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Secretory production of recombinant human C-reactive protein in *Escherichia coli*, capable of binding with phosphorylcholine, and its characterization

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Abstract

Recombinant human CRP (rhCRP) was secreted into culture supernatant of *Escherichia coli* by co-expressing *kil* gene that has a function to secrete colicin E1 outside the cell. Highly purified 5 g rhCRP was produced from 180 L culture supernatant by affinity chromatography. The purified rhCRP was indistinguishable from the native one with respect to Ca^{2+}-dependent binding ability to phosphorylcholine, electrophoretic behavior, N-terminal amino acid analysis, and immunochemical properties. The molecular weight of rhCRP monomer was determined to be 23059.7 Da by TOF/MS analysis. These results indicate that rhCRP has the same protein structure as native one and that rhCRP has the potential as a reference material and/or calibrator of high-sensitivity CRP assay to predict the risk of cardiovascular disease.

Keywords: C-reactive protein; *kil* gene; Phosphorylcholine; High-sensitivity CRP assay; Cardiovascular disease

C-reactive protein (CRP) was first discovered in 1930 by Tillet and Francis [1] as a substance precipitated with the C-fraction of the patient's serum. CRP is well known as an acute phase reactant that reflects high-grade inflammation, being classified as Ca^{2+}-binding pentameric protein, because it consists of five non-covalently bound, identical, spherical subunits with a molecular weight of 23,048. Each subunit consists of 206 amino acids without glycosylation and is arranged in a cyclic, symmetric, planar pentameric disk [2,3]. CRP has the ability to bind to various substances such as C-polysaccharides, phosphorylcholine, and complement proteins in a Ca^{2+}-dependent manner [4]. The primary structure of human CRP was determined by protein sequencing and subsequently corrected by DNA sequencing of the cloned gene [5,6]. Recently, Rifai et al. [7] found that serum level of CRP reflecting low-grade inflammation is promising to diagnose a variety of cardiovascular diseases. For the immunoassay of the low-grade inflammation, namely high-sensitivity CRP assay, accurate measurement of CRP is necessary on the basis of highly qualified reference material such as recombinant human CRP.

Materials and methods

Bacterial strains and reagents. Escherichia coli (*E. coli*) JM109 and NM522 were used as host strains. All the reagents were purchased from Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Culture medium. LB medium and glucose minimum medium were used. LB medium was composed of 1% Bacto-Tryptone, 0.5% yeast extract, and 0.5% NaCl. Glucose minimum medium was composed of 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl per liter, containing 1 mL of 20% $MgSO_4 \cdot 7H_2O$, 0.5 mL of 1% thiamin chloride, and 10 mL of 20% glucose. Agar (1.5%) was added to glucose minimum medium or LB medium to prepare solid medium. Ampicillin was added to the medium at a final concentration of 50 μg/mL. High-density cultivation was performed according to the method of Uchida et al. [8].

Polymerase chain reaction. Using human placenta-derived genome DNA (Clontech) as template, the gene encoding mature protein region of human CRP was amplified by polymerase chain reaction (PCR). PCR was performed using GeneAmp DNA Amplification Reagent Kit (Perkin–Elmer Cetus) and AmpliTaq Recombinant Taq DNA Polymerase. Sequences of sense and antisense primers used in this study

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were as follows: 5′-GGAATTCATGCAAACAGATATGTCAAG TAAAGCTTTTGTATTTCCCAAAGAGTCGGA-3′ for sense primer and 5′-GGACGTCTCAGGGCCACAGCTGGGGTTT-3′ for antisense primer. The underlined bases indicate the mutation near N-terminal region of the protein for the optimum CRP gene expression in *E. coli* without changes of amino acids.

DNA sequencing. ssDNA was prepared from *E. coli* JM109 carrying plasmid pTZ18U or pTZ19U to which CRP gene was cloned after being infected with helper phage M13KO7. Using the ssDNA as template, the DNA sequence was determined by 370A DNA Sequencer (ABI).

