14). Electrophoresis was run with 5% acrylamide and Tris-glycine buffer (pH 9.5) for 90 min at room temperature in the presence or absence of 1 mM CaCl₂.

Polyacrylamide Gel Isoelectric Focusing—Polyacrylamide gel isoelectric focusing was performed on 5% polyacrylamide gel as described by Finlayson and Chrambach (15). Gels contained 2% Ampholine (pH 3–10). The anolyte and catholyte were 20 mM phosphoric acid and 1 M NaOH, respectively. Samples were applied on the cathodic side of the gel and were focused at 100 V for 1 h then at a constant voltage of 200 V until the end of a run (5 h total time). Focused gels were stained with 0.1% Coomassie Brilliant Blue G250. Standard pI markers were purchased from Oriental Yeast Co., Osaka, Japan.

RESULTS

Purification of CRP from CRP-Positive Human Sera by DEAE-Cellulose Column Chromatography—A simple and effective purification procedure for human serum CRP was developed using DEAE-cellulose (Whatman DE52) column chromatography.
DEAE-cellulose column chromatography of human CRP-positive sera. (A) Human sera containing CRP (10 ml) were dialyzed against 500 ml of 0.01 M sodium phosphate buffer (pH 8.1), and loaded onto a DEAE-cellulose column (1.5 x 12 cm) equilibrated with the same buffer. Chromatography was carried out at 4°C. The column was washed with 100 ml of 0.01 M sodium phosphate buffer (pH 8.1) (E_1), 180 ml of 0.04 M sodium phosphate buffer (pH 5.9) (E_2), 150 ml of 0.1 M sodium phosphate buffer (pH 5.8) (E_3), 160 ml of 0.4 M sodium phosphate buffer (pH 5.2) (E_4), and 50 ml of 0.4 M sodium phosphate buffer (pH 4.5) containing 2 M sodium chloride (E_5). The effluent was collected in 5 ml fractions. The protein concentration (O) of each fraction was determined by measuring the absorbance at 280 nm, and the CRP activity (●) was measured by the method described in "MATERIALS AND METHODS." (B) Rechromatography of human CRP on a DEAE-cellulose column. CRP fraction obtained from the first DEAE-cellulose column was applied to the second DEAE-cellulose column (1.5 x 8 cm) and eluted successively with 80 ml of 0.4 M sodium phosphate buffer (pH 5.2) (E_4) and 50 ml of 0.4 M sodium phosphate buffer (pH 4.5) containing 2 M sodium chloride (E_5). The effluent was collected in 3 ml fractions.

It was found that CRP was strongly adsorbed on DEAE-cellulose and was eluted with an acidic buffer (high ionic strength). A typical procedure for purification of CRP was as follows: CRP-positive serum (10 ml) was dialyzed against 500 ml of 0.01 M sodium phosphate buffer (pH 8.1) and then applied to a DEAE-cellulose column (1.5 x 12 cm), which was equilibrated with the same buffer. Stepwise elution was carried out with sodium phosphate buffers (Fig. 1A). All of the CRP activity was recovered in a fraction eluted by 0.4 M sodium phosphate buffer (pH 4.5) containing 2 M NaCl. Only two bands other than CRP were detected in this fraction when it was examined by SDS-polyacrylamide gel electrophoresis. Upon rechromatography of the fraction showing CRP activity on a DEAE-cellulose column, all of the CRP activity was again eluted only with 0.4 M sodium phosphate buffer (pH 4.5) containing 2 M NaCl (Fig. 1B). The purity was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 2), polyacrylamide gel disc electrophoresis, and polyacrylamide gel isoelectric focusing. The molecular weight of CRP was estimated to

