Establishment of proven molecular evolution method PLM through the acquisition of Aβ42 aggregation-inhibitory/cytotoxicity-preventive peptides

（Aβ42 重合阻害／細胞毒性阻止ペプチドの取得などを通じての分子進化戦略 PLM 法の確立）

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Establishment of proven molecular evolution method PLM through the acquisition of Aβ42 aggregation inhibitory/cytotoxicity-preventive peptides

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To

My family

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Abstract

There is a great demand for the development of novel therapeutic molecules that combine the high specificity and affinity shown by biologics with the bioavailability and lower cost of small molecules. Since peptides (typically less than 100 amino acids in size) possess high binding affinity and specificity comparative to antibodies and have the high possibility to penetrate cells due to their smallness, peptides are ideal therapeutic candidates to bridge the gap between small molecules and biologics. Although many research studies are going on to develop the therapeutic peptides, there is a great deal of room to develop much better therapeutic peptides. This situation motivated us to develop the novel peptides with high binding affinity, specificity and functional activity towards different therapeutic targets. So, the objectives of this study are i) to develop novel peptides which have a high binding affinity and specificity towards beta amyloid 42 (Aβ42); ii) to screen the peptide-based inhibitors of Aβ42 aggregation and cytotoxicity so that these peptides can be therapeutic candidates for Alzheimer`s disease (AD) iii) to prove that progressive library method (PLM) can be an established molecular evolution method to engineer the peptides with high binding affinity, specificity and potential functional activity against different therapeutic targets, e.g. Aβ42, cathepsin E and etc.

Several decades of cumulated research evidence has proven that Aβ42 is the main cause of neuronal death in the brain of the patient with AD. Therefore, inhibition of Aβ42 aggregation holds great promise for the prevention and treatment of AD. Although several drugs based on Aβ immunotherapy and small molecule have been developed

and recently much effort has been given to develop drugs based on the peptides/peptide aptamers, none of them has yet translated into new medicines. To this end, it was aimed to develop the peptides which have high binding affinity and specificity to Aβ42.

To achieve the first two objectives of this study, the mRNA (cDNA) display technique and the *progressive library method* (PLM) were used. Once evolved peptides by the second stage all-steps-all-combinations (ASCS) method were further evolved by the third stage paired peptide (PP) method. The PP library generated from the second library selection products was subjected to *in vitro* selection against Aβ42 by using cDNA display method and then cloning and sequence analysis was performed to select the most frequently occurred PPs in the library.

By using surface plasmon resonance experiment, it was found that two PPs, P84 and P131 had higher binding affinity for the Aβ42 peptide (K_d value of 20 nM and 12 nM, respectively) than the previously reported Aβ42 binding peptides. To the best of our knowledge, this is the first report showing the development of randomly evolved peptide aptamers with the highest binding affinity to Aβ42. Then the functional characterization of P84 and P131 was performed to check their potential of inhibiting aggregation and cytotoxicity of Aβ42 peptide by using thioflavin T assay, atomic force microscopy assay and PC12 cells based cytotoxicity assay. It was found that both P84 and P131inhibited the aggregation of Aβ42, leading to the reduction of the cytotoxic effect of Aβ42 on PC12 cells. Therefore, it is believed that these PPs can be the potential therapeutic seeds for the AD.

As of now, PLM consists of the evolution of the first, second and third library and each library selection can be assigned as module finding, module shuffling and module pairing, quite similar phenomena occurring in natural evolution of proteins. As the last step in PLM, in this study the fourth library was introduced based on the point mutation together with the DNA shuffling method to further improve the affinity and functional activity of the most improved paired peptide evolved from the third library. So, the P109 peptide, which showed the strongest binding affinity to cathepsin E at neutral pH (K_d value of 2 nM), was selected and a randomly base-substituted library (fourth library) was generated by inserting point mutations in the sequence of P109. One mutant containing single point mutation of P109, here after named SK1, showed 1.6 fold improved binding affinity to cathepsin E at neutral pH compared to the affinity of non-mutated P109. SK1 also showed significantly improved functional activity (5% increased functional activity compared to that of P109).

In this study, highly functional peptides with a strong binding affinity towards the respective peptides were successfully developed from the third and fourth library, demonstrating the effectiveness of the progressive library method (PLM) in the evolution of peptides/proteins.

Since the natural evolution of proteins is based on point mutation and recombination, the evolution strategy for proteins, i.e., PLM seems to be completed by the introduction of the fourth library of point mutation-based one following the recombination-based second and third libraries.

List of figures

List of tables

List of abbreviations

ASAC	all-steps-all-combinations
AD	Alzheimer`s disease
APP	amyloid precursor protein
ANS	8－Anilinonaphthalene-1-sulfonate
AFM	atomic force microscopy
Aβ	beta amyloid
Aβ40	beta amyloid 40
Aβ42	beta amyloid 42
BBB	blood brain barrier
CCK-8	cell counting kit-8
DMSO	Dimethyl Sulphoxide
DMEM	Dulbeco`s modified eagle medium
D-PBS	Dulbeco`s phosphate buffer saline
eRAPANSY	evolutionary rapid panning and analysis system
FAD	familial AD

K_d	dissociation constant
µM	micro molar
nM	nano molar
PAGE	poly acrylamide gel electrophoresis
PLM	progressive library method
PP (s)	paired peptide (s)
PS1/2	presenilin 1/2
SDS	sodium dodecyl sulphonate
SPR	surface plasmon resonance
ThT	Thioflavin T
WST-8	water-soluble tetrazolium salt-8
YLBS	Y-ligation based block shuffling

Table of contents

Chapter 1

Introduction and literature review

1. Background

Current drug industry has been dominated by the drugs based on small molecules and biological macromolecules (biologics) (Verdine *et al*., 2007). The small molecules are generally synthetic organic compounds with a simple chemical structure and molecular size less than 1000 Dalton (less than 100 atoms). Small size, low cost and low price, oral bioavailability, ready synthesis, membrane-penetrating ability and stability are the advantages of small molecules. However the drugs based on small molecules have the major disadvantage of low specificity and higher attrition rate in the clinic (Craik *et al*., 2013). On the other hand, biologics are mainly protein or carbohydrate based (like antibodies, insulins etc). Unlike small molecules, biologics have a very high affinity and specificity to the targets so there is increasing demand of drugs based on biological macromolecules (Reichert *et al*., 2005). The main hurdle with biologic drugs is their very poor cell permeability due to the very big size of biologics (50 KDa -150 KDa). Due to this nature, 90% of drug targets are not accessible for targeting (Verdine *et al*., 2007). In addition, only 10% of proteins encoded in the genome are estimated to be amenable to small-molecule targeting and another 10% by biologic drugs, and thus 80% of proteins are currently termed as `undruggable` (Schlippe *et al*., 2012). Moreover, biologic drugs are very costly due to the complexity of production in large quantities. Furthermore, biologics are very sensitive to various environmental factors like heat, light, agitation etc. From the above mentioned facts about small drugs and biologic

drugs, it is clear that there exist a big gap between the small molecules and biologic drugs. Therefore, the time has now come to discover new drug leads that fit between small molecules and biologics with the goal of combining advantages of small molecules and biologics.

1.2 Peptide as a therapeutic drug

An important goal of drug discovery is to develop nonmacromolecular species that retain the favorable molecular properties of antibodies and small molecules. Since peptides (usually less than 50 amino acids in size) possess high binding affinity and specificity comparable to antibodies and have the high possibility of cells/tissue penetration due to their smallness, these peptides are the ideal therapeutic candidates to bridge the gap between small molecules and biologic drugs (Zhang *et al*., 2000). Since peptides are much smaller than antibodies, peptides can be readily synthesized, optimized, evaluated and do not cause serious immune responses. Moreover, peptides are potent and could be metabolically cleaved and readily cleared from the body minimizing their toxic side effects.

Traditionally, therapeutic peptides can be obtained from three different sources such as i) natural or bioactive peptides produced by plants, animal or human (naturally occurring peptide hormones or from fragments of larger proteins); ii) peptides discovered from chemical libraries and iii) peptides isolated from genetic/recombinant libraries. Since bioactive peptides are selected by nature to have a stable fold, they have key contact residues to impact function and they have increased *in vivo* stability against proteases. However, there are only limited bioactive peptides against the specific target proteins. Until few years back, the peptide based drugs were mainly dominated by the

bioactive peptides due to the lack of the technology to develop the peptides as potent as bioactive peptides (Sato *et al*., 2006). However the development of the recombinant display technologies with the ever-increasing levels of diversity, it has been possible to find novel peptides as rival of those found in nature (Sato et al., 2006; Landon *et al.*, 2005). Over the past decade, recombinant display technologies have been essential tools in the discovery of peptide and protein ligands and have played a vital role in the directed molecular evolution of therapeutic peptides.

1.3 Directed molecular evolution

Directed molecular evolution mimics natural evolution of proteins/ peptides in the laboratory (*in vitro*) but operates on molecular level and focuses on specific molecular properties. Directed molecular evolution results novel peptides/proteins through generation and selection of combinatorial libraries of nucleic acids and peptides. Molecular selection technologies involve the construction of large, diverse DNA molecules and the establishment of associated selection mechanisms for identifying and isolating those that encode the relevant peptides, which bind with high affinity and specificity to a given target molecule (McGregor *et al*., 2008). These methods depend on the physical linkage of each nucleic acid with its encoded peptide. Moreover, the molecular selection methods depend on different display technologies for the development of novel peptides.

Based on the mode of selection of the library, there are *in vivo* and *in vitro* display technologies. The *in vivo* display technologies include phage display, bacterial display and yeast display. Phage display was developed by George P. Smith in 1985. Bacterial display systems were first introduced by Freudl *et al*. and Charbit *et al*. in 1986 where

as yeast display was developed by Borden and Wittrup in 1997. Since the invention, these display methods have been successfully applied to many different areas of research, including immunology, cancer research, drug discovery, epitope mapping, protein-protein interactions, plant science and infectious diseases, targeting a broad range of protein families. Though phage display has shown great potential in the discovery of new therapeutics and in recent years, the best known peptide display is the phage display technology itself (McGregor *et al*., 2008; Huang *et al*., 2012).

Phage display technology is based on the construction of a polypeptide library fused to a bacteriophage coated protein. In the phage display technique, a DNA library is genetically fused to a filamentous bacteriophage (commonly used bacteriophage is M13) so that co-infection by the bacteriophage, newly emerging phage particles encase individual gene sequences while displaying the corresponding gene-encoded polypeptides on their outer surfaces (Huang *et al*., 2012). In this way phage particles provide the physical link between genes and their encoded polypeptides. Usually phage display results about 10^{10} different polypeptides in the library which is incubated with an immobilized target molecule. All the non-binding library members are removed by washing and those rare species of the library which bind to the target are eluted. Some of the peptides derived from phage display are currently in clinical and preclinical trials (Giuliani *et al*., 2007).

Although phage display is now playing a significant role for the discovery of novel therapeutic peptides (Giuliani *et al*., 2007), there are some limitations of this technology. As already mentioned that Phage display has the peptides displayed on the surface of the bacteriophage, it allows only the screening and enrichment of peptides based on

natural amino acids, resulting the reduced diversity of the selected sequences. Furthermore, the library size is limited by the transformation efficacy of the bacteriophage and also the displayed peptide size is small.

1.4 mRNA/cDNA display technology

In order to overcome the limitations of phage display technology, two display technologies, mRNA (cDNA) display and ribosome display technologies, were developed at the end of the 1990s. The mRNA display technology was first described by Roberts and Szostak in 1997 and the same principle was reported concomitantly under the name of *in vitro* virus by Nemoto *et al*. in 1997 whereas ribosome display technology was first described by Hanes and Pluckthun in 1997. Since both mRNA and ribosome display technologies are based on the *in vitro* selection of the library, the library size is as large as 10^{13} different variants. Both display technologies begin with the library of DNA sequences coding for the random peptides. In case of mRNA display, the translated peptide is associated with the progenitor mRNA via a puromycin linker (Roberts *et al*., 1997 and Nemoto *et al*., 1997). Puromycin is an analog of the 3' end of a tyrosyl-tRNA with a part of its structure mimicking a molecule of adenosine, and the other part, a molecule of tyrosine. Since puromycin has a non-hydrolysable amide bond, it interferes with translation resulting the release of translation products (Roberts *et al*., 1997). In case of ribosome display, the translated peptide is associated with the progenitor mRNA and the stalled ribosome.

Although both mRNA display and ribosome display technologies have the great merit of very high diversity of the library and synthesis of proteins/peptides by using both natural and unnatural amino acids, mRNA display is superior over ribosome display

technology (Roberts *et al*., 1999). In case of ribosome display, selection stringency is limited to keep ribosome-mRNA-polypeptide in a complex because of the noncovalent ribosome-mRNA-polypeptide complex. Furthermore, ribosome is very large in size so that there might be unwanted interaction between selection target and the ribosome, and this may lead to a loss of potential binders during the selection cycle. On the other hand, puromycin linker used in mRNA display system is much smaller comparing to ribosome so that this linker may have less chance to interact with an immobilized selection target. Therefore, the mRNA display technology is more likely to give less biased results.

