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Recombinant canine C-reactive protein as a reliable calibrator for different types of cCRP assays

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Introduction

C-reactive protein (CRP) is a major acute phase protein in dogs. Its concentration increases rapidly during the systemic inflammation and subsequently, decreases quickly after the elimination of the source of inflammation. In healthy animals cCRP concentration in the blood is about 0.5-2 mg/L, while during an acute inflammatory response cCRP concentration increases to more than several hundreds mg/L. As the cCRP level in the blood correlates well with the severity of the inflammation it is used as an inflammatory marker in dogs.

A number of cCRP assays based on monoclonal or polyclonal antibodies as well as CRP ligand phosphocholine have been developed. In order to minimize between-assay discrepancy, choice of the calibration material would be important. Due to the heterogeneity of the native protein it would be preferable to use recombinant protein as a common calibrator.

The aim of present study was to express, purify and compare immunochemical and biochemical properties of recombinant cCRP (cCRPrec) with the endogenous cCRP (cCRPend).

Methods

cCRPrec was expressed in insect cells. The amino acid sequence of cCRPrec was identical to that of cCRPend. cCRPend was purified from serum of dogs with inflammation.

Monoclonal antibodies (mAbs) cCRP1 and cCRP11 were raised against the native cCRP. Sandwich immunoassay using these mAbs was used to compare immunoreactivity of cCRPend and cCRPrec. Detection mAb was labeled with a stable europium chelate.

Pro-Q Emerald 300 (Thermo Scientific) was used for staining sugar residues. SYPRO Ruby protein gel stain (Thermo Scientific) was used for staining both glycosylated and non-glycosylated cCRP subunits.

cCRPend and cCRPrec were analyzed by MALDI-TOF/TOF method (Bruker UltrafleXtreme).

Gel-filtration (GF) was performed on Superdex 200 10/300 GL (GE Healthcare) column to confirm cCRP oligomerization.

Results

Purified cCRPrec and cCRPend were analyzed by the GF. The immunological activity of cCRP in fractions was detected after GF with the pair of monoclonal antibodies cCRP11-cCRP1 (Fig. 1). Both proteins eluted in a single major peak after GF. Immunoreactivity coincided with the absorption peaks.

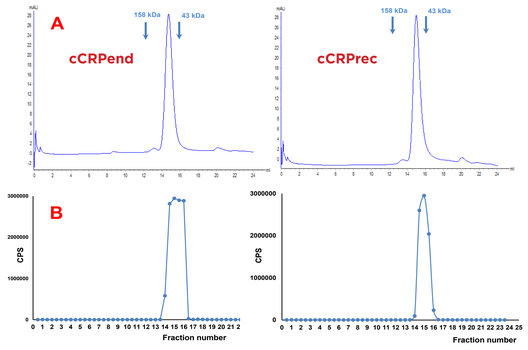

FIGURE 1. Gel filtration of purified cCRPrec and cCRPend.
A: Optical density profile of eluate at 280 nm.
B: Immunoreactivity was determined in sandwich ELISA using an assay **cCRP11-cCRP1**.

Native PAGE confirmed that cCRPrec corresponds to the oligomeric form like the native protein (Fig. 2A). It was shown that both cCRPend and cCRPrec consist of glycosylated and non-glycosylated subunits (Fig . 2B).

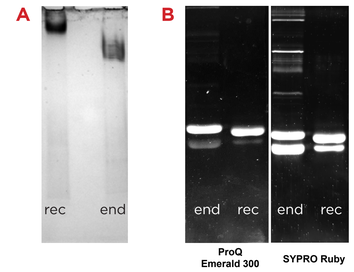

FIGURE 2. Gel electrophoresis of cCRPrec and cCRPend.
A: Native PAGE of cCRPrec and cCRPend.
B: Staining of cCRPrec and cCRPend for sugar residues (Pro-Q Emerald 300) and for total proteins (SYPRO Ruby). SDS-PAGE, denaturing conditions.

MS studies (Fig. 3) revealed that cCRPend comprises two major peaks with molecular weights 23 209 Da and 25 429 Da. cCRPrec comprises two major peaks 23 208 Da and 24 400 Da. Lower mass corresponds to non-glycosylated while heavier mass corresponds to glycosylated subunits. The difference in molecular masses of heavy subunits is explained by the difference in the glycosylation pattern.