Cloning of human CRP gene and gene construction. Human CRP gene is localized on chromosome 1 and consists of two exons. First exon consists of 64 bp, 57 bp of which encodes 18 amino acids of signal peptide gene and 7 bp of which encodes N-terminal amino acid of mature CRP. Second exon encodes most of the CRP gene. Primers were designed to hybridize each end of the second exon. Amplified DNA was cloned into pTZ18U and pTZ19U and sequenced. The sequence was the same as that reported by Woo et al. [6]. Signal peptide gene of *E. coli* alkaline phosphatase was chemically synthesized and connected to upstream of CRP gene. For secretion of expressed protein from *E. coli* cells into culture medium, *kil* gene derived from plasmid pMB9 which originated from pMB1 plasmid was inserted into downstream of CRP gene to construct expression plasmid in which CRP gene and *kil* gene were co-expressed under the same *trp* promoter (Fig. 1). Resulting human CRP expression plasmid was used to transform *E. coli* NM522.

E. coli transformation and culture. Transformation of *E. coli* was performed according to the method of Chung et al. [9]. *E. coli* NM522 was transformed with control plasmid or the human CRP expression plasmid and then cultured in LB medium containing 50 μg/mL ampicillin at 37 °C for 24 h. Expression of human CRP was detected by immunostaining by the following method. *E. coli* transformants were cultured in LB medium until absorbance at 600 nm reached up to 0.8. Total protein prepared from bacterial cells was solubilized by SDS and subjected to SDS–PAGE (polyacrylamide gel electrophoresis), followed by electrotransfer to polyvinylidene difluoride membrane. This membrane was subjected to immunostaining using rabbit anti-human CRP antiserum (Oriental Yeast, Tokyo) as capture antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) as secondary antibody. The detection of human CRP in the culture supernatant was performed by immunoassay using a DRI-CHEM 3000 colorimetric analyzer (Fuji Photo Equipment, Kanagawa).

Large-scale fermentation. Single colonies of transformant isolated on the agar plate were cultured in 1 L LB medium at 30 °C for 16 h and used as seeds. Using 250 L fermenter IF-250 (New Brunswick Scientific), the seed mentioned above was added to 180 L LB medium containing 0.5% glycerol and 0.05% $CaCl_2$ and cultured at 35 °C for 65 h.

Chromatography. ECH–Sepharose 4B produced by covalent linkage of 6-aminohexanoic acid to Sepharose 4B using an epoxy coupling method was purchased from Amersham Biosciences. It is shown that CRP has the ability to bind with the adsorbent in a Ca^{2+}-dependent manner and to be eluted by EDTA [10]. ECH–Sepharose (50 mL bed volume; 5 × 2.6 cm) was equilibrated with 20 mM Tris–HCl (pH 7.5) (buffer A) containing 2 mM $CaCl_2$. After washing with 20 mM Tris-HCl (pH 7.5) containing 0.14 M NaCl, 0.05% NaN_3, and 2 mM $CaCl_2$ (buffer B), protein fraction bound to the column was eluted with buffer A containing 0.14 M NaCl, 0.05% NaN_3, and 5 mM EDTA.

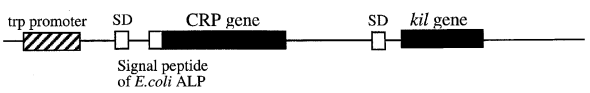

Fig. 1. Gene construction for the production of rhCRP. The gene constructed was composed of *trp* promoter, SD (Shine–Dalgarno) sequence, signal peptide of *E. coli* ALP (alkaline phosphatase), CRP gene, SD sequence, *kil* gene, and terminator.