Although the mRNA display system was originally developed for evolutionary protein engineering based on *in vitro* translation system, they were subsequently used for various purposes like for the discovery of therapeutic peptides from natural and/or non-natural and cyclic and/or linear amino acid libraries, for functional analysis including, protein-protein, drug-protein, peptide-protein and antigen-antibody interactions (Takahashi *et al*., 2003; Wada *et al.*, 2013; Schlippe *et al.*, 2012). Previous studies have revealed that *in vitro* selection of peptides from wide variety of libraries is a powerful approach for the development of new therapeutics against target molecules like GPCR which is a major drug target (Wada *et al*., 2013).

1.5 Evolutionary rapid panning and analysis system (eRAPANSY)

cDNA display (Tabuchi *et al*., 2001), a modified version of the mRNA display has been successfully applied in evolutionary rapid panning and analysis system (eRAPANSY) to develop novel peptide drug candidates against different targets like cathepsin E (acidic protease) (Kitamura *et al*., 2009). The eRAPANSY consists of three

primary steps; i) generation of huge diversity (10^{13}) library of molecules in the form of cDNA display by employing a Y-ligation based block shuffling (YLBS) technique (Kitamura *et al.*, 2002), ii) selection of peptides either based on selection-by-function or selection-by-affinity and iii) generation of progressive libraries by progressive library method (PLM), which allows the generation and selection of clones with greater activity and affinity than in the previous library, through the use of the YLBS. Novel peptides with a strong binding affinity (K_d in the pM range) has been successfully applied against the protease cathepsin E proving the effectiveness of this technique *in vitro* molecular evolution of therapeutic peptides (Kitamura *et al*., 2012; Madhu *et al*., 2011; Komatsu *et al*., 2012).

1.6 Progressive library method (PLM)

PLM is one of the important strategies for systemic i*n vitro* evolution method to develop advanced peptides against a specific target protein (Kitamura *et al*., 2012). PLM was first introduced by Kitamura *et al*. in 2009, which consists of generation of progressive libraries through the use of the Y-ligation-based-block shuffling (YLBS) method (Kitamura *et al*., 2002). In PLM, the successfully selected peptides from the preceding library are used to build the successive library. The successfully selected peptides (8 amino acids in length) from the secondary library by using *in vitro* cDNA display method showed improved functional activity and binding affinity against the protease cathepsin E at acidic pH compared to that of the peptides selected from the primary library. The peptides thus obtained from the secondary library were randomly shuffled to generate the third library (paired peptide (PP) library). The library was then used for *in vitro* selection by affinity or by function. The successfully selected paired

peptides (PPs) exhibited highest functional activity and binding affinity to cathepsin E at physiological pH and acidic pH (Biyani et al., 2011, Kitamura et al., 2012 and Komatsu et al., 2012). The general scheme of progressive library method is summarized in Fig.1.1 (Komatsu et al., 2012).

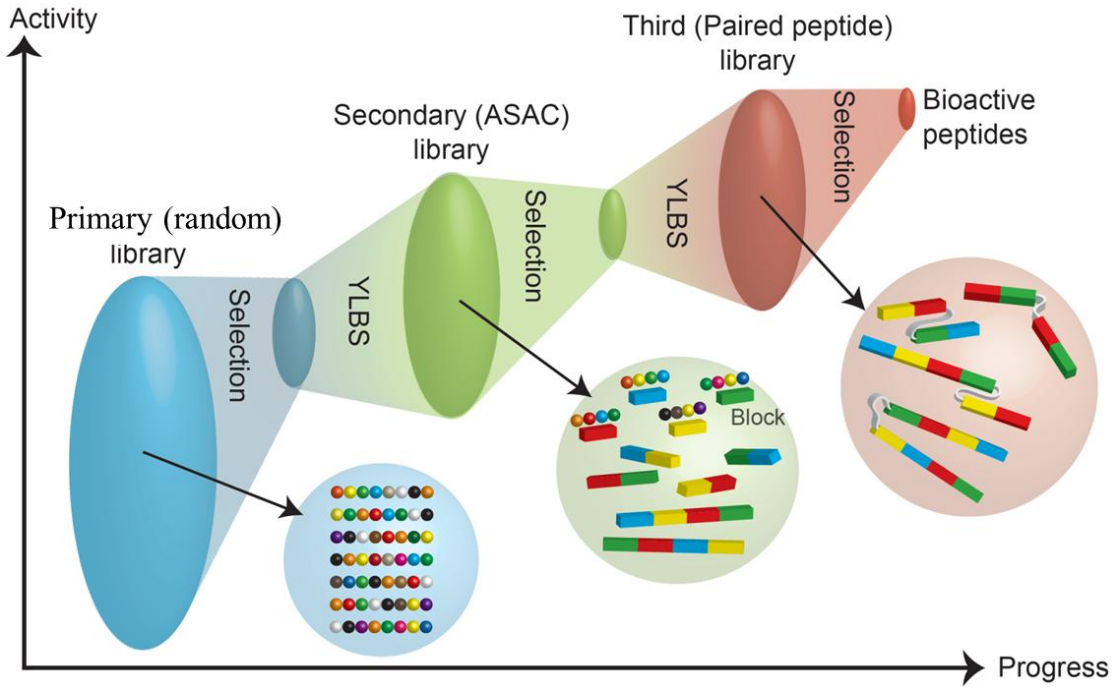

Figure 1.1. Progressive library method. Functional peptides were identified from the first random peptide library. The second library was generated by combining peptide blocks obtained from the primary library and subjecting them to the next round of selection. The tertiary library was constructed by pairing two peptides selected from the second library (Komatsu *et al*., 2012).

The primary, secondary and tertiary library selections used in PLM can be regarded as module finding, module shuffling and module pairing respectively, quite similar phenomena occurring in natural evolution of proteins. The functional activity and affinity of PPs against cathepsin E under different conditions proved the methodological

effectiveness of PLM in the *in vitro* evolution of therapeutic peptides. These previous studies on cathepsin E also demonstrated the proof of principal that PLM can be used to develop the therapeutic peptides against a vast array of targets like Aβ42 which is proven to be a main pathological hallmark of Alzheimer`s disease.

1.7 Research objectives and methodology

The plethora of research evidence has proven that Aβ42 plays a key role in Alzheimer`s disease pathogenesis (Selkoe DJ, 2001). Moreover, several lines of research evidences have proven that aggregation of Aβ42 is a main pathological hallmark of AD (Hardy and Selkoe, 2002). However, as of now there is no cure of AD. So, development of nM affinity peptides which can inhibit or reverse the aggregation and cytotoxicity of Aβ42 holds a great potential of therapeutic value for the treatment of AD. Although several peptides and small molecules as Aβ42 aggregation inhibitors have been reported by many research groups, none of them have been developed as a drug. To this end, PLM was used in previous study to find out the novel peptides against Aβ42 (Ueno-Tsuji *et al*., 2011) through *in vitro* selection of primary and secondary library. However, the best peptide from secondary library showed the moderate level of binding affinity to Aβ42 (K_d value in μM range) which is far below the binding affinity needed to be used as a potential therapeutic candidate for Aβ42. Since the novel PPs evolved from tertiary library of PLM showed strongest binding affinity and functional activity against cathepsin E (Komatsu *et al*., 2012; Kitamura *et al*., 2012), this fact motivated us to generate and screen the paired peptide (PP) library which is the third library of PLM to develop novel peptides with improved binding affinity to Aβ42. Therefore, the objectives of this study are listed below.

i) Development of novel peptides with higher binding affinity and specificity to Aβ42.

ii) Functional screening of novel peptides which can inhibit Aβ42 aggregation and cytotoxicity so that these peptides can be the therapeutic candidates for Aβ42

iii) Establishment of the progressive library method (PLM) as an effective *in vitro* molecular evolution method to engineer novel peptides with high binding affinity, specificity and functional activity against different therapeutic targets, e.g. Aβ42, cathepsin E and etc.

In order to fulfill the first objective of this study, the third library (paired peptide library) was generated by random pairing of peptides selected from the second library using YLBS method. Once evolved peptides by the all-steps-all-combinations (ASCS) method introduced in secondary library were further evolved by the paired peptide (PP) method. The PP library generated from the third library selection products was subjected to *in vitro* selection against Aβ42 by using cDNA display method and then cloning and sequence analysis was performed to select the most frequently occurred PPs in the library. After 4th round of successive in vitro selection by cDNA display method, the potential PPs were selected from the cloning and sequence analysis. The surface plasmon resonance (SPR) analysis was performed to confirm the binding affinity of the selected PPs to Aβ42. In order to fulfill the second objective, different biophysical characterizations based on thioflavin T assay, atomic force microscopy assay, polyacrylamide gel electrophoresis and *in vitro* bioassay were performed. Indeed, the introduction of PP approach in case of Aβ42 can provide strong support that PLM is an effective *in vitro* evolution method. Furthermore, in order to fulfill the third objective of this study, the fourth library was introduced by generating random mutagenesis library

of cathepsin E activating paired peptide P109 (K_d value of 2 nM) based on single point mutation together with DNA shuffling approach.

1.8 Thesis organization

The whole thesis has been divided into four chapters. Chapter 1 is about the introduction and literature review of this study. The objectives of this study are mentioned at the end of this chapter.

Chapter 2 is about the establishment of proven molecular evolution method PLM through acquisition of Aβ42 aggregation-inhibitory/cytotoxicity-inhibitory peptides. This chapter consists of the major research findings of this study. Identification of novel PPs with highest binding affinity to Aβ42 has been described in this chapter. Similarly, inhibition of Aβ42 aggregation and cytotoxicity by PPs has been shown by performing different biophysical characterizations like thioflavin T assay, atomic force microscopy and PC12-cells based bioassay.

Chapter 3 is about the improvement of binding affinity of cathepsin E activator P109 peptide. A fourth library was introduced based on the point mutation together with the DNA shuffling method. A randomly base-substituted library (fourth library) was generated by inserting point mutations in the sequence of P109. The library was used for affinity-based *in vitro* selection using cDNA display method. The results mentioned in this chapter suggested that fourth library is the last step of PLM, supporting that PLM is an established *in vitro* molecular evolution method to develop new therapeutic peptides against different target proteins/peptides.

Chapter 4 is about the overall conclusions and prospects to the future study. The major findings of this study and future prospects of the study have been described in this chapter.

Chapter 2

Acquisition of Aβ42 aggregation-inhibitory/cytotoxicity-preventive peptides using PLM

2.1 Abstract

Several decades of cumulated research evidence has proven that aggregation of beta amyloid 42 (Aβ42) is the main cause of neuronal death in the brain of patient with Alzheimer's disease (AD). So, Inhibition of Aβ42 aggregation holds great promise for the prevention and treatment of AD. Although several drugs based on Aβ immunotherapy and small molecule have been developed, none of them has yet translated into new medicines. The main problem with Aβ immunotherapy drugs is their failure to cross the blood brain barrier. On the other hand, small molecule drugs have the potential of off target specificity and high clinical attrition rate. Since peptides have the high affinity and specificity comparable to the immunotherapy drugs and also peptides have the high possibility of crossing the blood brain barrier due to their small size compared to immunotherapy drugs, recently much effort has been given to develop drugs based on the peptides/peptide aptamers. Despite intensive research studies on the peptide candidates for AD therapeutics, there is a great deal of room for improvement in the design of much better peptides. So, in this study, systematic *in vitro* evolution method called progressive library method (PLM) was used and novel peptides with nano-molar affinity to Aβ42 were developed. Particularly, two peptides, P84 and P131 showed about 26 fold and 48 fold, respectively, higher binding affinity to Aβ42 compared to the peptide with strongest binding affinity to Aβ42 developed from

previous library. Furthermore, both paired peptides showed strong inhibition of Aβ42 aggregation, resulting the recovery of PC12 cells from the cytotoxic effect of aggregated Aβ42. Our results suggest that these novel peptides can be the potential therapeutic seeds for Alzheimer`s disease.

2.2 Introduction

2.2.1 Alzheimer`s Disease

Alzheimer`s disease (AD) is an age-related neurodegenerative disorder and is the most common cause of dementia among aged people (Brookmeyer *et al*., 2007). AD was named after the name of German neuropathologist Alois Alzheimer who first described this disease in 1906 (Berchtold *et al*., 1998). Clinically, the symptoms of AD include the loss of memory, thinking ability and eventually leading to the loss of ability to carry out the simplest tasks (Selkoe DJ, 2001). The main affected area of the brain by AD is the hippocampal area which is the main region responsible for memory formation. It has been predicted that AD will affect 1 in 85 people worldwide by 2050 (Brookmeyer *et al*., 2007). The histopathological features of AD include the presence of amyloid beta plaque, neurofibrillary tangles and neuronal atrophy (Hardy and Selkoe, 2002). Although the exact cause of AD remains unknown and most of the reported evidences are rather sporadic, a small fraction of AD patients (less than 1%) genetically inherit AD through autosomal dominant inheritance which results disease onset before the age of 65. Mutations that increase the accumulation of beta amyloid (Aβ) deposition are associated with familial AD (FAD). Mutations in amyloid precursor protein (APP), presenilin 1 (PS 1) and presenilin 2 (PS 2) are proven to be responsible for the onset of FAD (Nunan and Small 2002). These mutations all result in an increased production of

Aβ42 and lead to AD neuropathology, suggesting that Aβ42 deposition is one of the pivotal steps in the pathogenesis of AD. This supports the amyloid cascade hypothesis which proposes that the increased production or decreased clearance of Aβ peptides causes the AD (Hardy and Selkoe, 2002).

