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学位論文題目	*In vitro* selection of anti-gliadin single-domain antibodies from a naïve library and its application for cDNA-display mediated immuno-PCR（ナイーブライブラリからの抗グリアジン一本鎖重鎖抗体の試験管内淘汰とそのcDNAディスプレイ-イムノPCR法への応用）
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論文の内容の要旨

The prevalence of wheat/gluten toxicity, along with adherence to a strict gluten-free diet, is becoming a serious dietary concern worldwide. Celiac disease (CD) has become one of the most common autoimmune diseases, resulting in lifelong intolerance to gluten in sensitized individuals. CD induces a T cell-mediated inflammatory process in the small intestine, which leads to an enteropathy with impairment of the mucosal surface and, consequently, substandard absorption of nutrients. In celiac subjects, the ingestion of gluten causes a variety of clinical symptoms, including typical gastrointestinal symptoms. People with gluten-related disorders have to remove gluten from their diet strictly, because there are no reliable medical treatments for food allergies to date. Hence, the assurance of the absence of gluten in food is of paramount importance.

The aim of this study was to develop high affinity gliadin binding antibodies which may used in gluten detection in the analysis of different gluten-free foods in future advancements. The experimental part of the study focuses on qualitative and quantitative studies on gliadin detection using immunological and electrophoretic analysis methods. Gliadin is the storage protein in wheat grain that is soluble in aqueous alcohols but is insoluble in water or neutral salt solutions. In general, gliadin is extracted with aqueous alcohol (60%–70%). In the present study, gliadin was extracted with 70% alcohol (with or without 10% DMSO) in PBS buffer. Insolubility in aqueous solutions/ buffers and incomplete extraction of the protein were some of the obstacles in the process of immobilizing gliadin on magnetic beads. Since gliadin is a complex mixture of proteins, it was also challenging to immobilize gliadin completely and evenly on the beads during each selection round. Gliadin proteins are divided into subgroups of α-, γ-, β- and ω-gliadins. The group of α- gliadins is generally the major group, comprising between 44 and 60% of the total gliadin content. The second largest group is γ- gliadins (31–46%), and together these groups account for about 80% of wheat gliadins. The α- and γ- gliadins are often combined and simply referred to as α- gliadins because of the high similarity of their N-terminal

amino acid sequences. After considering several bead immobilization methods, the antigenic gliadin proteins were covalently coupled to NHS-activated carboxylic acid beads to achieve maximum immobilization. This method allowed approximately 60%-70% immobilization of medium-molecular weight gliadin groups only on the beads.

Recently, huge attention has been made towards the single variable domain on a heavy chain antibodies (VHH). They are antigen binding fragment of Heavy chain only antibodies (HcAb), which are naturally produced in camelids. VHH antibodies are approximately 10-fold smaller (~ 15 kDa) than conventional antibodies (~ 150 kDa). Moreover, the core of the antigen recognition site, i.e., the CDR3 loop, of VHH antibodies is often longer than that of conventional antibodies. Altogether, they exhibit several advantageous features like high stability, versatility, unique refolding capacity, reduced aggregation tendency and high affinity to their cognate antigens. Recently, several artificial and naïve VHH libraries from llamas have been developed. Naïve libraries are derived from primary B-cells of non-immunized donors which gives large diversity to the library with functional properties. Hence in the present study, alpaca derived naïve VHH library has been used to screen specific antibodies against the target gliadin protein. Lymphocytes were isolated from the blood of alpacas. Total RNA was extracted with and used to synthesize cDNAs, and the synthesized cDNAs were amplified using VHH specific primers. Recombinant VHH DNA fragments were designed with the DNA fragments consisting of a T7 promoter, omega (Ω) enhancer, Kozak consensus sequence, VHH gene, His-tag, and linker hybridization region (Y tag) in order to become comparable in following assays. The obtained full-length, VHH DNA library was used in the subsequent assays.

The cDNA display method is an immunization-free *in vitro* selection tool which is important when comes to obtaining antibodies that can recognize targets which cannot be used in immunizing animals due to their low immunogenicity or toxicity. The present study aimed to select VHH antibodies against wheat gliadin from an alpaca derived naïve VHH library. Since gliadin is not immunogenetic in all animals, it is difficult to ensure the formation of suitable antibodies by immunization techniques. Besides, some studies have indicated that the immunological response to antigen vaccines in children, even those with CD, does not differ markedly from that of the general population. Hence, *in vitro* selection techniques are necessary for screening antibodies against variable toxins, such as gliadin. The key feature of the cDNA display method is the easy to use preparation using a unique puromycin linker, which is hybridized and photo-ligated to the 3'-end of mRNA. The method proved to screen many affinity peptides/VHHs successfully without using experimental animals. The cDNA display enables the successful selection of high-affinity VHH from synthetic libraries, with a library size of over 10^{13}. This method has several characteristics that assist in improving many drawbacks (e.g. instability, limited diversity or small library size, cell toxicity of proteins, effect of fused coat proteins, and difficult handling) that are occur either cell dependent (i.e., phage display, bacterial display, yeast display) or cell-independent (i.e., ribosome display, mRNA display) in vitro selection techniques.