Determination of molecular weight of rhCRP monomer. Recombinant human C-reactive protein (rhCRP) was analyzed by a JMS-LDI 1700 (JEOL, Linear type, Tokyo) matrix-assisted laser desorption-induced time-of-flight (MALDI-TOF) mass spectrometer equipped by a nitrogen laser operating at 337 nm. TOF spectra were produced at 4.8 kV acceleration voltage by averaging 50 single spectra. A matrix containing 100 mM sinapinic acid was used. rhCRP (0.5 mg/mL) was diluted 5-fold with 50% methanol to a final concentration of 0.1 mg/mL (0.9 pmol/μL) before applying into the mass spectrometer.

Ca^{2+}-dependent phosphorylcholine binding assay. Ca^{2+}-dependent binding ability to phosphorylcholine was analyzed either by column method using ECH–Sepharose [10] or ELISA method using bovine serum albumin conjugated with phosphorylcholine (PC–BSA) [4]. In the case of column method, 1 mg/mL CRP samples in buffer B was applied to ECH–Sepharose 4B (1 mL) and the adsorbed CRP was eluted with buffer A containing 0.14 M NaCl, 0.05% NaN_3, and 5 mM EDTA. CRP concentration of the eluted fraction was determined from absorbance at 280 nm. In the case of ELISA method, PC–BSA was solubilized with 20 mM Na_2CO_3 buffer (pH 9.6) at a concentration of 250 ng/mL and 50 μL of this solution was applied to each well of 96-well microtiter plate in the presence or absence of 2 mM $CaCl_2$, allowed to stand for 1 h. The wells were washed with 200 μL wash buffer [50 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 10 mM $CaCl_2$, and 0.1% Brij 35] and 50 μL of CRP samples was added, allowed to stand for 1 h. After washing with the wash buffer (0.2 mL × 3), rabbit anti-human CRP antiserum bound with CRP was detected using goat anti-rabbit IgG conjugated with horseradish peroxidase. Orthophenylene diamine was used as coloring dye by measuring absorbance at 492 nm.

Immunoreactivity and protein assay. Immunoreactivity of rhCRP was assayed by a Lpiaace autoanalyzer (Iatron Lab., Tokyo) using rabbit anti-rhCRP antiserum sensitized latex. Polyclonal rabbit antibodies were raised against native human CRP and rhCRP. Protein concentration was determined by the method of Smith et al. [11] using BSA as standard. SDS–PAGE was performed by the method of Laemmli [12] using 10–20% gradient gel (Daiichi Pure Chemicals, Tokyo; 84 mm height × 90 mm width × 1 mm thickness). Proteins were stained by Coomassie brilliant blue R-250 or silver staining. Amino acid sequence was determined by a 477A protein/peptide sequencer with HPLC Model 120A (ABI).

Results and discussion

Expression of CRP into culture supernatant by co-expressing kil gene in E. coli

Based on the finding that colicin E1 is secreted into culture supernatant when *kil* gene is properly expressed in *E. coli* [13], expression system co-expressing human CRP and *kil* genes was constructed to enable human CRP to be secreted into culture supernatant with the aid of *kil* gene (Fig. 1). The *kil* gene was inserted at downstream of CRP gene and the expression vector was transformed in *E. coli*. Expression vector pTRP has thermosensitive replicational origin to increase the plasmid copy number with increase of temperature. Therefore, the transformant was cultured in a 100 mL flask to high density at 30 °C by holding the plasmid copy number low and then the temperature was increased to reach high copy number. Under these