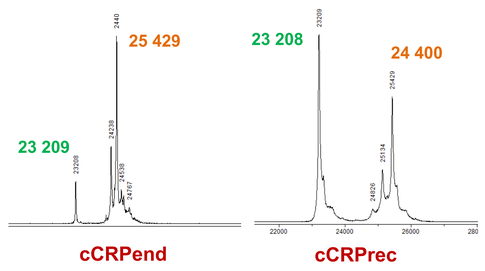

FIGURE 3. Mass spectrometry analysis of cCRPend and cCRPrec.

Both cCRPend and cCRPrec revealed similar immunoreactivity in a sandwch type immunoassay (Fig. 4A). cCRPrec can be used in systems for cCRP immunodetection based on the interaction between cCRP and phosphocholine (Fig. 4B).

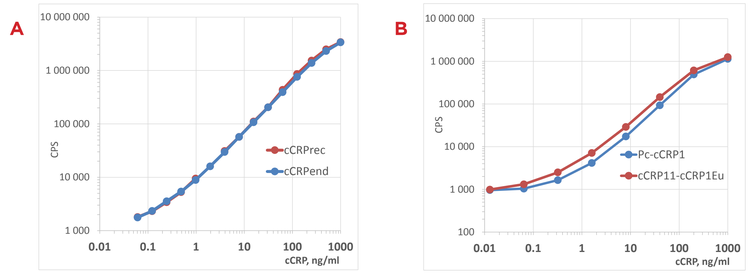

FIGURE 4. Titration curves for cCRPend and cCRPrec.
A: The pair of mAbs **cCRP11**(capture)-**cCRP1**(detection).
B: cCRPrec immunodetection in two types of sandwich immunoassays: **phosphocholine** (capture)-**cCRP1** (detection) (blue) and **cCRP11** (capture)-**cCRP1** (detection) (red).

While cCRP in animal blood is presented as a homopentamer for its immunodetection it is possible to use a sandwich immunoassay utilizing one and the same antibody for capture and detection (Fig. 5). Our analysis revealed that cCRPrec is recognized by such "homosandwich" immunoassay, thus suggesting that it is presented in preparation in the oligomeric form.

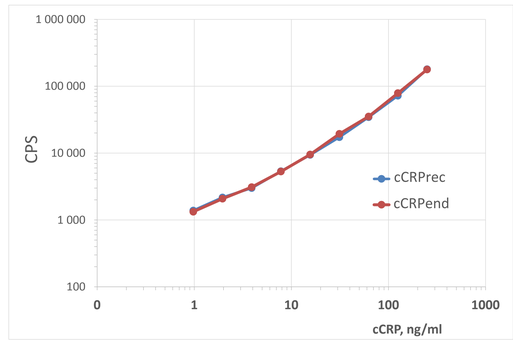

FIGURE 5. Calibration curves for cCRPend and cCRPrec in a "homosandwich" immuno-assay. mAb **cCRP11** was used as a capture and detection antibody.

Stability studies revealed that cCRPrec is a highly stable molecule that is resistant to multiple lyophilizations, freeze/thaw cycles, and long-term storage (Fig. 6).

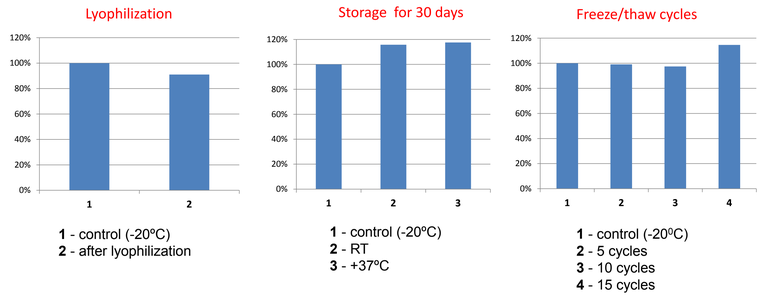

FIGURE 6. cCRPrec immunoreactivity measured in cCRP11 – cCRP1 immunoassay after lyophilization, long-term storage, and repeated freeze/thaw cycles.

Conclusions

cCRPrec obtained in the current study:

- Like cCRPend comprises glycosylated and non-glycosylated subunits
- Reveals immunoreactivity similar to the immunoreactivity of cCRPend
- Retains immunoreactivity after lyophilization, repeated freeze/thaw cycles and long-term storage
- Is a promising candidate for the development of a reliable standard/calibrator for cCRP immunoassays

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