A DNA library encoding VHH was transcribed in vitro to yield mRNA. The mRNA was annealed to the puromycin linker and photo-crosslinking was performed. The cross-linking efficiency of the puromycin linker with the mRNA was estimated to be more than 90% according to the band intensity ratios visualized on PAGE analysis. Higher ligation efficiencies of mRNA and puromycin linker DNA helps to overcome remaining free linkers in the protein translation solution which may end up in inefficient cDNA display formation. The successful formation and purification of cDNA display molecules of VHH was confirmed by SDS-PAGE (5.6%). Although the formation yield of the cDNA display molecules seems to be low, it was in line with our previous studies, in which the formation efficiency of the cDNA display for small polypeptides was observed to be approximately 5%–25%. However, it is important to improve the formation efficiency of the cDNA display molecule. Here, *in vitro* affinity selection was performed using constructed

VHH-coding cDNA display molecules toward immobilized gliadin, and three candidate VHHs were successfully selected. When the number of selections was increased, the elute quantity was also increased and reached a plateau, indicating enrichment of the binders. The band intensities of the last selection round were graphically compared with the parallel negative selection band intensities. The intensity of the positive selection elution band was considerably higher (by approximately 11-fold) than the negative selection elution band. This result indicated high specific and low nonspecific binging of candidate VHHs. Following the in vitro selection, all the selection elutes were analyzed by NGS, instead of analyzing the last selection elute only, to generate more comparable sequence data. According to the percentage abundance (frequency) of the NGS data, the top three VHH sequences, which were not appeared in the negative selection data pool were selected as candidate VHH sequences.

Determination of the interactions between the selected VHHs and the target gliadin is an essential step toward understanding the affinity. Protein interactions were determined using two conventional assays (pulldown assay and ELISA assay) and also with novel cD-IPCR assay. In the pulldown assay, specificity of the anti-gliadin VHH towards gliadin were confirmed by comparing with control proteins including a single chain peptide (BDA) and a single domain antibody (anti-GFP VHH). The results confirmed that VHH1 is specifically bound with gliadin and gliadin only, and the observed binding was not from potential stickiness to the gliadin. In the study, data indicated that anti-gliadin VHH appeared to recognize several toxic protein components of the target gliadin. Gliadin is a large family of proteins with closely similar amino acid sequences, but different molecular weights. These proteins have been classified as α, β, γ, and ω gliadins. This multiple protein binding is important because the developed VHHs may be able to recognize almost all the toxic gliadin components present in food. Similar result was observed in several other anti-gluten antibodies developed to date. The differences in the CDR regions are likely to be responsible for this multiple binding, but further studies are required for confirmation.

In ELISA assay, results indicated increasing binding of VHH at higher concentrations. Also, higher detection signals were observed for VHH1 and VHH2 candidates compared to VHH3, which was comparable with the pulldown assay and cD-IPCR assay results. Moreover, data indicated the non-specific binding of anti-gliadin VHH to BSA protein was considerably low and at the same time there was no association of anti-BSA VHH to gliadin. However, in ELISA was not able to show any saturated signal even when the concentration reached to 10 μg/mL.

As a novel immuno-qPCR application, we applied our previously developed cD-IPCR assay successfully in this study to understand the binding patterns and specificity of selected VHHs towards gliadin. Compared with other immuno-PCR methods, the cD-IPCR method is unique because of the covalent conjugation mechanism. In this method, the peptide is always conjugated covalently to DNA at a ratio of 1:1. Because of the sensitivity of the assay, binding of the VHH to very low gliadin concentrations (i.e., 0.001 μg/mL) was able to be detected with a statistical significance ($p < 0.01$, *t*-test). VHH1 and VHH2 were the best binders according to the immuno-PCR assay and the results were similar to the results of other assays. In the specificity confirmation assay for the VHH1 against two other control proteins (BDA and anti-GFP VHH), the data indicated that VHH1 is uniquely bound to gliadin protein. The data was in line with the results observed in pulldown assay.