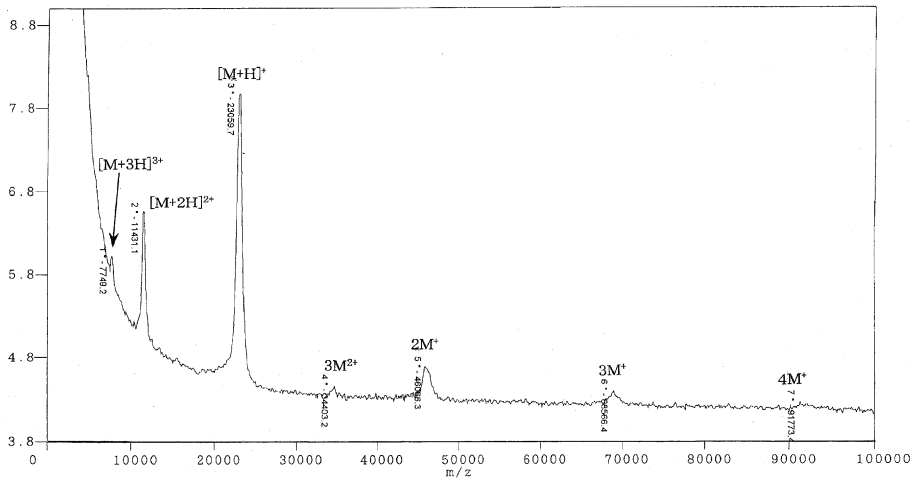

Fig. 2. MALDI-TOF mass spectra of rhCRP. rhCRP was subjected to MALDI-TOF/MS analysis. A matrix containing 100 mM sinapinic acid was used. m/z Values of the peaks numbered are (1) 7749.2, (2) 11431.1, (3) 23059.7, (4) 34403.2, (5) 46066.3, (6) 68566.4, and (7) 91773.4. Peak 1 is corresponding to trivalent formed by binding three protons with CRP, peak 2 to divalent formed by binding two protons, and peak 3 to monovalent formed by binding one proton. Peaks 4–7 are corresponding to cluster ions, respectively. The data were obtained under the technical support by Dr. Akira Shimizu (Osaka Medical College, Japan).

conditions, 80% of human CRP expressed was secreted into culture supernatant at high levels of 50–60 mg/L. However, when the transformant without *kil* gene was cultured, human CRP was hardly secreted, but localized in periplasm as inclusion body.

Single-step purification of rhCRP

After centrifugation of fermented culture medium under large-scale fermentation, culture supernatant was subjected to purification by ECH–Sepharose affinity chromatography using Ca^{2+}-dependent adsorption and EDTA-dependent elution. Five grams of rhCRP was purified from 180 L culture supernatant of fed-batch culture at a concentration of 30 mg/L.

Biochemical properties of rhCRP and stability

Purified rhCRP was subjected to SDS–PAGE under reducing conditions. The dye staining of 1 μg protein or silver staining of 0.1 μg protein showed the single band at the same migration position as native CRP monomer (23 kDa). rhCRP also showed a similar behavior on non-SDS–PAGE to native CRP. N-terminal amino acid analysis revealed that rhCRP had the same amino acid (glutamine) as deduced from DNA sequence of native CRP. From MALDI-TOF mass spectra of rhCRP (Fig. 2), the molecular weight of rhCRP monomer was determined to be 23059.7 Da. This value was in excellent agreement with the theoretical mass calculated from the known CRP amino acid sequence (23046.7 Da). The spectra indicate that rhCRP thus obtained is a high-quality material. Purified rhCRP was bound to PC–BSA in the presence of Ca^{2+} and unbound by adding 5 mM EDTA. Ca^{2+}-dependent binding ability of rhCRP to phosphorylcholine was hardly distinguishable from that of native CRP (Table 1). There has been no report that denatured CRP or monomer CRP shows the binding ability. In addition to the behavior on ECH–Sepharose 4B, these results suggest that rhCRP forms pentameric structure as native CRP does. Stability of rhCRP at 4 °C or at −20 °C was examined by measuring Ca^{2+}-dependent binding ability to phosphorylcholine (Table 2). When stored at 4 °C in buffer B, the stability was maintained for 6 months, and even after 16 months, 96% of Ca^{2+}-dependent binding ability to phosphorylcholine was observed. rhCRP was found to be stable for 16 months without any loss of ability, when stored at −20 °C.