2.2.2 Beta amyloid 42 (Aβ42) as a therapeutic target for AD

The Aβ peptide, which consists of 40 to 42 amino acids, is generated from the β – amyloid precursor protein (APP) by the subsequent proteolytic activities of β- and ϒ-secretases (Haass *et al*., 1992; Seubert *et al*., 1992 and Shoji *et al*., 1992). Extensive research studies strongly support the central role of amyloid beta (Aβ) in the pathogenesis of Alzheimer`s disease (Hardy and Selkoe, 2002). Based on molecular genetics, exploration of mutations in APP and direct alteration of Aβ production in AD experimental model animals further supports the key role of Aβ in AD pathogenesis (St. George-Hyslop *et al*., 2000 and Jonsson *et al*., 2012). At the early days of AD research, the extracellular amyloid fibril formation was regarded as the main cause of AD (Hardy *et al*., 1992) however the recent studies have proven that progressive accumulation of soluble Aβ oligomers play a vital role in the pathogenesis of AD by causing long term potentiation impairment and synaptic dysfunction (Lue *et al*., 1999; McLean *et al*., 1999; Haroutunian *et al*., 2000; Walsh *et al*., 2007; Shankar *et al*., 2008 and Benilova *et al*., 2012).

Aβ40 and Aβ42 peptides are the two major peptides present in AD patients. Although Aβ40 is produced in significantly large amount compared to Aβ42 by enzymatic cleavage from the APP, Aβ42 has been found more neurotoxic due to its higher

hydrophobicity, which leads to faster oligomerization and aggregation (Walsh *et al*., 2007 and Blennow *et al*., 2006). Furthermore, it has been reported that Aβ42 oligomers are more toxic than protofibrils or fibrils (Ahmed *et al*., 2010). Thus, Aβ42 has become a major therapeutic target with different anti- Aβ strategies being accompanied. One of the strategies includes lowering the production of the Aβ peptide by inhibiting the enzymes responsible for Aβ generation (Jasvinder *et al*., 2011). Similarly, another strategy is to prevent the formation of Aβ aggregates and to increase the rate of Aβ clearance from the brain. This strategy can be accomplished by Aβ immunotherapy which uses anti- Aβ antibodies generated following vaccination or introduced passively and over the last one decade, Aβ immunotherapy has entered for clinical trials in humans (Lemere *et al*., 2010).

Until today, there is no cure of AD and the available medications can temporarily slow the worsening of symptoms. Although great effort and cost has been invested to understand the pathophysiology of AD and to develop the Aβ immunotherapy drugs, this process has not yet translated into new medicines due to the failure of drugs in different phases of clinical trials (Barry *et al*., 2012 and Salkoe *et al*., 2011). Similarly, despite the invaluable role of antibodies in medical diagnostics and biomedical research, antibodies are tedious and expensive to generate, and it is very difficult to produce them in very large quantities. To this end, much effort has been devoted to design small molecules and peptides capable of inhibiting Aβ aggregation and to neutralize Aβ cytotoxicity. Most of these small molecules have been identified by screening thousands of already available small molecules (Angela FM, 2012) whereas small peptides were generated as a partial fragment of the Aβ42 peptide by rational design or by

combinatorial libraries (Takahashi *et al*., 2010; and Baine *et al*., 2010). Despite the intensive research studies on such small molecules and peptides as a potential candidate for AD therapeutics, there is lot of space to design much better small peptides with high binding affinity and specificity to Aβ42 peptides. This fact motivated us to perform this study.

2.2.3 Objectives relevant to this study

The objectives of the study described in this chapter are i) to develop novel peptides with high binding affinity and specificity to Aβ42 and ii) to develop potential therapeutic seeds to inhibit and reverse the misfolding and aggregation of Aβ42.

In order to fulfil these objectives, it was aimed to develop high-affinity Aβ42 binding peptide aptamers based on evidence supporting the use of peptide aptamers for biological and therapeutic applications (Li *et al*., 2011). Here, the evolution of novel peptide aptamers using the mRNA (cDNA) display technique [Nemoto *et al*., 1997; Robert *et al*., 1997] and paired peptide (PP) method (Kitamura et al., 2012; Komatsu et al., 2012) is described. In the previous study, peptides with a moderate binding affinity to Aβ42 (K_d in μM) were explored from the preceding libraries by the all-steps-all-combinations (ASAC) method (Tsuji-Ueno et al., 2011). In the present study, thus evolved peptides were further evolved by the PP method. The evolved peptide aptamers, hereafter called paired peptides (PPs), contained two peptide moieties which were arbitrarily combined by linking through a definite length of a linker sequence. The selected two peptides had higher binding affinity for the Aβ42 peptide than previously reported Aβ42 binding peptides (Tsuji-Ueno et al., 2011). Furthermore, different

biophysical experiments were performed to show that the novel peptides with improved binding affinity can inhibit the aggregation of Aβ42 leading to the reduction of the cytotoxic effect of Aβ42 in PC12 cells.

2.3 Materials and Methods

Chemicals were obtained from Wako (Japan), unless otherwise stated.

2.3.1 Generation of a paired peptide library and cDNA-peptide fusion product

A paired peptide (PP) library was generated by following the Y-ligation-based block shuffling (YLBS) method (Kitamura et al 2002) with slight modifications. In brief, cDNAs coding for 8 different types of peptide blocks selected from the first and second libraries (Tsuji-Ueno *et al.*, 2011) and two types of linkers (Table 2.1) were synthesized and purified (> 95%) by JBios, Japan. Before starting the first round of YLBS, phosphorylation of 5` end of 3`-half spacer and 3`-half Y-Tag was performed by incubating the mixture of 100 µM 3`-half spacer mixture or 3`-half Y-Tag (1 µl), 10× PNK buffer (1 µl), 10 mM ATP (1 µl), 1 µl of PNK (10,000 U/ml) (NEB) and water up to 10 µl at 37^{o}C for 1 h. Then, the reaction mixture was heat inactivated at 85^{o}C for 15 min.

After phosphorylation reaction, the first round of YLBS was performed. For this purpose, 10 pmol/µl 5`-half peptide blocks mixture was mixed with 10 pmol/µl 3`-half phosphorylated mixture, 5µl of 2× ligation buffer (specially prepared for YLBS, Kitamura *et al*., 2002) and 1 mM ATP (1 µl) and then the mixed reagents were used for annealing reaction by incubating the reaction mixture for 2 min at 94^{o}C, for 10 min at

60^{o}C and then for 10 min at 40^{o}C. The lowering of temperature should be very gradual (0.1^{o}C/sec). After that, as shown in Figure 2.1 below, the first round of YLBS was performed with biotin-labeled 5′-half peptide blocks and 3′-half linker blocks by adding 2 µl T4 DNA ligase (Takara) to the annealing reaction and incubating at 25^{o}C for overnight (up to 16 hours). The YLBS product was then digested with the MboI restriction enzyme (NEB) and then purified using Dynabeads MyOne Streptavidin C1 (Invitrogen) to obtain single-stranded 5′-half DNA sequence blocks coding for "D5-peptide-spacer blocks." Then, a second round of YLBS was performed with D5-peptide-spacer blocks and a 3′-half peptide blocks to obtain DNA sequences that code for PPs composed of "peptide-spacer-peptide" blocks by following the protocols as mentioned above. The required cDNA construct to use for cDNA display (Xa-paired peptide-Y-Tag) was prepared by PCR using forward primer b-5`-half-stem (5`-*GGCTCGCGAATA CTGCGATTGAAGGTCGT*-3`) and reverse primer 3`-stem rev (5`-*GGCTCGCGAATACTGCGAAGG AGTGAG*-3`). In this experiment, at first two PP libraries were generated by using short and long linker separately and only the final product obtained after PCR was mixed together resulting the PP library. The reason of doing so was to minimize the possible biased diversity of PPs during YLBS which is supposed to be more efficient with short linker compared to that with long linker. Theoretically, the PP library consisted of 128 different variants (8 different types of 5'-half peptide blocks x 2 types of linkers x 8 different types of 3'-half peptide blocks). However, the actual library diversity was assumed to be much higher due to the possible substitution and indel mutations during library generation process (Komatsu *et al.*, 2012).

Table2.1. Peptide blocks and spacers used for constructing the third library (Paired peptide library)

Name	(5` -> 3`) Nucleotide sequence	Length (bp)	Amino acid sequences
5-half peptide blocks			
P5001	D5-GGC AGC AGC CCT CCT GGC CTG TGC	53	GSSPPGLC
P5002	D5-TGC TGC AAG CCT TCC TGT TGC TGT	53	CCKPSCCC
P5105	D5-TGC GGC ATT CTC GAT CCC ATC CCT TGG	56	CGILDPIPW
P5109	D5-GGC TGC CCA TGC ATT GGC ATT ATT	53	GCPCIGII
P5128	D5-CCA AAC CCA CCA GAT CAA ATC CCC ACC	56	PNPPDQIPT
P5226	D5-CCA AAC CCA CCA GAT CCC ATC CAT TGG	56	PNPPDPIHW
P5241	D5-GAC GGC AAA TCC ATT GGC CCA ATT	53	DGKSIGPI
P5268	D5-GAC TGC TCC TCC GAT CTC ACT CCT TCG	56	DCSSDLTPS
3-half linker blocks			
L1	D3-GGA GGC TCC GGA GGC AGC	48	GGSGGS
L2	D3-GGC GGA GGC TCC GGT GGA GGG AGC GGA GGC GGT TCT	66	GGGSGGGG GGS
3-half peptide blocks			
P5001	D3`-GGC AGC AGC CCT CCT GGC CTG TGC	76	GSSPPGLC
P5002	D3`-TGC TGC AAG CCT TCC TGT TGC TGT	76	CCKPSCCC
P5105	D3`-TGC GGC ATT CTC GAT CCC ATC CCT TGG	79	CGILDPIPW
P5109	D3`-GGC TGC CCA TGC ATT GGC ATT ATT	76	GCPCIGII
P5128	D3`-CCA AAC CCA CCA GAT CAA ATC CCC ACC	79	PNPPDQIPT
P5226	D3`-CCA AAC CCA CCA GAT CCC ATC CAT TGG	79	PNPPDPIHW
P5241	D3`-GAC GGC AAA TCC ATT GGC CCA ATT	76	DGKSIGPI
P5268	D3`-GAC TGC TCC TCC GAT CTC ACT CCT TCG	79	DCSSDLTPS

D5 : GGC TCG CGA ATA CTG CG **ATT GAA GGT CGT (29 bp)**

D3 : GAT CTC ACT CCT T CGC AGT ATT CGC GAG CC (30 bp)

D3`:*AGG ACG GGG GGC GGC GGG GAA A* GAT CTC ACT CCT T CGC AGT ATT CGC GAG CC (52 bp)

The underlined sequences are complementary to eachother. Sequences in bold letters in D5 code for Xa factor recognition sequence (IEGR). The sequences written in italic in D3` (Y-Tag sequences) are the sequences for puromycin linker binding site. LI: Liker 1; L2: Linker 2