A preliminary study was performed to assess the applicability of the developed VHHs in detecting gliadin in food to determine whether VHHs can recognize gliadin in the presence of other food components. The cD-IPCR method was used as a competitive immunoassay, and the data indicated a positive detection pattern with increasing gliadin concentrations. When the amount of spiked gliadin reached 5% of the food sample (5 ng/ mL spiked gliadin in 100 ng/ mL rice), the detection was statistically significant ($p < 0.01$). However, the ΔCt values of lower spiked concentrations

(0.5% and 0.05%) were not significant enough and further optimization is necessary to improve sensitivity. Food extracts contain a mixture of compounds, which can act as nonspecific binders with the detector antibodies. Hence, it is a critical and difficult task to perform detection assays in actual food.

As discussed here, a naïve VHH DNA library was used successfully for selecting VHH candidates that specifically bound to toxic gliadin, without immunizing animals, using the cDNA display assay. The presence of several components in the gliadin protein mixture was a challenge as this might cause uneven immobilization of a one particular component on the beads during each selection round. Three candidate VHHs were selected at the end of the in vitro selection. VHH1 and VHH2 were the best binders toward gliadin compared with VHH3 in all assays performed, including the cD-IPCR assay. Even though the candidate VHH1 and VHH2 showed considerable affinities toward gliadin, more investigation and optimization are required before they can be used in practical applications. However, this study opens new paths to combine advanced technologies, such as the cDNA display method and cD-IPCR, to obtain effective binders for toxic and complex proteins, which is difficult to achieve using conventional methods. Also, these immunization-free affinity antibody preparation technologies may reach the ethics and principles of modern scientific experimentation, especially in terms of animal protection. Thus, cDNA display-based assays will open new avenues for gluten quantification. Nevertheless, we believe that the cD-IPCR method can be applied with even higher sensitivity levels after making improvements to the binders. Although still in its infancy, we believe that gliadin detection using selected VHHs will undoubtedly continue to advance.

論文の審査結果の要旨

The prevalence of wheat/gluten toxicity, along with adherence to a strict gluten-free diet, is becoming a serious dietary concern worldwide. Celiac disease (CD) has become one of the most common autoimmune diseases, resulting in lifelong intolerance to gluten in sensitized individuals. CD induces a T cell-mediated inflammatory process in the small intestine, which leads to an enteropathy with impairment of the mucosal surface and, consequently, substandard absorption of nutrients. People with gluten-related disorders have to remove gluten from their diet strictly, because there are no reliable medical treatments for food allergies to date. Hence, the assurance of the absence of gluten in food is of paramount importance. Recently, huge attention has been made towards the single variable domain on a heavy chain antibodies (VHH). They are antigen binding fragment of Heavy chain only antibodies (HcAb), which are naturally produced in camelids. VHH antibodies are approximately 10-fold smaller (~ 15 kDa) than conventional antibodies (~ 150 kDa).

Moreover, the core of the antigen recognition site, i.e., the CDR3 loop, of VHH antibodies is often longer than that of conventional antibodies. Altogether, they exhibit several advantageous features like high stability, versatility, unique refolding capability, reduced aggregation tendency and high affinity to their cognate antigens. The cDNA display method is an immunization-free in vitro selection tool which is important when comes to obtaining antibodies that can recognize targets which cannot be used in immunizing animals due to their low immunogenicity or toxicity. The key feature of the cDNA display method is the easy to use preparation using a unique puromycin linker, which is hybridized and photo-ligated to the 3'-end of mRNA. The method proved to screen many affinity peptides/VHHs successfully without using experimental animals. The cDNA display enables the successful selection of high-affinity VHH from synthetic libraries, with a library size of over 1013. Thus, in the present study, alpaca derived naïve VHH library has been used to screen specific antibodies against the target gliadin protein using cDNA display method. The aim of this study was to develop high affinity gliadin binding antibodies which may use in gluten detection in the analysis of different gluten-free foods in future advancements.

The experimental part of the study focuses on qualitative and quantitative studies of gliadin detection using in vitro selected antibodies with immunological and electrophoretic analysis methods. Here, gliadin was extracted with 70% alcohol in PBS buffer. Since gliadin is a complex mixture of proteins, it was also challenging to immobilize gliadin completely and evenly on the beads during each selection round. Gliadin proteins are divided into subgroups of α-, γ-, β- and ω-gliadins. The group of α- gliadins is generally the major group, comprising between 44% and 60% of the total gliadin content. The second largest group is γ- gliadins (31–46%), and together these groups account for about 80% of wheat gliadins. After considering several bead immobilization methods, the antigenic gliadin proteins were covalently coupled to NHS-activated carboxylic acid beads to achieve maximum immobilization. This method allowed approximately 60%-70% immobilization of medium-molecular weight gliadin groups only on the beads.