Fig. 1. DEAE-cellulose column chromatography of human CRP-positive sera. (A) Human sera containing CRP (10 ml) were dialyzed against 500 ml of 0.01 M sodium phosphate buffer (pH 8.1), and loaded onto a DEAE-cellulose column (1.5 x 12 cm) equilibrated with the same buffer. Chromatography was carried out at 4°C. The column was washed with 100 ml of 0.01 M sodium phosphate buffer (pH 8.1) (E_1), 180 ml of 0.04 M sodium phosphate buffer (pH 5.9) (E_2), 150 ml of 0.1 M sodium phosphate buffer (pH 5.8) (E_3), 160 ml of 0.4 M sodium phosphate buffer (pH 5.2) (E_4), and 50 ml of 0.4 M sodium phosphate buffer (pH 4.5) containing 2 M sodium chloride (E_5). The effluent was collected in 5 ml fractions. The protein concentration (O) of each fraction was determined by measuring the absorbance at 280 nm, and the CRP activity (●) was measured by the method described in "MATERIALS AND METHODS." (B) Rechromatography of human CRP on a DEAE-cellulose column. CRP fraction obtained from the first DEAE-cellulose column was applied to the second DEAE-cellulose column (1.5 x 8 cm) and eluted successively with 80 ml of 0.4 M sodium phosphate buffer (pH 5.2) (E_4) and 50 ml of 0.4 M sodium phosphate buffer (pH 4.5) containing 2 M sodium chloride (E_5). The effluent was collected in 3 ml fractions.

Fig. 2. SDS-polyacrylamide gel electrophoresis of CRP purified by DEAE-cellulose column chromatography. Purified CRP was subjected to SDS-polyacrylamide gel electrophoresis for 10 h at 5 mA per gel using 10% polyacrylamide gel. The gel was stained with 0.05% Coomassie Brilliant Blue R250 in a mixture of 10% acetic acid (v/v) and 30% isopropanol (v/v), and was then destained with 10% acetic acid (v/v).
be 21,000, as found by Gotschlich and Edelman (6).

Table I shows the result of amino acid analysis of CRP purified by DEAE-cellulose column chromatography. The amino acid composition of CRP was consistent with that of CRP purified from ascites fluid, which was described by Oliveira et al. (7).

The purification procedure is summarized in Table II. CRP was purified approximately 1,000-fold with a fairly good yield.

**TABLE I.** Amino acid composition of CRP purified by DEAE-cellulose column chromatography.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Present work a</th>
<th>Oliveira et al. (7)</th>
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<tr>
<td>Asp</td>
<td>15</td>
<td>15</td>
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<tr>
<td>Thr</td>
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</tr>
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<td>Ser</td>
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</tr>
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</tr>
<tr>
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<td>2</td>
</tr>
<tr>
<td>Val</td>
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</tr>
<tr>
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<td>9</td>
</tr>
<tr>
<td>Leu</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Thr</td>
<td>8d</td>
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<tr>
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</tr>
<tr>
<td>Lys</td>
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</tr>
<tr>
<td>His</td>
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<td>2</td>
</tr>
<tr>
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<tr>
<td>Trp</td>
<td>—e</td>
<td>5</td>
</tr>
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</table>

a The values were calculated by assuming the number of leucine residues to be 14. b Determined as cysteic acid. c Value obtained at 72 h was used. d Extrapolated to 0 time of hydrolysis. e Not determined.

**TABLE II.** Purification of human serum CRP by DEAE-cellulose column chromatography. For details of the purification procedure, see "MATERIALS AND METHODS." One unit of CRP was defined as the quantity of CRP required for inducing the agglutination of lipid suspension measured as 0.2 change of $A_{380}$.

<table>
<thead>
<tr>
<th></th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
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</thead>
<tbody>
<tr>
<td>I Original sera</td>
<td>961.5</td>
<td>385</td>
<td>0.4</td>
<td>—</td>
</tr>
<tr>
<td>II First DEAE-cellulose</td>
<td>1.38</td>
<td>239</td>
<td>172</td>
<td>62</td>
</tr>
<tr>
<td>III Second DEAE-cellulose</td>
<td>0.52</td>
<td>192</td>
<td>370</td>
<td>50</td>
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For large scale purification of CRP, 500 ml of dialyzed CRP positive serum was placed on a DEAE-cellulose column (6 x 20 cm), and then eluted successively with one liter of 0.4 M sodium phosphate buffer (pH 5.3) and 300 ml of 0.4 M sodium phosphate buffer (pH 4.5) containing 2 M NaCl. Pooled CRP fraction was rechromatographed on a DEAE-cellulose column. The procedure gave purified CRP as judged from the results of SDS-polyacrylamide gel electrophoresis.

