博 士 論 文　(要 約)

論 文 題 目　A study on enzymatic synthesis of stable cyclized peptides which inhibit protein-protein interactions

(蛋白質間相互作用を阻害する安定な
環状化ペプチドの酵素合成に関する研究)

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My PhD thesis is composed of six chapters.

In Chapter 1, general introduction including background information and objective & strategy of this thesis was described.

Protein-protein interactions (PPIs) are involved in almost all cellular processes and their defectiveness accounts for dysregulated signaling and the resulted diseases, thus representing an important class of targets for the treatment of various diseases. Inhibitors of PPIs are crucial tools for developing therapies for human diseases. Peptides, which combine the favorable properties of both small molecules and protein therapeutics, represent a promising class of therapeutics to disrupt specific protein-protein interactions. However, it's still challenging to make effective peptide inhibitors, and the generally low half-lives of peptides *in vivo* have probably caused this lack of success.

In my study, with the aim to make peptides more effective at inhibiting specific PPIs: to improve their stability and to evaluate their inhibitory potency *in vitro* and *in vivo*, a combined strategy was employed: the strategy of bioactive sequences grafting into the scaffold of natural cyclic peptides was combined with the strategy of SrtA-mediated synthesis of grafted cyclic peptides, optimization of the grafting site and grafted sequence, as well as BRET-based assay. The advantages of the above strategies are as follows: peptide-grafting strategy provides one of the most effective routes to make peptides less susceptible to proteolytic degradation; SrtA can catalyze protein/peptide cyclization easily and efficiently *in vitro*, moreover, it's promising to extend the applications of SrtA to *in vivo*; BRET assay is applicable both *in vitro* and *in vivo*.

I first selected SFTI-1 to provide the stable scaffold for bioactive peptides grafting, considering its outstanding proteolytic stability, small size, and tolerance to mutagenesis. To explore the possibility of SFTI-1 as the scaffold for developing stable peptide inhibitors, I selected a tetrapeptide ESDV as the model sequence, which is known to be the starting point for developing drugs for the treatment of ischemic brain diseases.

In Chapter 2, the strategy of "enzymatic synthesis of stable cyclic peptide-inhibitors of protein-protein interactions" was investigated *in vitro*. The tetrapeptide ESDV was grafted into the framework of SFTI-1 by replacing the original residues FPDG in the smaller loop which is suggested to be a "plug and play" cassette. The head-to-tail cyclization of the grafted peptide was catalyzed by SrtA, after assembled with the recognition sequences of SrtA.

It shows that cyclization of the grafted peptide catalyzed by SrtA proceeded efficiently, and the cyclic peptide was obtained in good yield. The produced cyclic peptide showed significantly enhanced stability against proteolytic degradation, with half life longer than 20 h in human serum. Interestingly, it is found that SFTI-1 scaffold even without a cyclic backbone could provide stability to a grafted peptide, likely due to the extra stability conferred by the hydrogen bond network, disulfide bond as well as the constrained structure of the scaffold. It is thus concluded that peptide sequences can be stabilized by grafting into SFTI-1 scaffold.

However, in the case of ESDV motif, peptide grafting and cyclization result in reduced binding ability to PDZ2 domain of PSD-95. By SPR analysis, the interaction between the cyclic peptide and PDZ2 domain could be clearly detected, but when the binding ability of the cyclic peptide to immobilized PDZ2 was compared with that of Tat-NR2B9c, which has been under clinical trials for the treatment of ischemic brain diseases, it is found that the response caused by cyclic peptide binding was ～4-fold lower. The reduced binding activity of the cyclic peptide was further confirmed by an ELISA-based competitive binding assay, in which it shows that even at much higher concentrations, the cyclic peptide could not completely inhibit its linear counterpart, which showed strong binding affinity to PDZ2, to bind to PDZ2.

Therefore, it is concluded from Chapter 2 that, SrtA-mediated synthesis of grafted cyclic peptide is efficient, and the produced grafted cyclic peptide shows remarkable stability and desired bioactivity, however, it shows reduced binding activity. With the aim to further address the inhibitory effect of the cyclic peptide *in vitro* as well as *in vivo*, and to optimize the cyclic peptide, the following work was carried out.

To facilitate the evaluation of the inhibitory effect of the cyclic peptide *in vivo*, e.g. in mammalian cells, cyclic peptide was synthesized in mammalian cells rather than delivered into the cells. To facilitate the intracellular synthesis of cyclic peptide, sortase reaction was introduced to mammalian cells.

In Chapter 3, an approach was developed to introduce sortase reaction to living mammalian cells for intracellular synthesis of cyclic grafted peptides. Transduction of *Streptococcus pyogenes* sortase A (SpSrtA) into mammalian cells by Bioporter reagent was confirmed, the function of transduced SpSrtA was analyzed, and the controllability of transduced SpSrtA-catalyzed reaction was studied.

It is found that, our approach, by exogenously introducing SpSrtA to mammalian cells, is easily dose- and time-controllable and shows very low cytotoxicity, but has lower cyclization efficiency because the transduction amount of SpSrtA was confirmed to be lower, when comparing with the reported co-transfection method where both of SpSrtA and its substrate were introduced into cells via DNA transfection. Considering that cyclic peptides should be synthesized in relatively high yield to facilitate the evaluation of their inhibitory effect *in vivo*, therefore, the co-transfection method was selected for intracellular synthesis of cyclic grafted peptides.