To generate the VHH antibody library, lymphocytes were isolated from the blood of alpacas. Total RNA was extracted and used to synthesize cDNAs, and the synthesized cDNAs were amplified using VHH specific primers. Recombinant VHH DNA fragments were designed with the DNA fragments consisting of a T7 promoter, omega (Ω) enhancer, Kozak consensus sequence, VHH gene, His-tag, and linker hybridization region (Y tag) in order to become comparable in following assays. The obtained full-length, VHH DNA library was used in the subsequent assays.

To generate cDNA display molecules, a DNA library encoding VHH was transcribed in vitro to yield mRNA. The

mRNA was annealed to the puromycin linker and photo-crosslinking was performed. The cross-linking efficiency of the puromycin linker with the mRNA was estimated to be more than 90% according to the band intensity ratios visualized on PAGE analysis. Higher ligation efficiencies of mRNA and puromycin linker DNA helps to overcome remaining free linkers in the protein translation solution which may end up in inefficient cDNA display formation. The successful formation and purification of cDNA display molecules of VHH was confirmed by SDS-PAGE (5.6%). Although the formation yield of the cDNA display molecules seems to be low, it was in line with our previous studies, in which the formation efficiency of the cDNA display for small polypeptides was observed to be approximately 5%–25%. Following five rounds of in vitro selection using constructed VHH-coding cDNA display molecules against immobilized gliadin, Next Generation Sequencing (NGS) was performed to select three candidate VHHs as affinity binders against target gliadin.

Determination of the interactions between the selected VHHs and the target gliadin is an essential step toward understanding the affinity. Protein interactions were determined using two commonly used assays (pulldown assay and ELISA assay) and also with novel cD-IPCR assay. In the pulldown assay, specificity of the anti-gliadin VHH towards gliadin were confirmed by comparing with control proteins including a single chain peptide (BDA) and a single domain antibody (anti-GFP VHH). The results confirmed that VHH1 is specifically bound with gliadin and gliadin only, and the observed binding was not from potential stickiness to the gliadin. In ELISA assay, results indicated increasing binding of VHH at higher concentrations. Also, higher detection signals were observed for VHH1 and VHH2 candidates compared to VHH3, which was comparable with the pulldown assay and cD-IPCR assay results. Moreover, data indicated the non-specific binding of anti-gliadin VHH to BSA protein was considerably low and at the same time there was no association of anti-BSA VHH to gliadin. However, in ELISA was not able to show any saturated signal even when the concentration reached to 10 μg/mL.

As a novel immuno-qPCR application, we applied our previously developed cD-IPCR assay successfully in this study to understand the binding patterns and specificity of selected VHHs towards gliadin. Compared with other immuno-PCR methods, the cD-IPCR method is unique because of the covalent conjugation mechanism. Here, the peptide is always conjugated covalently to DNA at a ratio of 1:1. Because of the sensitivity of the a ssay, binding of the VHH to very low gliadin concentrations (i.e., 0.001 μg/mL) was able to be detected with a statistical significance ($p < 0.01$, t-test). VHH1 and VHH2 were the best binders according to the immuno-PCR assay and the results were similar to the results of other assays. In the specificity confirmation assay for the VHH1 against two other control proteins (BDA and anti-GFP VHH), the data indicated that VHH1 is uniquely bound to gliadin protein. The data was in line with the results observed in pulldown assay. A preliminary study was performed to assess the applicability of the developed VHHs in detecting gliadin in food to determine whether VHHs can recognize gliadin in the presence of other food components. Moreover, an attempt was taken to detect gliadin using selected anti-gliadin VHH1 in commercially available food samples using the novel cD-IPCR method.

In conclusion, this study opens new paths to combine advanced technologies, such as the cDNA display method and cD-IPCR, to obtain effective binders for toxic and complex proteins, which is difficult to achieve using conventional methods. Also, these immunization-free affinity antibody preparation technologies may reach the ethics and principles of modern scientific experimentation, especially in terms of animal protection. These cDNA display-based assays will open new avenues for gluten quantification. Nevertheless, the cD-IPCR method could be applied with even higher sensitivity levels after making improvements to the binders and protein-capture beads.