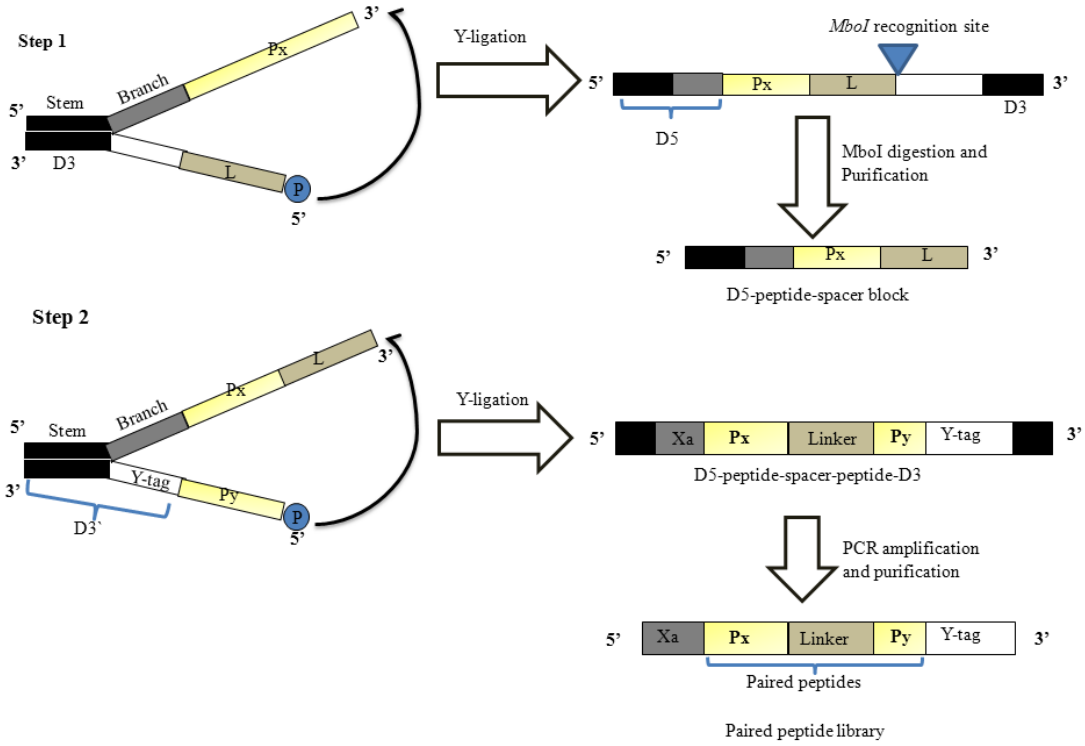

Figure 2.1. **Schematic representation of generation of paired peptide (PP) library by Y-ligation-based block shuffling (YLBS) method.** Note that 8 different types of peptide blocks (Px as 5`-half peptide blocks and Py as 3`-peptide blocks) and two types of linkers (L) were used in this study. The first step YLBS was performed with Px and two types of linkers (L). The YLBS product was digested with the *Mbo*I restriction enzyme and then purified using Dynabeads MyOne Streptavidin C1 to obtain single-stranded 5′-half DNA sequence blocks coding for "D5-peptide-spacer blocks". Then, second step YLBS was performed with 5`-half "D5-peptide-spacer blocks" and Py to obtain DNA sequences that code for paired peptides composed of "D5-peptide-spacer-peptide-D3`" blocks. Here, Xa represents the Xa factor recognition site (IEGR which is a part of D5). D5 represnts the device sequences of 5` half (stem and brach part) which consists of cDNA sequences coding for Xa factor recognition site; D3 represents the device sequences of 3` half of first step YLBS; D3` represents the device sequences including Y-Tag of 3` half for second step YLBS; Y-Tag represents the cDNA sequences for puromycin-linker binding site. The black box of each device represents the complementary cDNA sequences.

This library was then used to design the full cDNA construct by using overlap extension

PCR. The purified cDNA construct containing paired peptides thus obtained by overlap

extension PCR was ready to use for *in vitro* transcription, puromycin linker ligation, and *in vitro* translation to generate cDNA-peptide fusion constructs as shown in Figure 2.2.

For *in vitro* transcription reaction, 1 pmol of cDNA construct was mixed with 3 µl of 25 mM each rA, U, C and GTP mixture, 2 µl of 5× T7 transcription and 1 µl of T7 enzyme mixture (Ribomax Large Scale RNA production system, Promega). The total mixture was gently mixed and incubated at 37^{o}C for 2 h. After that, 1 µl of RQ1 RNase free DNase (1 U/µl) was added and incubated at 37^{o}C for 15 min.Then, RNA purification was performed by using RNase minielute purification kit by following the manufacturer`s protocol (Promega).

The mRNA thus obtained was used for puromycin linker ligation. For this purpose, 50 pmol mRNA procuct was mixed with 60 pmol of streptavidin puromycin liker, 2.5 µl of 10× T4 RNA ligase buffer (Takara) and RNase free water up to total volume of 25 µl. All the reagents were mixed gently and incubated for ligation reaction (the ligation conditions were 2 min incubation at 90^{o}C followed by 2 min incubation at 72^{o}C, 30 s incubation at 25^{o}C and holding at 4^{o}C). Then 1 µl of T4 PNK enzyme (9 U/µl, Toyobo) and 1 µl of T4 RNA ligase (40 U/µl, Takara) were added to the ligation mixture and then incubated at 25^{o}C for 2 h.

The ligation product was then used for *in vitro* translation reaction. Retic Lysate IVT Kit from Ambion was used for *in vitro* translation. 10 pmol of puromycin linker ligation product was mixed with 1.25 µl of 20× Master Mix-Met deficience reagent, 1.25 µl of 20× Master Mix-Leu deficiene reagent, 34 µl of reticulocyte lysate and RNase free water was added for up to 50 µl. The total reaction mixture was mixed gently and incubated at 30^{o}C for 30 min. Then, 20 µl of 3 M KCl (RNase free) and 6 µl of 1 M $MgCl_2$ was added, mixed gently and incubated at 37^{o}C for 1 and half hour.

Finally, the *in vitro* translation product was checked by using SDS PAGE. The *in vitro* translation product (mRNA-peptide fusion construct) was then used for reverse transcription.

The reverse transcription (RT) of the mRNA-peptide fusions was performed on Dynabeads MyOne Streptavidin C1 by using RT kit ((ReverTraAce –alpha, Toyobo) following manufacturer`s protocol. The RT product was digested with 500 U RNase T1 (Ambion) in order to cleave the biotin-site on the SBP linker. Then, the cDNA-peptide fusions released from the beads were isolated using His-Tag purification kit (HiS Mag Sepharose Ni, GE healthcare). Finally, cDNA-peptide fusion constructs were generated by trimming unwanted peptide portion of this construct using factor Xa protease treatment (1 U/ µl, Invitrogen) followed by Xa removal using BioSpin columns (Qiagen). The cDNA-peptide fusion constructs thus obtained were used for in vitro selection process as shown in Figure 2.2.

2.3.2 *In vitro* selection of novel peptides

The cDNA-peptide fusion constructs obtained from the PP library (Figure 2.2) were mixed with Dynabeads MyOne Streptavidin C1 and incubated at room temperature for 30 min to remove any non-specific binding peptides. The supernatant was collected in a new tube containing biotin-LC-Aβ42 (AnaSpec Inc., U.S.A) and incubated at room temperature for 20 min and 10 min for first and second rounds of selection, respectively, while the incubation was done for 5 min at room temperature and at 37^{o}C for the third and fourth rounds of selection, respectively. The used biotin-LC-Aβ42 was monomerized and stored at -80^{o}C prior to use according to the method of Tsuji-Ueno *et al* (2011). Then, Dynabeads MyOne Streptavidin C1 were added and incubated for

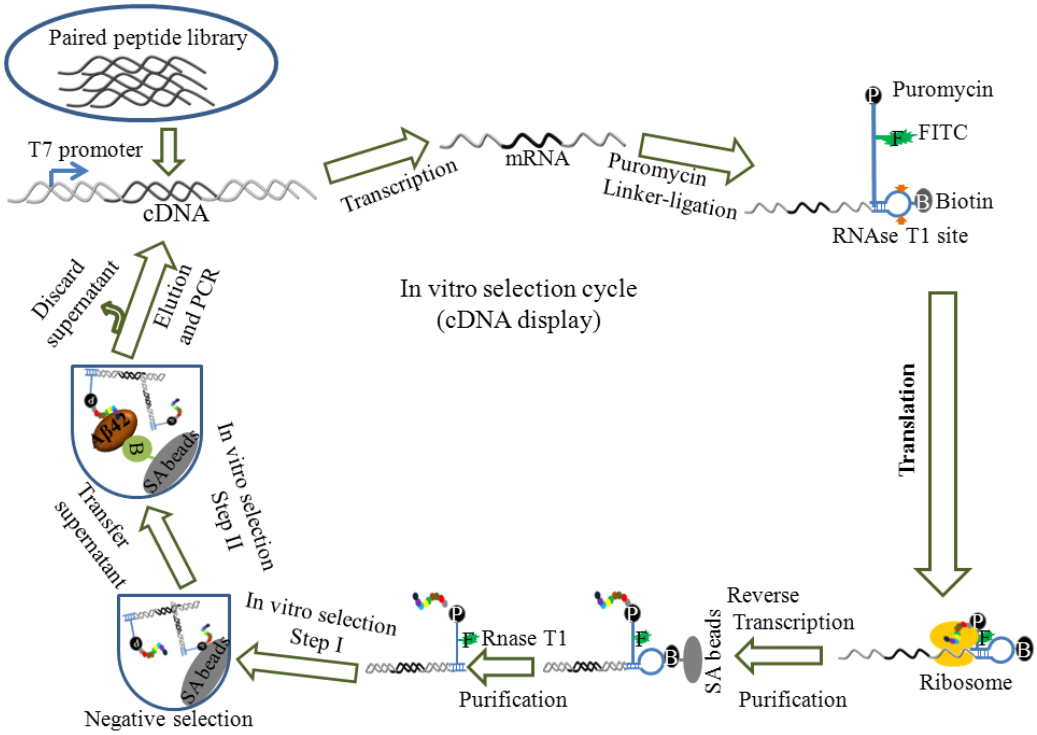

Figure 2.2. Scheme for *in vitro* peptide selection. A paired peptide library was used for this purpose.

30 min to collect the Aβ42-binding PPs.

The beads were washed with D-PBS (-) buffer containing 0.5% tween-20. The Aβ42-binding peptides were eluted with a 25% ammonia solution at 65°C for 15 min. The eluted solution was ethanol precipitated, and the pellet was resuspended in double distilled water. The cDNAs coding for the PPs were cloned and sequenced to select the most readily available novel peptides for chemical synthesis and further testing.

2.3.3 Preparation of Aβ42 aggregates

Aβ42 peptide was purchased from AnaSpec Inc., U.S.A. Monomerized Aβ42 peptide was prepared as described previously (Tsuji-Ueno *et al.*, 2011). Just before use, the

monomerized Aβ42 peptide that was stored at -80^{o}C was thawed and dissolved in 50 mM NaOH to obtain a 0.5 mM Aβ42 peptide solution that was further diluted with 1× D-PBS (-) to a final concentration of 200 μM to neutralize the NaOH. The peptide solution was sonicated for 3 min and then ultra-centrifuged at 15,000 rpm for 10 min. Finally, the supernatant of the peptide solution was collected to obtain a completely dissolved peptide solution. This solution was stored at -80^{o}C and it was considered a monomerized peptide solution. For the surface plasmon resonance (SPR) experiment, the Aβ42 samples were prepared by following the protocol described by Tsuji-Ueno *et al.* (2011). Aβ42 fibrillization was performed by incubating 100 μM monomerized Aβ42 peptide solutions in the presence or absence of different concentrations of PPs at 37^{o}C with shaking at 350 rpm for up to 48 h. The chemically synthesized PPs with purity more than 95% were obtained from SCRUM, Japan. Similarly, Aβ42 oligomerization was performed by incubating 50 μM Aβ42 peptide solutions in the presence or absence of different concentrations of PPs at 37^{o}C without shaking. After incubation, the samples were then diluted to the concentrations required for the different experiments.

2.3.4 Measurement of binding affinity

The binding affinity (the dissociation constant [K_d]) of each peptide for Aβ42 was determined at 25^{o}C by SPR using the Biacore 2000 (GE Healthcare). PPs were immobilized on different lanes of a CM5 Biacore sensor chip (GE Healthcare, UK) by a general amine coupling method using immobilization buffer (pH 8.5) containing 10 mM borate and 1 M NaCl. The reference lane was prepared without PPs. Then, different concentrations of monomerized Aβ42 (2.5 μM, 5 μM, 10 μM, 20 μM, and 40 μM) were

added to determine the binding affinity of the PPs for Aβ42. For all samples, PBS (-) buffer (pH 7.4) was used as the running buffer and a 50 µM NaOH solution was used to remove Aβ42. The sensograms obtained were fit to a 1:1 Langmuir binding model and the K_d values were determined using BIA evaluation software (GE Healthcare).

2.3.5 Thioflavin T (ThT) binding assay

At various time points, an aliquot of the 100 µM Aβ42 peptide solution prepared for fibrillization and a 50 µM Aβ42 peptide solution prepared for oligomerization were taken and diluted to 10 µM. 50 µL of the diluted peptide solutions was placed in each well of a 96-well plate (Black with clear, flat bottom; Costar) along with 50 µL of a 100 µM ThT (Sigma-Aldrich, India) solution (prepared in 50 mM glycine buffer, pH 8.5). The same concentration of ThT solution without Aβ42 samples was used for background fluorescence measurement. Fluorescence was measured at 490 nm using a Tecan Infinite M200 microplate reader. At first, the background ThT fluorescence was subtracted from the ThT fluorescence of samples. Then, the ThT data in the absence and presence of PPs were normalized to the ThT data of fibrillized Aβ42 sample in the absence of PPs and the values were expressed in percentage. So, at each time point, ThT data of Aβ42 sample in the absence of PPs was set to 100% in order to calculate the percentage of inhibition of ThT fluorescence (i.e., in order to calculate the percentage of inhibition of fibrillization) of Aβ42 sample in the presence of varied concentrations of PPs.

2.3.6 Atomic force microscopy

Aβ42 peptide fibrillization and oligomerization solutions were diluted to 25 µM. Then 20 µL of the diluted sample was deposited on freshly cleaved mica. The mica was

incubated at room temperature for 30 min and then rinsed with ultra-pure water and any remaining water was removed under inert gas. Then, the sample was dried in a desiccator overnight. Atomic force microscopy (AFM) images were obtained using a NanoWizard3 ultra (JPK Instruments AG). A Cantilever AC 160TS (Olympus) was used for AC-mode imaging.

