論文の内容の要旨
Abstract of Dissertation

Title of Dissertation ： Phage display selection of antibody fragments for noncompetitive detection of thyroid hormones
（論文題目　甲状腺ホルモンの非競合的検出のための抗体断片のファージ提示選択）

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Immunoassay has been extensively used as a diagnostic tool in biotechnology and biomedical field. Though there are so many methods exist, recently, open-sandwich immunoassay (OS-IA) for the non-competitive detection of haptens has been developed which is based on the phenomenon of increased association of two antibody variable domains (V_H and V_L) upon binding with antigens. In this study we established OS-IA for thyroxine (T4; 3, 3', 5, 5'-tetraiodo-thyronine), which is the most commonly measured thyroid hormone for diagnosis of thyroid function. Stimulation of thyroid gland by the pituitary hormone (TSH) causes the release of T4 in bloodstream. It exists either as a complex with carrier protein or as a free form in blood. Only the free portion (unbound) of T4 is responsible for the biological action.

So on the first chapter of PhD thesis I overviewed this area and made introduction to the following two topics: 1) Direct construction of open-sandwich enzyme immunoassay for one-step noncompetitive detection of thyroid hormone T4, and 2) Micro open-sandwich ELISA to rapidly evaluate thyroid hormone concentration from serum samples.

In Chapter 2, direct construction of open-sandwich enzyme immunoassay for one-step noncompetitive detection of thyroid hormone T4 is described. To obtain antibody fragments suitable for OS-IA swiftly by-passing time-consuming conventional hybridoma technology, direct acquisition of such genes from immunized mice by phage display technology was attempted. After obtaining antibody V region genes from the spleen of immunized mice, we successfully isolated anti-T4 antibodies using a Fab phage display system (pDong system) with an estimated diversity $1.5x10^5$. To estimate the affinity of the two isolated clones, indirect competitive ELISA with immobilized T4-BSA was performed. For the both clones, unconjugated T4 completely inhibited the binding of Fab to T4-BSA in a dose-dependent manner. In both cases, the obtained IC_{50} was about 5-13 ng/mL, indicating their high antigen binding affinity. Also, the clones showed similar cross-reactivity to another thyroid hormone ,3,3',5-triiodothyronine (T3).

After the deletion of C_H1 gene from pDong1 by *Sgr*A1 digestion and self ligation, the resulting phagemids were used for the expression of V_H-displaying phage (V_H-phage) and free light chain (V_L-C_L) for OS- ELISA The culture supernatant containing V_H-phage and L chain was added to the microplate immobilized with goat anti-human kappa chain

antibody, and the bound phages were detected with anti-M13 HRP conjugate. As a result, D11 showed a clear antigen-dependent increase in signal, suggesting its suitability to OS-IA. According to the dose-response, a significant signal at 1 ng/mL of free T4 was detected. On the other hand, F11 showed higher signal even in the absence of antigen, but low signal in the absence of immobilized anti-light chain antibody, suggesting stronger V_H/V_L interaction of this clone than D11.

The results of phage OS ELISA indicated the superiority of D11 in OS-IA than F11. With the intent of clarifying this reason, we determined the nucleotide sequences of the two clones. To our surprise, from the deduced amino acid sequences of the V_H/V_L both clones share very similar primary structure even within three CDRs for both V_H and V_L. However, two residues (37 and 74) in V_H and four located in V_L (31, 94, 96 and 100) are different between the two, which make them distinctive. Among them, the residues H37, L96 and L100 are located at the V_H-V_L interface and could be attributable to the stronger V_H / V_L interaction in F11. Especially, the H37 of D11 is small Ala, while that of F11 is Val. This residue is also in the interchain interface, and potentially playing an important role in V_H/V_L interaction. However, preliminary experiment to exchange V_H/V_L sequences of D11 and F11 in pDong1 indicated that both V_H and V_L are important in deciding the V_H/V_L interaction strength in the absence of antigen.

To further confirm the T4-dependency in phage OS ELISA of clone D11, we attempted to reproduce the assay with purified fusion proteins. As in our previous studies, we used *E. coli* maltose binding protein (MBP) as a fusion partner for the ease of soluble expression the fusion proteins and also their immobilization/conjugation. Hence, the obtained purified proteins were either immobilized directly (MBP-V_L) to the microplate, or used as a conjugate with horseradish peroxidase (HRP-MBP-V_H) to probe its binding activity to the other variable region fragment (MBP-V_L). The result indicated similar or superior antigen (free T4)-dependent association of purified HRP-MBP-V_H conjugate to immobilized MBP-V_L than V_H-displaying phage, which successfully detected less than 0.1 ng/mL T4 in PBS solution. Furthermore, we tried to apply OS-ELISA to estimate T4 concentration in serum using the recombinant proteins. The estimated total T4 concentration using an ethanol-extracted pooled serum was 90 ng/mL (9 µg/dL). The value was in good agreement with the normal adult range of 5-12 µg/dL.

In the third chapter of this thesis, micro open sandwich ELISA to rapidly evaluate thyroid hormone concentration from serum was described. Since the patients suffering from thyroid diseases are increasing, rapid and sensitive assays are strongly desired. To answer such demand, here we tried to integrate the sensitive OS-IA into a microfluidics-based analytical system. The resultant system is expected to hold the promise of integrating an entire laboratory onto a single chip (i.e., lab-on-a-chip) equipped with a thermal lens microscope (TLM), requiring less reagents, sample volumes and short analysis time (20 min). The two variable domains (V_H and V_L) of the anti-T4 antibody D11 were expressed as fusion proteins with *E. coli* maltose binding protein (MBP) and MBP-V_H HRP were used to perform the os(open-sandwich) ELISA in a portable micro-ELISA system (µELISA IMT-501). Greater than 99% of T4 is reversibly bound to plasma proteins, while approximately 0.03% of T4 exists free in the blood. To precipitate the plasma proteins, a

brief ethanol extraction was carried out both the control (T4/T3 deficient, Sigma, Germany) and test sera (healthy human donor).

Using the glass microfluidic chip equipped with a dam structure to keep microbeads for the reaction, first we optimized the measurement conditions for antigen T4 in PBS. MBP-V_L in PBS was mixed with polystyrene beads for overnight, which was added with a blocking agent in PBS (PBS-IB) to block the remaining surface. The detection limit was influenced by the incubation/washing conditions and the blocking agent concentration in these reactions. The reactions of HRP-labeled MBP-V_H, T4 and MBP-V_L beads were also carried out in the presence of various concentrations of PBS-IB, and final concentration of 5% gave the highest signal/background (S/B) ratio. Hence we used these conditions in the following microchip experiments. Finally, HRP-MBP-V_H at various concentrations was introduced into the microfluidic chip at various flow rates and the resulting S/B ratios were evaluated. Consequently, it was found that the reaction with 0.5 µg/mL HRP-MBP-V_H at 2 µl/min gave the best result and we successfully detected less than 1 ng/ml of free T4. The total serum concentration is a specific and sensitive index of thyroid function. Based on the established condition, we estimated the total T4 concentration of a healthy individual using ethanol extracted sample sera. The obtained value was 80 ng/ml (= 8 µg/dl), which coincided well with the normal adult range for the total T4 in serum of 5-12µg/dl.

In the forth chapter of the thesis**,** conclusion and perspective are described. This would be the first micro open-sandwich ELISA constructed with antibody fragments directly selected from immunized mice. This system will be applied to the sensitive detection of many diagnostic markers.