Isoelectric Focusing of CRP—Isoelectric focusing of partially purified CRP in 1% Ampholine (pH range 3–10) is shown in Fig. 3A. The activity of CRP showed a single peak having an isoelectric point (pI) of 7.4. The collected fraction with CRP activity showed a single band on SDS-polyacrylamide gel electrophoresis (M.W. 21,000).

As shown in Fig. 3B, however, when 1% Ampholine with a different pH range (4–6) was used as the carrier ampholite, CRP activities were separated into at least two peaks. These peaks have pI values of 5.3 and above 7.0. The latter peak may correspond to the peak showing pI 7.4 in the Ampholine (pH 3–10). Alteration of the protein concentration did not affect the ratio of the peak showing pI 5.3 to the peak showing pI 7.4. In both cases, a contaminating protein, which was identified as albumin by SDS-polyacrylamide gel electrophoresis and isoelectric focusing, showed a constant pI of 4.7. The variation of pI was reversible, since the protein which was recovered from the fraction showing pI 5.3 showed a pI of 7.4 on polyacrylamide gel isoelectric focusing (Ampholine pH 3–10). We obtained nearly the same result even when another brand of carrier ampholite, Pharmalyte (Pharmacia, Sweden), was used as a carrier ampholite.

Fluorescence Measurement of CRP-ANS Complex—In 10 mM Tris-malate buffer (pH 7.4), excitation of ANS at 413 nm resulted in a low fluorescence emission with a maximum at 510 nm (a in Fig. 4A). Addition of CRP caused a marked increase in fluorescence intensity, which was accompanied by a blue shift in the emission maximum to 480 nm (b in Fig. 4A). Fluorescence intensity was further increased when the pH of the solution was decreased to 5.5 (c in Fig. 4A). In the presence of Ca²⁺, no appreciable pH-dependent change of fluorescence intensity was observed (Fig. 4B). The solubility of the protein was not affected by pH change under the present experimental conditions (the concentration of CRP was...

Fig. 4. Effect of pH on the fluorescence of the CRP-ANS complex. The excitation wavelength was 413 nm. The relative emission curve of 10 µM ANS was measured in the presence and absence of CRP (10 µg) purified by DEAE-cellulose column chromatography. The fluorescence of CRP-ANS complex was measured in 10 mM Tris-malate buffer at pH 7.4 (line b, ---) and at pH 5.5 (line c, ----). Line (a) (------) shows the fluorescence of free ANS. The fluorescence was measured in the presence (B) and absence (A) of calcium ions (10 µM).

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20 μg/ml). It is interesting to note here the previous observation that CRP became insoluble in buffer below pH 7.0 (16). The pH-dependent solubility change of CRP was observed when the concentration was above 1 mg/ml. The critical pH was found to be 6.4 as determined from the pH-dependent turbidity change of CRP solution (data not shown).

Effect of Ca\(^{2+}\) on CRP Molecules—As shown in Fig. 4, the fluorescence intensity of CRP-ANS complex at pH 7.4 was affected by Ca\(^{2+}\), suggesting that Ca\(^{2+}\) may induce a conformational change of the CRP molecule.