In Chapter 4, the strategy of "enzymatic synthesis of stable cyclic peptide-inhibitors of protein-protein interactions" was investigated *in vivo*. By using the approach decided in Chapter 3, peptide and SpSrtA were introduced into mammalian cells, and intracellular peptide cyclization catalyzed by SpSrtA was examined. It shows that, by using the strategy of sortase A-catalyzed peptide cyclization, production of grafted cyclic peptide *in vivo* is feasible, thus facilitating the screening of optimal cyclic peptide-inhibitors *in vivo*.

In Chapter 5, the inhibitory potency of various cyclic peptides was evaluated by BRET

assay. The possible reasons for the decreased binding activity were first explored. The crystal structure of a peptide-complexed PDZ domain reveals that the peptide interacts with PDZ domain mainly via hydrogen bonds (H-bonds), importantly, the C-terminal valine plays a critical role in the interaction by forming four H-bonds via the carboxyl group and making Van der Waals contacts via the side chain. This provides one explanation for the reduced binding activity of the cyclic peptide grafted with ESDV sequence, as the carboxyl group of valine was lost after grafting. Other possibilities are also involved, considering that the binding pocket of PDZ domains has a relatively small surface area, whereas, the ESDV-grafted cyclic peptide has a relatively large size. Accordingly, the grafted peptide sequence as well as the grafting site needs to be optimized to improve the binding activity of the cyclic peptide.

To improve the binding activity of cyclic peptides to PDZ2, different peptide sequences were designed by optimizing the grafting site as well as the grafted peptide sequence. The inhibitory effect of these cyclic peptides was first assessed by an *in vitro* BRET assay, where Rluc-PDZ2 and YPet-NR2B9c fusion proteins were mixed in the absence or presence of cyclic peptides; and except three cyclic peptides, which caused no or little reduction, the addition of other cyclic peptides caused a similar and significant reduction in BRET signal.

The inhibitory potency of these cyclic peptides was further examined in a cellular context, which provides an ideal background for the selection of specific inhibitors, by a cell-based BRET assay, where Rluc-PDZ2 and YPet-NR2B9c fusions were co-expressed and the interaction between PDZ2 and NR2B9c enabled the generation of specific BRET signals. To introduce the cyclic peptides into cells co-expressing the BRET pairs, cells were co-transfected with the DNA of SpSrtA and the linear peptide precursors, and cyclic peptides were synthesized after SpSrtA-catalyzed cyclization. The inhibitory potency of the cyclic peptides can be reflected by the level of decrease in the BRET signal. It is demonstrated by the cell-based BRET assay that cells with cyclic peptides were detected with similar levels of BRET signal.

However, when comparing the results of cell-based BRET assay with *in vitro* assay, inconsistency was observed. Considering that there are still some uncertainties with cell-based BRET assay, *in vitro* BRET assay thus is the better choice for the evaluation of the inhibitory effect of various peptides. Whereas, taking into account of the attractive advantages of cell-based BRET assay, for instance, cellular environment provides the ideal background for the selection of specific inhibitors as well as it is cost effective because cyclic peptides don't need to be chemically synthesized, cell-based BRET assay will be improved for future use.

Finally, to account for the differences in the binding activity of various cyclic peptides, the binding mode of different cyclic peptides to PDZ2 binding pocket was modeled. However, about two thirds of the modeling were unsuccessful; meanwhile, as the modeling was conducted in vacuum state, it's possible that the modeled binding mode is very different from

reality, as a result, more complete studies, e.g. molecular docking, need to be performed to explore the binding modes of different cyclic peptides to PDZ2, thus interpreting their difference in inhibitory potency.

In Chapter 6, a conclusion of my study was made, and future prospect was described.

In summary, by employing the combined strategy of peptide grafting into the scaffold of natural cyclic peptides and SrtA-catalyzed synthesis of grafted cyclic peptides, cyclic peptides with remarkable stability and desired inhibitory activity can be generated; production of such cyclic peptides is efficient *in vitro* and feasible *in vivo*, thus facilitating the screening of optimal cyclic peptide-inhibitors. In addition, BRET assay is a useful tool to evaluate the inhibitory effect of various cyclic peptides *in vitro* and *in vivo*.

In spite of the above favorable findings, there are still some issues to be solved, for instance, cyclic peptides generated in living cells should be quantified in order to more clearly assess and compare their inhibitory potency; the expression levels of the BRET pairs should be fixed by using stably expressing cell lines or normalized to keep the expression levels of BRET pairs consistent among different assays.

In the future, to obtain optimal cyclic peptide-inhibitors, library screening will be performed by combining virtual screening, e.g. molecular docking-based, and biological evaluation, e.g. by BRET assay.

Meanwhile, other bioactive peptides, which are involved in diseases like angiogenesis, can be stabilized by using the same strategy, grafting the sequences into the framework of naturally occurring cyclic peptides like SFTI-1. The grafting site as well as grafted sequence can be further optimized under the guidance of computationally simulated interaction studies, to seek for potential drug leads for the treatment of diseases. Furthermore, the functional activities and selectivity of the potential drug leads can be assessed based on the validated BRET assays.