2.3.7 TRICINE-SDS-PAGE

FAM-labeled Aβ42 peptide (AnaSpec Inc., U.S.A) was mixed with unlabeled Aβ42 peptide at a molar ratio of 1:1 at a final concentration of 50 μM Aβ42 peptide, and was then incubated under the oligomerization conditions as described above. An aliquot of the peptide solutions from these oligomerization reactions was diluted with 1× PBS (-) at different time intervals prior to tricine-SDS-PAGE. A 16% running gel and a 4% stacking gel were prepared following a previously established protocol (Schägger, 2006). A 0.1 μM Aβ42 peptide solution was applied in the wells of the stacking gel. A protein marker (Precision Plus protein standard; Biorad) was added to one well. Electrophoresis was carried out at 30 V for approximately 30 min until the sample completely entered the stacking gel. Then, the voltage was increased to 90 V and the electrophoresis was run at this voltage until it was finished. Gel images were obtained with a Scanner (Biorad) and the fluorescence intensity was quantified by using Quantity One software (Biorad).

2.3.8 8-Anilinonaphthalene-1-sulfonate (ANS) fluorescence analysis

A 50 μM solution of Aβ42 with or without 10 μM or 100 μM PPs in PBS (-) was incubated at 37^{o}C without agitation for up to 48 h. Then, 50 μM ANS (Sigma-Aldrich,

Switzerland) was added to assess the conformation of 10 μM Aβ42. The ANS fluorescence spectra (λex = 380 nm, bandpass 10 nm) was measured from 420 nm to 570 nm emission using a Tecan Infinite M200 microplate reader.

2.3.9 Cell –based assay

Rat pheochromocytoma (PC12) cells were purchased from the RIKEN BioResource Center, Japan and the cells were cultured in DMEM medium supplemented with 10% horse serum, 10% fetal bovine serum, and 1% penicillin-streptomycin. All cell culture reagents were purchased from GIBCO, U.S.A. unless otherwise stated. PC12 cells (2,500 cells/well in 25 μL of medium) were cultured in 384-well tissue culture-treated plates for 24 h at 37°C. Aβ42 peptide fibrillization and oligomerization samples incubated in the absence or presence of PPs were added to the cells at a final concentration of 10 μM and mixtures were cultured for up to 48 h at 37°C. The NaOH carryover from the Aβ peptide solution was 0.6 mM per well. Cells treated with 0.6 mM NaOH without the peptide samples were used as a control. Cell viability was measured using the cell counting kit-8 (CCK-8) cell proliferation and cytotoxicity assay (Dojindo, Japan) following the manufacturer's instructions. Briefly, CKK-8 solution was diluted 1:2 in PBS (-) buffer and 5 μL of the diluted solution was added to the cells. The cells were incubated at 37°C for 2 h. Then, the absorbance of water-soluble tetrazolium salt-8 (WST-8) was measured at 450 nm using the Tecan Infinite M200 microplate reader. The absorbance values were normalized to the control.

To detect caspase-3/7 activity induced by Aβ42 sample in the absence or presence of different concentrations of PPs, an Apo-ONE Homogeneous Caspase-3/7 Assay Kit (Promega, USA) was used according to the manufacturer`s protocol. Caspase activity

was detected by fluorescent emissions with excitation at 485 nm and emission at 530 nM. The PC12 cell culture and cell treatment with Aβ42 samples was same as that of cell viability assay. The Aβ42 concentration used for cell viability and caspase assay was the same. However, only 10 µM and 100 µM PPs were used in case of caspase assay.

2.4 Results

2.4.1 *In vitro* selection of novel peptides from the PP library

The PP library was constructed by random shuffling of eight peptide blocks selected from the previous libraries (Table 2.1) using YLBS (Kitamura *et al*., 2009 and 2012; Komatsu *et al*., 2012) as shown in Figure 2.1. The PPs were then used to construct the mRNA ready to be used for in vitro transcription followed by puromycin-linker ligation, *in vitro* translation and *in vitro* reverse transcription process to make the mRNA (cDNA) display product as shown in Figure 2.2. Then, in vitro selection using the mRNA display (Figure 2.2) provided PPs containing peptide blocks linked via a linker. The PPs had different length and block-diversity (Table 2.2). The combination of PPs was completely random (Table 2.2 and Figure 2.3), and in addition some PPs had indel and frame shift mutations due to the relaxed nature of the restriction enzyme (MboI) used, which expanded the diversity of the mutant library. Furthermore, most of the PPs were linked via a short linker (6 amino acids in length). Although the theoretical size of the PP library was 128 variants, the actual diversity of the library was found to be much bigger. One of the reason is that the designed molecular diversity of the paired peptides is very small (in this case, 128 variants) due to the limited number of blocks, which is

far smaller than the number of molecules used for the selection (usually about 1012) (Kitamura *et al*., 2012)

Table 2.2. Paired peptides (PPs) obtained from *in vitro* selection of the thirdly generated library

Clone number	**Name**	**Amino acid sequence**	**No. of amino acids**	**Occurrence**
#1	P1	PNPPDPIHWSGGGSGGGSTHQIPSI	25	1
#59	P59	WCKPSCGGSGGSDCSSDLSR	20	1
#68	**P68**	**DCSSDLTPSGGSGGSDCSSDLTPS**	**24**	**2**
#73	**P73**	**CGILDPIPWGGSGGSPNPPDQIPT**	**24**	**2**
#82	P82	PNPPDPIHWEGAEAVHCSSDLTPS	24	1
#84	**P84**	**CGILDPIPWGGSGGSCGILDPIPW**	**24**	**3**
#90	P90	PNPPIPSIGAEAPVEGAEAAKNPPDPIHW	29	1
#91	P91	QTHQIPSIGEAPEAATANPLAQL	23	1
#92	P92	GCPCIGIIGGSGGSRCSSDLTPS	23	2
#96	P96	PNPPDPIHWSGGGSGGGSQNPSDQIPT	27	1
#104	P104	PKPTRSHPLGGSGGGSALRSHSF	23	1
#106	P106	PNPPDPIHWERRRDCSSDLTPS	22	1
#107	P107	PNPPDPIHWGGGRCKPSCCC	20	1
#113	P113	PNPPDPIHWERRRFHQIPSIGG	22	1
#116	P116	PNPTRSHPLGGSGGGSHPLED	21	1
#127	P127	PNPPDQIPTGGGSGGGSGGGSDCSSDLTPS	30	1
#128	P128	PNPPDPIHWGGSGGSPNPPDQIPT	24	1
#129	P129	PNPPDPIHWGGSGGGSCCKPSCCCGGSGGSDCSSDLTP	38	1
#131	**P131**	**GCPCIGIIGGSGGSDCSSDLTPS**	**23**	**2**
#135	P135	DGKSIGPIGCCKPSCCC	17	2
#139	P139	PKPTRSHPLVRWRERRRFDCSSDLTPS	27	1
#141	P141	PNPPDPIHWERRRFDRSSDLTPS	23	1
#143	P143	PNPPDPIHWRKRRPNPPDPIHW	22	1
#146	P146	PNPPDPIHWERRRDGSSDLTPS	22	1
#147	P147	PNPPDPIHWERRRFHQIPSI	20	1
#152	P152	DGKSIGPIGGSGGSPNPPDQIPT	23	2

The PPs with full block sequences are indicated by bold letters .

Furthermore, these four PPs were selected for chemical synthesis.

It was also observed the deletion of long linker (12 amino acids in length), resulting 6 to 8 amino acids in length. Based on the full block sequence information of parent blocks used for YLBS and based on the frequency of occurrence, four PPs were selected for further characterization (Table 2.3).

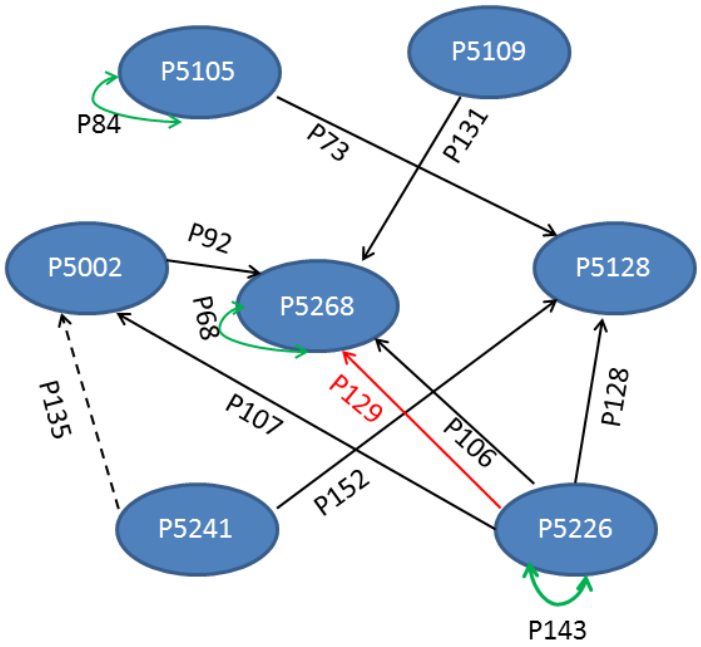

Figure 2.3. **Random combination of peptide blocks selected from the first and second library resulted PP library**. Single straight arrow head shows that two peptides (N -> C) are connected via a linker. Double curve arrow head shows the self-paired peptides via a linker. Note that dotted arrow head means the linker is partially deleted; black arrow head for short linker and red arrow head for long linker.

Table 2.3. Paired peptides selected and their properties

Name	Systematic code name	Amino acid sequence	Source peptide blocks representation	No. of amino acids	Kd (M)
P68	(pp(Aβ42)7068)	DCSSDLTPS*GGSGGS* DCSSDLTPS	P5268-L1-P5268	24	No binding
P73	(pp(Aβ42)7073)	CGILDPIPW*GGSGGS* PNPPDQIPT	P5105-L1-P5128	24	3.7×10^{-6}
P84	(pp(Aβ42)7084)	CGILDPIPWGGSGGSCGILDPIPW	P5105-L1-P5105	24	2.0×10^{-8}
P131	(pp(Aβ42)7131)	GCPCIGII*GGSGGS* DCSSDLTPS	P5109-L1-P5268	23	1.2×10^{-8}

a The GRAVY value is calculated by dividing the sum of the hydropathy values for each residue by the length of the sequence (Kyle and Doolittle; 1982).

2.4.2 Improved binding affinity of the novel peptides to Aβ42

The four chemically synthesized peptides (Tables 2.2 and 2.3) were used for SPR experiments to determine their binding affinity for Aβ42. As shown in Figure 2.4 below, P68 did not bind to the target Aβ42 peptide, whereas the other three peptides did. Of these three peptides, P84 and P131 had stronger binding affinities than the peptides selected from the previous library (Tsuji-Ueno *et al*., 2011). The binding affinities were measured as dissociation constants (K_d) as shown in Figure 2.4, and the K_d of each peptide is shown in Table 2.3. The binding affinities of P84 (20 nM) and P131 (12 nM) were approximately 26 fold and 44 fold stronger than that of the peptide with the strongest binding affinity (P5105, K_d value 536 nM) selected from the previous library (Tsuji-Ueno *et al*., 2011).

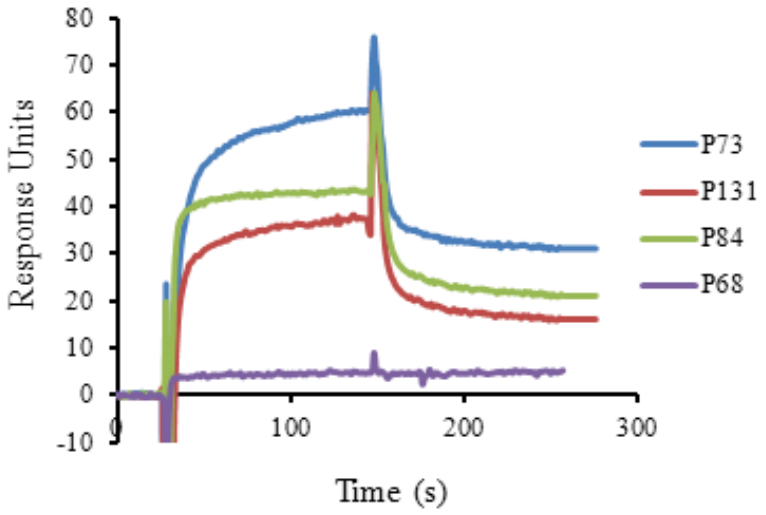

Figure 2.4. Determination of the binding affinity of the PPs to Aβ42 by surface plasmon resonance (SPR). Representative binding curves were obtained by running 40 μM Aβ42 over 0.5 mM PPs immobilized on the CM5 sensor chip.