DISCUSSION

We have developed a simple procedure for purification of CRP from human sera. The method described here gives better yields with fewer steps than other methods reported so far for purification of CRP from ascites fluid. There are several advantages to our method: 1) CRP binds strongly to DEAE-cellulose, whereas almost all other components were easily eluted, 2) the second purification step requires only a relatively small bed volume of DEAE-cellulose to remove the contaminating proteins, 3) simple buffer systems are adequate for the purification. CRP thus prepared was homogenous as examined by SDS-polyacrylamide gel electrophoresis, polyacrylamide gel disc electrophoresis, and polyacrylamide gel isoelectric focusing. James et al. (17) recently purified CRP from pooled human sera, using PC-Sepharose, DEAE-cellulose, and Sephacryl S-200. In their procedure, the protein was eluted from DEAE-cellulose using a linear NaCl gradient with constant pH. In the present experiment, we eluted the protein stepwisely by changing the pH and ionic strength simultaneously. The elution buffer system might be critical for effective separation of CRP from other proteins. In our preliminary experiment, CRP behaved in a different way on a DEAE-Toyopearl column. CRP was eluted from the column with 0.4 M sodium phosphate buffer (pH 5.2) without NaCl. This difference may suggest that CRP has affinity for the cellulose moiety. In fact, CRP is reported to bind to polysaccharides such as galactan (18). Interaction between CRP and sugar residues of DEAE-cellulose may also participate in the binding of CRP to DEAE-cellulose.

The isoelectric point of CRP has been reported to be 4.8 (19) and 7.9 (20). We found, however, that the isoelectric point changed depending upon the experimental conditions. It was 7.4 in the system of Ampholine (pH range 3-10), whereas two isoelectric points (5.3 and 7.4) were observed in the same Ampholine with a different pH range (4-6). The difference between the two experimental conditions was only in the starting pH values, which were 7.0 and 5.5 in Ampholine pH range 3-10 and 4-6, respectively. It is likely that the conformational structures of CRP showing pi 5.3 and pi 7.4 may be different from each other. It was reported that conformational change of proteins affected their isoelectric points (21).

In the Ampholine (pH range 4-6), CRP activity was recovered from both fractions showing isoelectric points of 5.3 and 7.4. The observed elution point (5.3) of CRP might be different from its real isoelectric point, since the protein in this fraction was apparently flocculated, and elution might be delayed (19). It is possible that CRP has at least two populations; one may change its conformation with lowering of the pH and the other may not, though both of them behave similarly on SDS-polyacrylamide gel electrophoresis. There is an alternative possibility that aggregation of the subunits of CRP may be affected by pH. This seems unlikely, however, since change of protein concentration had no appreciable influence on the behavior of the protein in isoelectric focusing.

The conformational change depending upon pH was supported by the fluorescence behavior of the complex of CRP and ANS. In the absence of Ca\(^{2+}\), the fluorescence intensity of CRP-ANS complex was affected by pH change, whereas the fluorescence of ANS in an aqueous solution was not. In the presence of Ca\(^{2+}\), however, pH-dependent change of fluorescence intensity was not observed. It was reported that enhancement and shift to shorter wavelengths of fluorescence were consistent with the removal of fluorescent dyes from an aqueous environment to a hydrophobic binding site on the protein (22). We conclude that the change of pH or Ca\(^{2+}\) concentration induces conformational change of one population of CRP, resulting in the change of isoelectric point and hydrophobicity. The present finding
confirms the previous observation that Ca\(^{2+}\) induced a change of CD spectra of CRP (23). It is interesting that another calcium-binding protein, calmodulin, changes in conformation when it binds Ca\(^{2+}\). These changes were detected as a change of the mobility on electrophoresis (24) or a change of fluorescence intensity of ANS (25).

Recently Potempa et al. (Potempa, L.A., Maldonado, B.A., Laurent, P., Zemel, E., & Gewurz, H., personal communication) also observed that there are two populations of purified CRP. On polyacrylamide gel isoelectric focusing analysis, the purified CRP was distributed into two forms in the presence of 8 M urea and absence of Ca\(^{2+}\); one slow-migrating and the other fast-migrating. In the presence of Ca\(^{2+}\), CRP did not split into two such populations. It is possible that CRP has at least two populations having different sensitivity to Ca\(^{2+}\). The chemical and conformational structures of the two populations of CRP should be further examined.

It was reported that in regions of inflammation and infection, the exudate pH dropped to 6.5 at 60 h after the start of the inflammatory reaction (26). The pH- and Ca\(^{2+}\)-dependent conformational change of CRP may have an important role in the maintenance of biological reactivity of CRP at the site of active inflammation.

REFERENCES


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