Simple and Efficient Purification of C-Reactive Protein from Canine Serum

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ABSTRACT. Simple purification method for canine serum, C-reactive protein (CRP) was established. By combining an affinity chromatography on Sepharose 4B coupled with o-phosphorylthanolamine and a gel filtration on a column of Sephacryl S-300, about 9 mg of purified CRP from 50 ml of canine serum was obtained. The method is simple and efficient to purify canine CRP.---KEY WORDS: affinity chromatography, canine serum, C-reactive protein.

C-reactive protein (CRP) was firstly found by Tillet and Francis in 1930 in the human serum of pneumococcus infection [14]. Thereafter, CRP has been recognized as an acute phase protein, which increases in serum in inflammations and in tissue injuries of human and other animals [12]. The increase in CRP in human serum has been studied clinically in relation to early diagnoses and prognoses in various disease [16]. Since Gotschlich [8] and Dillmann and Coles [3] reported the presence of CRP in canine serum, the CRP has also been studied for the similar purposes in canine diseases [3, 4, 6, 13]. However, the reported CRP-levels in canine serum varied depending on laboratories and clinics and seems not yet to be practically applicable for canine clinical pathophysiology, because a few methods for determination of CRP has been standardized only recently [15]. To determine CRP level in canine serum and to correlate it with canine diseases, it is essential to prepare purified canine CRP and anti-canine CRP IgG by simple and efficient procedure. For this purpose, affinity chromatography column coupled with phosphorylcholine as a ligand has been reported [7]. However, the purification method is time-consuming and complex, because the affinity column is not yet available from commercial source. In the present study, therefore, we attempted to establish the method for purification of canine CRP by using an affinity column coupled with o-phosphorylthanolamine (PEA), which is available from commercial source and has been for the purification of human CRP [11].

To prepare canine serum CRP, 0.5 ml turpentine oil per kg of body weight was injected intramuscularly into two clinically healthy mongrel dogs. After 48 hr, the animals were bled and the serum was prepared by centrifuging the clotted blood firstly at 3,500 × g for 15 min and then the supernatant at 8,000 × g for 30 min. The serum was stored at -20°C until used. Agarose coupled with PEA through divinyl sulfone bond (PEAD-agarose) was purchased from Sigma Chem., Co., St Louis, MO, U.S.A. Protein content was determined at 280 nm in UV spectrophotometer. The concentration of CRP was determined by single radial immunodiffusion method with rabbit anti-canine CRP serum. The protein concentration of purified canine CRP was determined at 280 nm by using the absorption coefficient of 17.0, which was reported for 1% human CRP [1]. Goat anti-canine IgG and anti-canine serum were purchased from Cappel Research Products, Durham, NC, U.S.A.

Table 1 shows the binding capacity and the purification rate in a chromatography of canine serum CRP on a PEAD-agarose column at pH 7.0 and 8.0. Both the binding capacity and the purification rate at pH 8.0 was higher than those at pH 7.0. The binding capacity of a PEAD-agarose column was higher than that of an affinity column coupled with phosphorylcholine as a ligand; the capacity of this column was reported to be 4.7 mg protein of canine serum CRP per ml of the gel [9]. We also examined the binding capacity of other three affinity columns for the CRP; a column of agarose coupled with PEA through epoxy bond (PEAE-agarose) (Sigma, Chem., Co.), CH- and ECH-Sepharose 4B columns (Pharmacia LKB Biotechnology, Uppsala, Sweden). The PEAE-agarose column bound 12.02 mg CRP per ml of the gel, whereas CH- and ECH-Sepharose columns were 3.57 and 1.02, respectively.

From these results, we purified canine serum CRP by an affinity chromatography on PEAD-agarose at pH 8.0 followed by gel filtration on Sephacryl S-300 superfine column (Pharmacia, Fine Chemicals, Uppsala, Sweden); the gel filtration was employed because the fraction eluted from the PEAD-agarose column showed several bands other than CRP in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Figure 1-A and -B show elution profiles of canine serum CRP from affinity chromatography on PEAD-agarose column.

Table 1. Efficiency of affinity chromatography of canine serum CRP on PEAD-agarose column

<table>
<thead>
<tr>
<th>Binding capacity&lt;sup&gt;a&lt;/sup&gt; (CRP mg/ml gel)</th>
<th>Purification rate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.0</td>
<td>pH 7.0</td>
</tr>
<tr>
<td>PEAD-agarose</td>
<td></td>
</tr>
<tr>
<td>12.4</td>
<td>10.42</td>
</tr>
<tr>
<td>234.8</td>
<td>166.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> The column (10×13 mm) was equilibrated with buffer A and 60 ml of canine serum was applied. After washing the column with the buffer, CRP was eluted with buffer B. The rate was calculated as the ratio of CRP concentration (mg/ml) per absorbance at 280 nm of the eluted fraction to that of canine serum applied.

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chromatography on PAED-agarose and subsequent gel filtration on Sephacryl S-300 column. The recoveries of CRP from the affinity chromatography and from the gel filtration (Fraction IV) were 96.2% and 84.8%, respectively; by the purification procedure developed in the present experiment, about 9 mg of purified CRP was obtained from 50 ml of canine serum. Figure 2 shows SDS-PAGE of the fractions from Sephacryl S-300 column. Only two protein bands in fraction IV were seen, whereas, in addition to these two main bands, several protein bands and a very faint band were observed in fractions I through III and fraction V, respectively. In agar gel double immunodiffusion test, the purified CRP (Fraction IV, Well No. 3 in Fig. 3) formed only one precipitin line with rabbit anti-canine CRP serum (Well No. 2 in Fig. 3). In agar gel double immunodiffusion and immunoelectrophoresis, the canine CRP cross-reacted with goat anti-human CRP (Nippon Biotest Lab., Co., Tokyo) (Data not shown).

It has been reported that canine CRP is comprised of 5 subunits, which are consisted of 2 subunits of 26 kD and 3 of 22 kD [2, 7]. In the present study, SDS-PAGE of the purified canine CRP showed only 2 bands, 28 kD and 24 kD, indicating that these 2 correspond to these subunits of the CRP. Moreover, in agar gel double immunodiffusion, the purified CRP showed one precipitin line with anti-canine CRP serum. This again shows that the canine CRP prepared by the present purification method was highly purified. The method is simple and efficient to purify canine CRP and is applicable for preparation of a large amount of the canine CRP, which is required to produce specific anti-canine CRP serum, because CRP is immunologically crossreactive among different mammalian species, the immunogenecity of CRP is low and a small amount of CRP always exists in blood circulation.
Fig. 3. Agar gel double immunodiffusion test of purified canine serum CRP. Center well, goat anti-canine IgG (20 µl). Lateral wells (1) and (4), canine serum IgG (prepared by ammonium sulfate precipitation followed by gel filtration on Sephacyrl S-300) (500 µg/ml); (2), rabbit anti-canine CRP serum; (3) and (6), purified canine CRP (1,000 µg/ml); (5), goat anti-canine serum. Agar gel, 1.5% agar in barbital buffer (ionic strength 0.05; pH 8.6); distance between every two wells, 3 mm. The gel plate was incubated at 20°C for 2 days and each well contained 20 µl of the samples. Rabbit anti-canine CRP serum was prepared by injected 0.3 mg of purified canine CRP emulsified with Freund’s complete adjuvant (Wako, Pure Chem., Co., Osaka) 3 times at 12-days interval and then 0.6 mg without the adjuvant 5 times at a week interval as booster injections.

REFERENCES