2.4.3 P84 and P131 inhibit the fibrillization of Aβ42

Since P84 and P131 showed stronger binding affinities for Aβ42, their effect on the aggregation of Aβ42 was examined by using a ThT assay and AFM analysis. Figure 2.5 shows the results from the ThT fluorescence assay of the Aβ42 samples incubated with or without these two PPs. Since the amount of ThT fluorescence decrease is proportional to the degree of Aβ42 fibril form covered or destroyed by PPs (only the fibril-bound form of ThT is fluorescent), the fluorescence decrease in the presence of PPs clearly indicates the inhibitory effect of PPs on Aβ42 fibrillization. The fibrillization of Aβ42 was reduced by about 10% when incubated for short time (up to 8 h) in the presence of 10 μM and 100 μM PPs whereas the fibrillization of Aβ42 was reduced by about 60% after 48 h of incubation in the presence of 10 μM and 100 μM P84 or P131. Both P84 and P131 at 0.1 μM and 1 μM concentrations of substoichiometric peptide/ Aβ42 ratios showed no significant inhibition of Aβ42 aggregation (data not shown). The reduction of fibrillization was dependent on the incubation time and the peptide concentration (Figure 2.5A and 2.5B). Contradictorily, the PPs at 1 mM concentrations did not show improved inhibition effect on Aβ42 fibrillization (Figure 2.5D) compared to the inhibition effect observed at 10 μM or at 100 μM concentrations of both PPs. From these ThT assay results, it was found that P84, particularly at its lower concentration (10 μM), was a stronger inhibitor of Aβ42 fibrillization than P131 which has a higher binding affinity than P84 (Table 2.3).

To further confirm the inhibitory effect of P84 and P131 on the aggregation of Aβ42, an AFM analysis was performed.

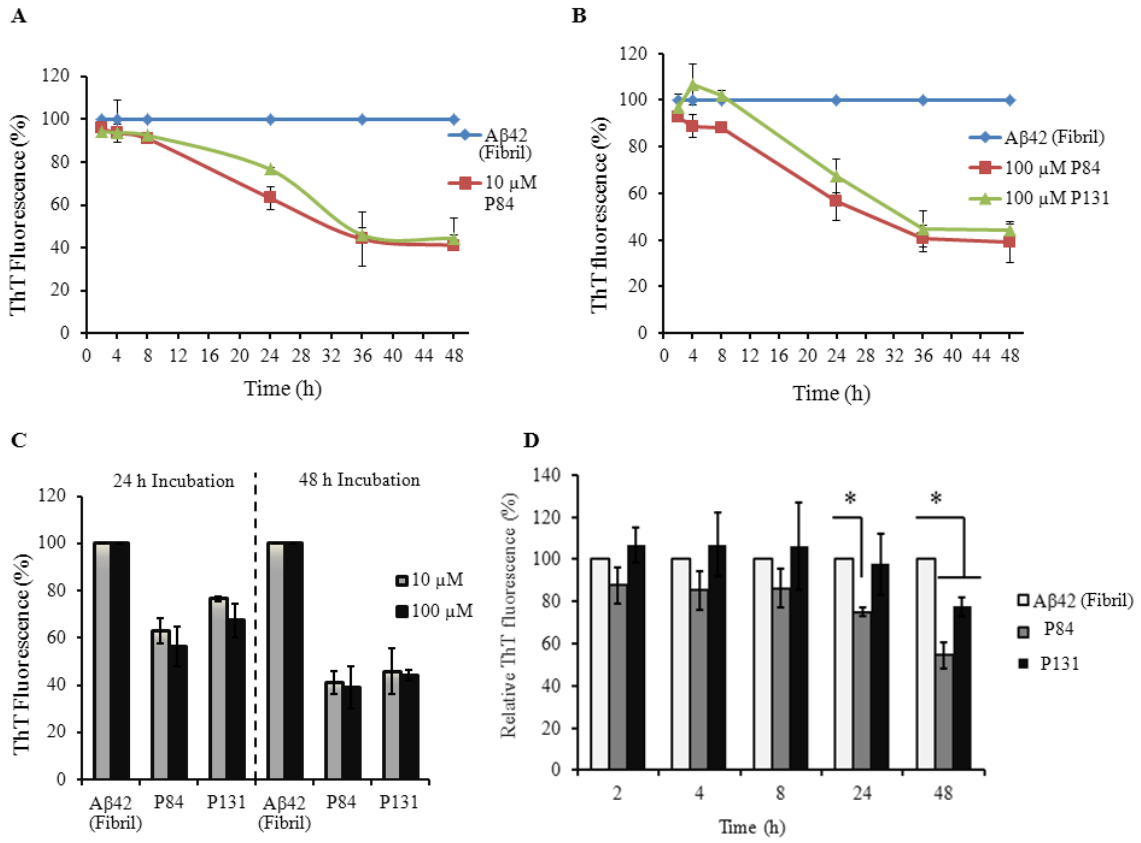

Figure 2.5. P84 and P131 inhibit the fibrillization of Aβ42. (A) A thioflavin T (ThT) binding assay showing the time-dependent inhibition of Aβ42 (100 μM) fibrillization when incubated with 10 μM P84 or P131 at 37°C for up to 48 h. (B) Time-dependent inhibition of Aβ42 (100 μM) fibrillization when incubated with 100 μM P84 or P131 at 37°C for up to 48 h. (C) Inhibition of Aβ42 (100 μM) fibrillization incubated with two different concentrations of PPs at 37°C for up to 48 h. (D) Time-dependent inhibition of Aβ42 (100 μM) fibrillization when incubated with 1000 μM (1 mM) P84 or P131 at 37°C for up to 48 h. The data were obtained from three independent assays, and each assay was performed in triplicate in 96-well microplates. Error bars represent the standard deviation. The symbol * indicates P value <0.05 with respect to the value of Aβ42 fibrils formed in the absence of PPs.

Since the ThT fluorescence of the Aβ42 samples incubated in the presence of 10 μM and 100 μM of PPs for 24 h to 48 h was not significantly different (Figure 2.5C), Aβ42 samples incubated with 10 μM PPs were selected to use in the AFM analysis.

Figure 2.6 shows the AFM images of the Aβ42 samples incubated in the absence or presence of 10 µM P84 and P131. The fibrillization of Aβ42 was significantly reduced when the 100 µM Aβ42 sample was incubated with 10 µM P84 and P131 for 24 h (Figure 2.6A). The Aβ42 filaments incubated with P131 were morphologically distinct (a bit thicker) from the Aβ42 filaments incubated alone and from those incubated in the presence of 10 µM P84. Interestingly, the numbers of Aβ42 fibrils formed in the presence of PPs after 24 h (Figure 3A) were roughly half compared to the control. Furthermore, the AFM images showed that longer incubation (for 48 h) with both PPs effectively inhibited the aggregation of Aβ42 (Figure 2.6B). Consistently with the results of the ThT assay, the AFM analysis showed that P84 more effectively inhibited the fibrillization of Aβ42 than P131.

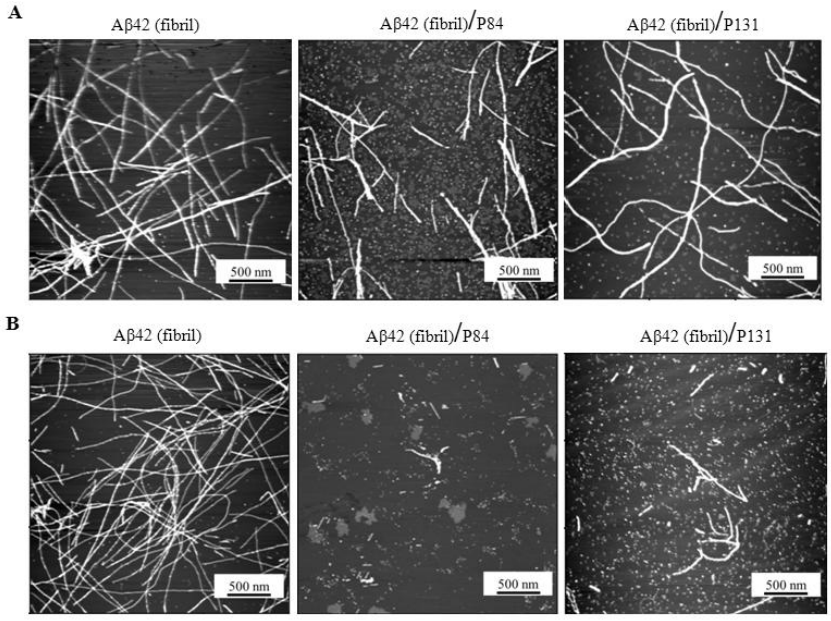

Figure 2.6. AFM images of Aβ42 incubated under modified conditions for fibrillization. Panels, (A) and (B) show the AFM analysis of Aβ42 samples incubated for 24 h and 48 h, respectively, in the absence or presence of 10 µM P84 or P131. The images are of 3 µm × 3 µm sections.

2.4.4 Effect of P84 and P131 on the oligomerization of Aβ42

Since both P84 and P131 could inhibit the fibrillization of Aβ42, next step was to know the effect of these two PPs on the oligomerization of Aβ42. Since, Aβ42 oligomers are of different sizes, the size of the Aβ42 oligomers was mapped by tricine-SDS-PAGE using a known protein marker as shown in Figure 2.7A.

The samples were prepared with an equal molar ratio of Aβ42 and FAM-labeled Aβ42 to simplify visualization of the Aβ42 oligomers in the gel after electrophoresis and to quantify the higher molecular weight Aβ42. Aβ42 oligomers with molecular weights greater than 100 kDa were observed as shown in zone A of Figure 2.7A. The presence of dimers or trimers in the monomeric Aβ42 sample may be due to either its storage in a highly concentrated form or its exposure to room temperature while preparing the samples for SDS-PAGE. As shown in Figure 2.7A and 8B, P131 neither inhibited nor promoted the oligomerization of Aβ42. In contrast, P84 clearly inhibited formation of the higher molecular weight oligomers (in zone A) of Aβ42. The percentage of the higher molecular weight oligomers in each sample lane in Figure 2.7B was measured by selecting the same region of interest in all the samples in Figure 2.7A using Quality One software (BioRad).

The AFM analysis showed that both the morphology and the size of the Aβ42 oligomers in the Aβ42 oligomerization assay samples prepared in the presence or absence of both PPs were different (Figure 2.7C). It was also observed that P84 more strongly inhibited the oligomerization of Aβ42 than P131, which supports the results of the tricine-SDS-PAGE analysis. Importantly, the increased particle numbers and decreased particle length and width were observed in the presence of P84 as shown in

the middle image of Figure 2.7C. Since the morphology of the Aβ42 oligomers formed in the presence or absence of PPs observed in the AFM images was different, ThT assay was performed to determine whether these different oligomers show different ThT fluorescence intensities as was observed for the Aβ42 fibrils. Interestingly, the ThT fluorescence intensity was the same regardless of whether the Aβ42 oligomers were formed in the absence or presence of P84 and P131 (Figure 2.7D) indicating that these oligomers formed under different conditions lacking cross-β structures which provide the binding sites to ThT reagent (Biancalana *et al*., 2010). Next, an ANS fluorescence assay was performed to test whether the oligomers formed in the presence of PPs have hydrophobicities that are similar to that of oligomers formed in the absence of PPs. The ANS fluorescence spectrum showed that the hydrophobicity of the Aβ42 oligomers formed in the presence of PPs was comparable to that of the oligomers formed in the absence of PPs (Figure 2.7E), which is consistent to the above ThT assay. All the oligomers had emission maxima around 508 ± 4 nm. Conveniently, the emission maximum of the Aβ42 monomers (the samples before oligomerization incubation) was more blue-shifted (about 40 nm) than that of the oligomers, providing a way for discriminating monomers and oligomers of Aβ42 peptide.

2.4.5 Effect of P84 and P131 on the cytotoxicity of Aβ42

The above mentioned results showed that P84 and P131 inhibited the aggregation of Aβ42. Next, the effect of these two PPs on the cytotoxicity of Aβ42 was explored. First, the effect of the P84 and P131 on the cytotoxic effect of fibrillized Aβ42 was assessed.