Table 1
Ca^{2+}-dependent phosphorylcholine binding ability of rhCRP using PC–BSA

CRP concentration (mg/mL)	Absorbance at 492 nm	
	Native CRP	rhCRP
0.01	0.03	0.01
0.05	0.03	0.05
0.1	0.23	0.27
0.2	0.55	0.53
0.5	0.94	1.00
5	1.73	1.71

Note. CRP samples were incubated with PC–BSA (bovine serum albumin conjugated with phosphorylcholine) previously immobilized in a 96-well microtiter plate in the presence or absence of 2 mM $CaCl_2$. After washing, CRP bound with PC–BSA was determined by colorimetric immunoassay using rabbit anti-CRP antibody (see the text in detail). Absorbance at 492 nm in the absence of 2 mM $CaCl_2$ was negligible with all the concentrations examined of either native CRP or rhCRP. Average values of triplicate are shown.

Table 2
Long-term stability of rhCRP as assayed by Ca^{2+}-dependent phosphorylcholine binding ability using ECH–Sepharose

Storage temperature (°C)	Ca^{2+}-dependent phosphorylcholine binding ability (%)				
	Storage (month)				
	0	4	6	12	16
4	100.0	99.0	102.0	97.0	96.0
−20	100.0	100.0	102.0	101.0	102.0

Note. Aliquot CRP samples (1 mg/mL) in buffer B were stored at each storage temperature indicated. Each CRP sample was applied to ECH–Sepharose 4B in the presence of 2 mM $CaCl_2$ and eluted with 5 mM EDTA buffer (see the text in detail). Relative average values of triplicate by taking the values at 0 month as 100% are shown.

Immunochemical properties of rhCRP

Immunoreactivity of rhCRP was examined with 500 samples of human serum by latex immunoassay using rabbit anti-rhCRP antiserum raised against rhCRP, being compared with anti-native CRP antiserum raised against native CRP. These values measured were highly correlated with each other, with a correlation coefficient of 0.998 (Fig. 3), indicating that rhCRP has the same immunochemical properties as the native CRP.

In conclusion, these results indicate that rhCRP has the same protein structure as native CRP does and that rhCRP has the potential as a reference and/or calibrator of high-sensitivity CRP assay for a prediction of the risk of cardiovascular disease.

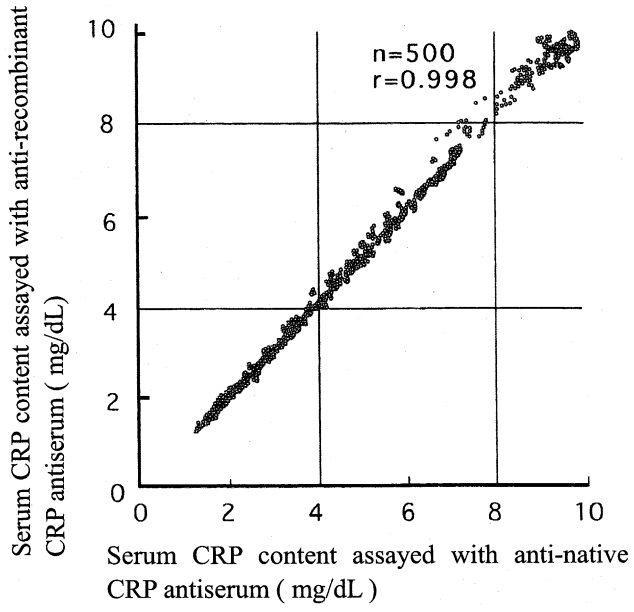

Fig. 3. Correlation coefficient in turbidoimmunoassay of serum CRP level with 500 samples. Human serum samples (500) were subjected to CRP assay using latex immunoassay kit. The average values of duplicate assay using anti-native CRP antibody raised against native CRP are plotted on the *x*-axis and those using anti-rhCRP antibody raised against rhCRP on the *y*-axis. These data were obtained from Dr. Koichi Suzuki (Iatron Lab.).

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