Epitope Analysis in development of novel type of CRP reagent with monoclonal antibody and clinical evaluation in patients serum

Hideki Kohno
Department of Applied Molecular Chemistry
College of Industrial Technology
Nihon University

Back ground

C-reactive protein(CRP) measurement has been used as acute phase marker and today, with the development of sensitive immunoassay it is renewed as risk factor of various disease such as myocardial infarction, arthritis rheumatoid and even to follow-up the control of diabetes. Moreover, in recent years high sensitive CRP has attracted attention because it enables us to predict of the coming disease. And at the aim of extensive utility , more sensitive and specific reagent will be needed for the diagnosis of diabetes with obesity and small infection in earlier stage(1).

Thus, our research with hot attention is to development of further improvement in sensitivity and specificity and accuracy is intensely required. In order to obtain more sensitive and specific immunoassay reagent we focused on epitope analysis of CRP protein to gain accuracy and specificity with the use of gene recombinant technology. Epitope, the region of approximately 5-8 amino acids residues, reacts to antibody directly. On the basis of epitope analysis, the application of synthetic short amino acids sequences enables us to clarify the reactive sites of the CRP reagent. In result, in this report, we like to document that our reagent based on that concept showed a great improvement both in sensitivity and specificity(2).

Materials and Methods

Extraction and amplification of CRP gene

At first stage, DNA has been derived from the white blood cell of human blood. With EDTA solution of threefold in volume was added to punctured bloods, it was added to glass fiber matrix (GFX) fixed column, and it was centrifuges at 13200 rpm for 30 sec.

After the beads was washed by the Tris- EDTA buffer , genome DNA was extracted and it was used for the template sequences for the polymerase chain reaction(PCR). We searched for in the database and seven types of primer (MKF-01, MKF-02, MKF-03,

MKF-04, MKF-05, MKF-06, MKR-01) were selected and designed. During the process we synthesized the above mentioned primer sequences to amplify the part of CRP sequence specifically one of five subunit of the whole sequences of CRP. Extracted genome was use as template and PCR was carried out with each primer. Six types pf gene sequence were obtained and PCR amplification products (Fig. 1) were purified by using a crystal violet gel.

Insertion of amplified gene into *E coli* (TOP 10)

In order to obtain gene recombinant protein , we used E. coli bacteria (E. coli) for the host cell. PCR gene sequence were sub cloned and inserted into pET-100/D-TOPO vector [Invitrogen] respectively, and mixed with TOP10 cell onto the LB plate with Ampicillin to select the objective cell line. Then it was incubated and grown at 37℃ for 15 h to harvest the cell body.

Inoculated colony based on PCR

To select gene recombinant cell into which plasmid has been introduced, PCR was done on the colony PCR bases with the use of before mentioned template. One the colony which indicated the positive reaction in PCR was selected and cloned to passage the LB medium with amplification.

Transformation of *E. coli* (BL21)

IPG which is similar to the structure of β-lactose, is a material greatly related to the transcriptional control of lactose (lac) operon of *E. coli*.
The production of the protein increases because of the inhibition of β-galactosidase .
The cultured colony was inoculated for 15h and it was divided into new LB medium..
IPTG was added to the other culture solution to become 1.0 mM in the final concentration, and the culture solution was collected respectively at every 1 hour (0 – 5 h). The collected culture solution was centrifuged (16,000×g, for 10 min) and the obtained pellet was frozen for the storage.

Specification of recombinant CRP proteins

The freeze and thawing procedure was repeated by dissolving the pellet collected from IPTG induction. It was centrifuged at 16,000×g for10 min and the obtained pellet suspension and supernatant were confirmed by the SDS-PAGE electrophoresis for the recombinant protein respectively.

Epitope analysis

The pellet that dissolved into lysis buffer was detected and measured by various immunoassay methods (ELISA, Western Blot, and Latex agglutination reaction). Epitope of each antibody was analyzed by using four types of anti-CRP which we prepared monoclonal antibody (MoAb) (Lot. 02010, Lot. 050921, Lot. 030700, Lot. CP80105) for various as immunoassay.

Clinical study of rebombinant CRP by use of latex agglutination

The WSC (water soluble carbodiimide) solution and the NHS (N-hydroxysuccinimide) solution were added to 1% carboxyl modified latex suspension and the carboxyl group was activated. Each type of ant-CRP MoAb was added and was combined with 1% of antibody solution. Afterwards, degenerated BSA was added into and the conjugated beads were blocked with 1% of denatured BSA solution. The prepared latex was hydrolyzed and was sensitized latex reagent were prepared. The working curve was mage with the anti-CRP MoAb sensitization latex reagent. In clinical study, Lever disease and type Ⅱ Diabetic sera were measured with each reagent. The comparative studies of these reagents were carried out. And we compared with two types of latex reagents which were anti CRP PoAb latex and anti CRP $F(ab')_2$ anti body latex.

Results and Discussion

Epitope analysis

The result of detection of expressed protein by Western Blot and ELISA are applied for the evidence. It proved that every Epitope of MoAb Lot. 020210, Lot. 030700 and Lot. CP80105 were located in the MK06 area (173 – 206 residues) of CRP from these results. It also proved that Epitope locate in 147 -172 residues of CRP different from other three type because MoAb Lot. 050921 showed no reactive to MK06 .

Latex agglutination reaction

The prepared four type of Latex reagents which were sensitized with CRP MoAbs and calibration curve for assay specimen by Latex agglutination reaction. The reactivity of the reagent was high in the order of MoAb Lot. 020210, Lot. 050921, Lot. CP80105 and Lot. 030700. Actually specimens were assayed for Latex agglutination reaction by Lot. 02010, Lot. 050921 and Lot. CP80105. Lot. 030700 reagent was low reactive and labile calibration curve. Two type of specimen (specimen of liver disease and that of diabetes type Ⅱ) were assayed by Latex agglutination reaction. As result, assay of liver

disease and type Ⅱ diabetes were high concentration of CRP as shown.
However, the value of Polyclonal IgG type has correlativity of 1:1 with PoAb $F(ab')_2$ reagent which is shown 7 . The result in diabetes type Ⅱ showed the similar trend in result. In the specimen assay the measurement has been done widely using PoAb $F(ab')_2$ reagent to prevent nonspecific reaction of the reagent. The type of comparison proved that a result of using moAb reagent has similar in measurement values to PoAb $F(ab')_2$ reagent. Therefore we were successful in improvement of in sensitivity and specificity using site specific MoAb.

References

(1)Wlfgang Ko0enig, Natakie Khuseynova, Jens Baumert and Christa Meisinger
Prospective Study of High-Sensitivity C-reactive protein as a Determinant of Mortality:Results from the MONICA/KORA Ausburg Cohort study, 1984-1998
Clinical Chemistry 54:2,335-342(2008)
(2)Claudia Marski, Lili Kazemi-Shirazi, Thomas Schickbauer, Stefan Winkler, Christian
Joukhadar, Oswald F. Wagner and Georg Endler
C-reactive protein and All-Cause Mortality in a Large Hospital-Based Cohort
Clinical Chemistry 54:2,343-349(2008)