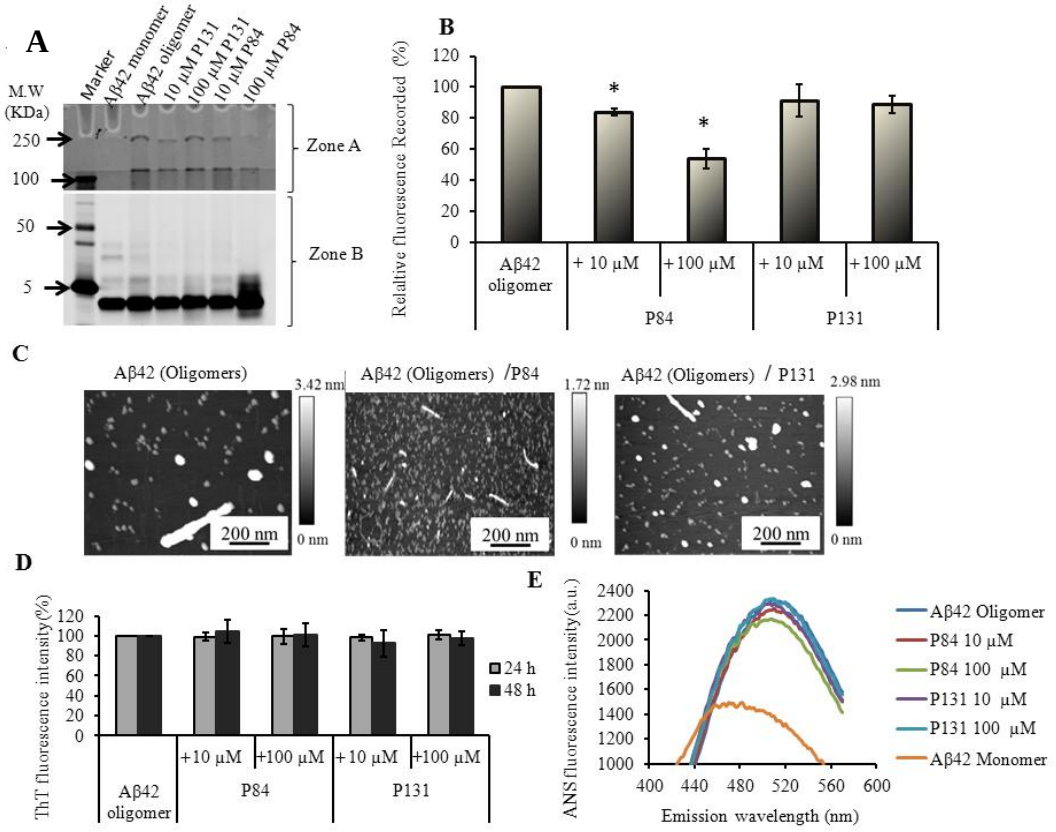

Figure 2.7. Effect of the PPs on the oligomerization of Aβ42. Aβ42 (50 µM) was incubated in the absence or presence of the PPs (10 µM or 100 µM) for 24 h or 48 h without shaking at 37°C. (A) Typical tricine-SDS PAGE image showing monomeric and oligomeric forms of Aβ42 incubated for 48 h in the absence or presence of the PPs. Note the reduction in the higher molecular weight Aβ42 oligomers in the presence of 100 µM P84. Each lane contains 10 pmol of Aβ42. Protein marker (horizontal lines in first lane) was used to quantify the size of Aβ42 oligomers as shown by arrow head. Monomeric Aβ42 was used as a control for the experiment. (B) The fluorescence intensity of Aβ42 oligomers distributed in the molecular range above 10 kDa within zone A as shown in Panel A. The fluorescence intensity was measured, and the values were normalized to the Aβ42 fibril (Panel A). The data were obtained from three independent images taken from three independent experiments. The symbol * indicates P value <0.05 with respect to the value of Aβ42 oligomers formed in the absence of PPs. (C) shows the AFM images of 50 µM Aβ42 incubated under oligomerization conditions for 48 h in the absence (left image) or presence of 10 µM P84 (middle image) or 10 µM P131 (rightmost image). Note the increased particle numbers and decreased particle length and width in the middle image. (D) ThT assay of an aliquot (10 µM) of the Aβ42 oligomerization samples, and (E) ANS fluorescence assay of aliquots (10 µM) of Aβ42 samples that were prepared by incubating 50 µM Aβ42 in the absence or presence of 10 µM or 100 µM PPs without agitation at 37°C for 48 h.

It was already explained that 10 µM or 100 µM PPs showed the time dependent inhibition of Aβ42 fibrillization (Figure 2.5A and 2.5B) however both PPs above 500 µM concentration showed less Aβ42 fibrillization inhibition effect compared to that of PPs at 100 µM concentration (Figure 2.5D). So, the effect of P84 and P131 at their lower (10 µM) and higher (above 200 µM) concentrations on the Aβ42 cytotoxicity was tested. For this purpose, PC12 cells were treated with 10 µM Aβ42 sample that was fibrillized in the presence of various concentrations of P84 and P131.

As shown in Figure 2.8A, both P84 and P131 inhibited the toxic effect of Aβ42 on PC12 cells. Rescue of the PC12 cells from the toxic effect of Aβ42 was dependent on the concentration of PPs and the time that the PC12 cells were exposed to the Aβ42 treated with the PPs. These results showed that 10 µM P84 or P131 significantly inhibited the cytotoxic effect of Aβ42 (20-30% cell viability increased). Furthermore, it was also observed that at paired peptide concentrations above 200 µM, inhibition of the Aβ42 cytotoxic effect was not improved compared to the effect of PPs observed at their 100 µM concentration (Figure 2.8A). On the other hand, the effect of 10 µM and 100 µM P84 and P131 on the toxicity of oligomerized Aβ42 was tested. As shown in Figure 2.8B, both at 10 µM and 100 µM, the PPs significantly inhibited the toxicity of the Aβ42 oligomers on PC12 cells (30-45% viability increased).

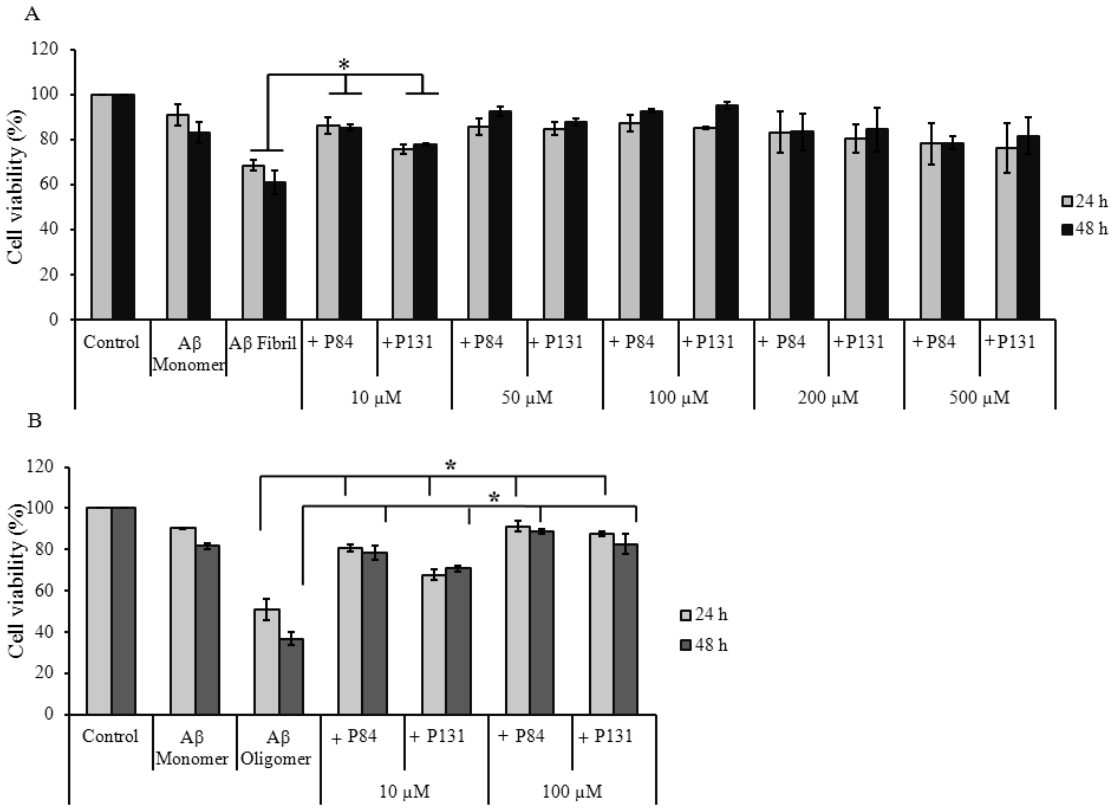

Figure 2.8. PPs inhibit the cytotoxicity of Aβ42. A CCK-8 assay was performed every 24 h for 2 days after treating PC12 cells with the 10 μM Aβ42 samples. Panel (A) shows the inhibition of the cytotoxic effect of fibrillized Aβ42 when incubated with different concentrations of P84 and P131 at 37°C with agitation for 24 to 48 h. Panel (B) shows the inhibition of cytotoxic effect of oligomeric Aβ42 when incubated with 10 μM and 100 μM P84 or P131 at 37°C without agitation for 24 to 48 h. The absorbance of WST-8 (see Materials and Methods) was measured at 450 nm to calculate cell viability. Data were obtained from three independent experiments. * indicates P value <0.05 with respect to the value of Aβ42 fibrils and Aβ42 oligomers formed in the absence of PPs.

Under both fibrillized and oligomerized conditions, inhibition of the toxicity by both P84 and P131 was more significant (around 20%) after the PC12 cells were exposed to the samples pretreated for 48 h than for 24 h. Although the data were not sufficient to calculate the accurate EC50 values for reducing the cell death by Aβ42, the preliminary ones for P84 and P131 were found to be 10.7 μM and 15.4 μM, respectively (Figure 2.9).

The above results showed that P84 (particularly at 10 µM concentration) more effectively protected the PC12 cells from the cytotoxicity of Aβ42 than by the P131, which is consistent to the Aβ42 aggregation inhibitory effect of P84 and P131.

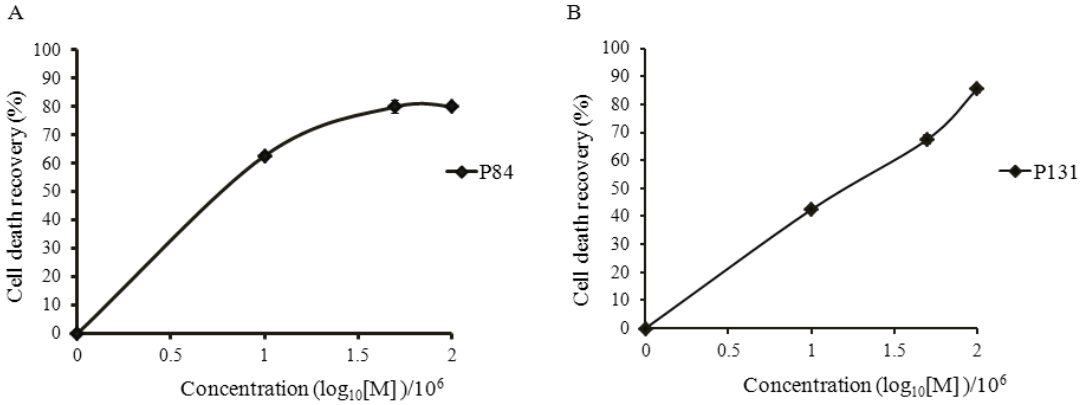

Figure 2.9. Tentative EC50 values of P84 and P131. The data used in this figure were extracted from the Figure 2.8A. When the cell viability of PC12 cells in the presence of 10 µM fibrillized Aβ42 alone was about 60% (See Figure 2.8A), that means 40% of cells were damaged when PC12 cells were exposed to 10 µM fibrillized Aβ42 in the absence of PPs for 48 h. So, in this case, percentage of cell damage recovery by PPs was 0. Then, the percentage of cell damage recovery by PPs at 10 µM, 50 µM, 100 µM (from Figure 2.8A) with respect to the percentage of cell damage caused by 10 µM fibrillized Aβ42 were calculated accordingly and data were plotted as cell death recovery (%) versus concentration of PPs in log scale. Panel (A) shows the cell damage recovery (%) when PC12 cells were exposed to 10 µM fibrillized Aβ42 in the presence of different concentrations of P84 for 48 h. Panel (B) shows the cell damage recovery (%) when PC12 cells were exposed to 10 µM fibrillized Aβ42 in the presence of different concentrations of P131 for 48 h. The tentative EC50 values of P84 (10.7 µM) and P131 (15.8 µM) were calculated by fitting the curves of Panels (A) and (B), respectively.

Several research evidences have proven that cytotoxic effect of Aβ42 is mediated by activation of caspase-3/7 (Eimer *et al.*, 2013). Therefore, in this study, a caspase assay was performed in order to check any effect of PPs on the activation of caspase-3/7

mediated by Aβ42. As shown in Figure 2.11 below, it was found that Aβ42 aggregates (under both oligomeric and fibrillar form) activated the caspase-3/7 compared to that of control sample and Aβ42 monomers.

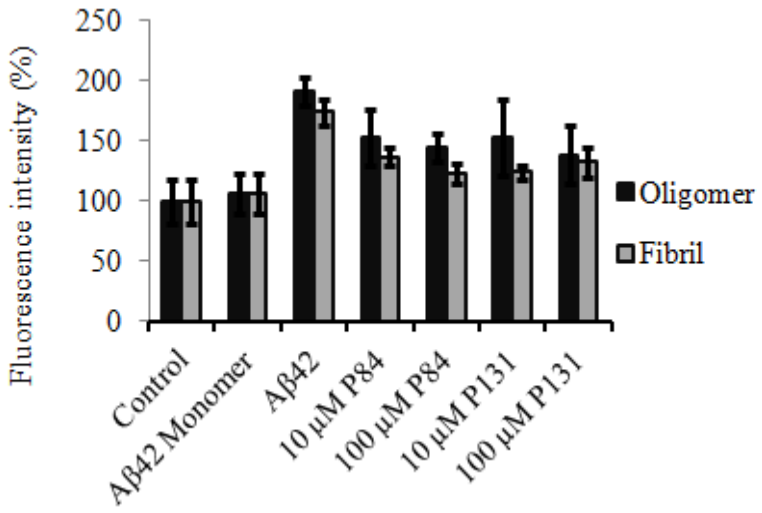

Figure 2.10. PPs inhibited the induction of caspase-3/7 activity by Aβ42. Caspase-3/7 assay was performed 24 h after treating PC12 cells with the 10 μM Aβ42 samples. The figure shows the inhibition of caspase-3/7 activity by Aβ42 when incubated with 10 μM and 100 μM P84 or P131 at 37°C without agitation for 48 h. The fluorescence (see Materials and Methods) was measured at 530 nm.

Then, activation of caspase-3/7 by Aβ42 fibril in the PPs is significantly less than that in the absence of PPs. However, no clear effect of PPs on Aβ42 oligomer was observed. This is the very first set of experimental data and further repetition of this experiment is needed in order to make the clear interpretation of the data. Although this is very preliminary data, it shows that the inhibition of cytotoxic effect of Aβ42 fibril in the presence of PPs may be mediated by inhibiting the caspase-3/7.

2.5 Discussion

The previous study obtained the Aβ42 binding peptides using the ASAC library method (Tsuji-Ueno *et al*., 2011). However the binding affinity of the peptides remained to be modest (Kd value in μM range). For therapeutic use, the peptide should have a nM affinity for the target protein/peptide (Chang *et al*., 2013). In this study the PP method (Kitamura et al., 2012; Komatsu et al., 2012) was adopted to evolve the Aβ42 binding peptides with a higher affinity. The selected novel peptides were constituted with either two identical or two different peptide blocks connected via mainly a short linker. This is because a long linker may interfere with the functional peptide region. Evidently not all paired peptides were effective in elevating Aβ42 binding but a limited number of them did (Kitamura et al., 2012; Komatsu et al., 2012). Sequence analysis of P68 and P131 suggested that some amino acids in the peptide block P5109 located at the N-terminus of P131 are contributable for binding to Aβ42. Similarly, sequence analysis of P73 and P5105 suggested that some amino acids in the peptide block P5105 located at the N-terminus of P84 are contributable for binding to Aβ42. To the best of our knowledge, P84 and P131 are the only peptides that have very strong binding affinities (about 10 nM of Kd) for Aβ42 among the many rationally designed and randomly designed Aβ42 binding peptides (Takahashi et al., 2010; Funke et al., 2012). Furthermore, P84 is the homodimer of P5105 which showed the strongest binding affinity among the peptide blocks reported in previous study (Tsuji-Ueno et al., 2011) apparently suggesting that the simple trick of creating homo-multivalency can win the selection. The mechanistic explanation at the molecular level for the improvement of binding affinity and inhibitory activity of those paired peptides is of great interest and open to succeeding studies.

The progressive improvement of binding affinity of peptides to Aβ42 developed from three successive libraries of PLM (Figure 2.11) and the sub-nM affinity peptide activators and inhibitors against cathepsin E (Kitamura *et al*., 2012 and Komatsu *et al*., 2012) strongly support that PLM is a very effective *in vitro* molecular evolution method to develop novel peptides with improved binding affinity against target protein/peptides.

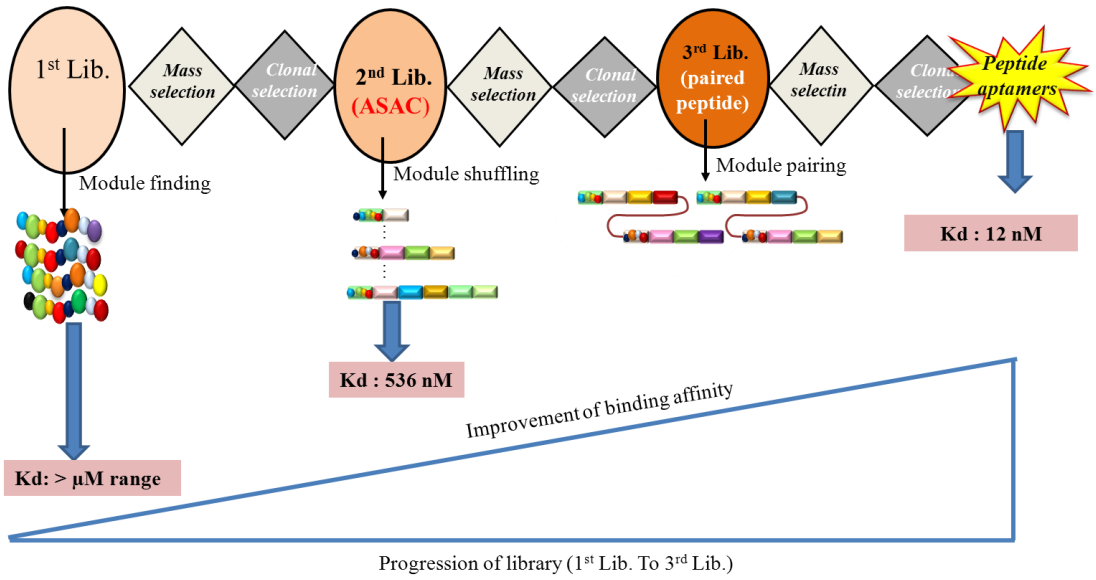

Figure 2.11. **Schematic representation to show the effectiveness of PLM to develop novel peptides with stronger binding affinity to the Aβ42.** Note the very strong (44 fold stronger) Kd value of the peptide selected from the 3rd library compared to the Kd value of the strongest peptide selected from the second library. The Kd value of peptides selected from first and second library were taken from the previous study (Tsuji-Ueno et al., 2011).

The improved binding affinity of the PP over the single peptide (as an example, comparison of Kd value of P84 and P5105, where P84 is a dimeric form of P5105) may be due to the synergetic binding of two peptide blocks of P84 to the two binding sites of target Aβ42, which supports the model of improved binding affinity of PPs proposed by Kitamura et al. (2012).

Protein misfolding and aggregation are very complex phenomena. Both fibrils and soluble oligomers of Aβ42 are considered toxic species that cause cognitive impairment leading to AD (Haroutunian *et al*., 2000; Shankar *et al*., 2008 and Haass *et al*., 2008). The molecular size of an Aβ42 oligomer is strongly correlated with its toxic properties. In addition, the composition, conformation, and surface of the Aβ42 oligomers are also considered to be important parameters of toxicity (Ahmed *et al*., 2010 and Ladiwala *et al*., 2012). The Aβ aggregation pathway must also be important for its toxicity. Several small aromatic molecules, Aβ42-binding peptoids, and chaperones have been shown to inhibit the aggregation and cytotoxicity of Aβ42 through different mechanisms, such as disruption of the cross-β structures and stabilization of nontoxic oligomeric forms (Luo et al., 2012, Sörgjerd et al., 2013 and Ladiwala et al., 2011).

Since the main aim of this study is to develop nM affinity peptide inhibitors of Aβ42 aggregation/cytotoxicity so that the peptides may be potential therapeutic seeds for the treatment of Alzheimer's disease, the effect of P84 and P131 on the aggregation and toxicity of Aβ42 under fibrillization and oligomerization conditions was explored. Both of the PPs at 10 μM were found to be able to clearly inhibit the fibril formation of Aβ42 as shown by the data presented in Figure 2.5. Interestingly, both P84 and P131 at 10 μM concentration at least up to 24 h showed the time dependent inhibition of Aβ42 fibrillization (Figure 2.5A). Furthermore, the evidence of Aβ42 fibrillization inhibition by P84 and P131 was clearly supported by the AFM images (Figure 2.6). On the other hand, only P84 could inhibit the generation of high molecular weight oligomers of Aβ42 (Figure 2.7A, 2.7B and 2.7C), whereas P131 did not have any significant inhibitory effect on Aβ42 oligomer formation. Moreover, our results (Figure 2.7C and

2.7D) strongly supported the fact that P131 did not promote, at least, the formation of higher molecular weight Aβ42 oligomers and protofibrils. Overall, P84 more effectively inhibited the aggregation of Aβ42 than P131, even though P84 had a weaker binding affinity for Aβ42 than P131. This observation confirms that peptides with higher binding affinities do not always have better functional properties. Further study is needed to compare the relationship between binding affinity and the functional properties of fibrillization inhibitors.

Although the exact mechanism underlying paired peptide-mediated inhibition of Aβ42 fibrillization is yet not known, we can offer a speculative scheme for this phenomenon as shown in Figure 2.12. This scheme considers the facts that the inhibition of Aβ42 fibrillization begins slowly at the initial stage and then proportionally proceeds depending on the time elapsed and finally reaches equilibrium (Figure 2.5A). Furthermore, the inhibition rate rather poorly depends on the concentrations of PPs at the higher concentration than 100 μM (in other words, saturation or in an excess amount against that of Aβ42, usually 50 μM) (Figures 2.5C and 2.8A). The essence of this scheme is in the slow step of the conversion from the fibril bound by PP to stably PP-bound Aβ42 state (though this is only a working hypothesis). The poor PP concentration-dependence can be rationalized by the saturation of the PP-binding sites on the fibril and the fixation of the monomer-state which can be proportional to the concentration of PP. The latter contributes to the fibril formation very weakly due to the relatively slow kinetics of the PP-binding to the monomer (which is also a hypothesis). At this moment, too many possibilities (diversities of members appearing in this phenomenon and each kinetic governing their conversions) exist in explaining the whole process observed in this experiment and thus

the future studies on these ambiguities are eagerly awaited. However, we think such a hypothesis as given here is still necessary for targeting the future works.

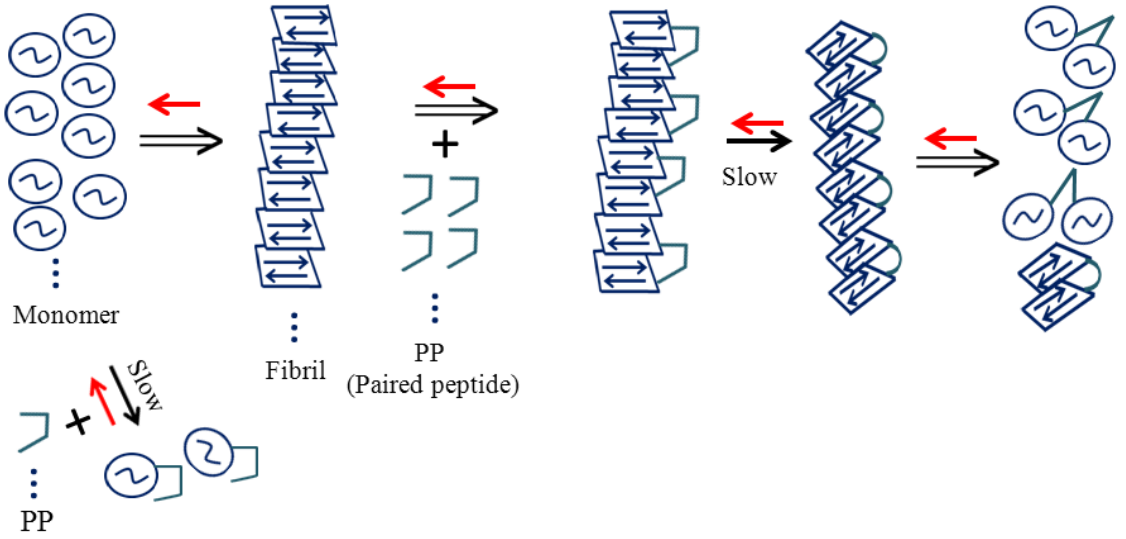

Figure 2.12. A hypothetical model for the aggregation inhibition by the PPs. This model considers the facts that the inhibition of Aβ42 fibrillization begins slowly at the initial stage and then proportionally proceeds depending on the time elapsed and finally reaches equilibrium. The essence of this scheme is in the slow step of the conversion from the fibril bound by PP to stably PP-bound Aβ42 state. The poor PP concentration-dependence can be rationalized by the saturation of the PP-binding sites on the fibril and the fixation of the monomer-state which can be proportional to the concentration of PP. The latter contributes to the fibril formation very weakly due to the slow kinetics of the PP-binding to the monomer. The kinetics used are only relative and the illustrative shapes of PPs represent their conformational differences.

In addition, the AFM images showed that the Aβ42 filaments were altered after incubation with P84 and P131, indicating that these two PPs may disrupt the fibrillization process through different pathways. It was observed that the numbers of Aβ42 fibrils in the presence of PPs after 24 h were roughly halved compared to the control. The reason may be the fact that inhibition of Aβ42 fibrillization begins slowly at the initial stage and then proportionally proceeds and reaches to 50% reduction (in

this case at 24 h) in the line of supporting the ThT assay (Figure 2.5). To determine these mechanisms, the exact stoichiometry of each peptide binding to Aβ42 should be determined.

It was found that P84 and P131 inhibited the cytotoxic effects of Aβ42 fibrils and oligomers in a concentration-dependent manner which is taken into consideration in Figure 2.5 by giving a shunt of the monomer-state to the PP-bound monomer. Interestingly, at paired peptide concentrations above 200 μM, it was observed that the Aβ42 cytotoxicity was not further inhibited by both PPs (Figure 2.8A) supporting the hypothesis that the effect of PPs to Aβ42 reaches to saturation at the higher concentration. Similarly, Aβ42 oligomers (not shown in Figure 2.12 for simplicity) formed in the presence of PPs were less toxic to PC12 cells than Aβ42 oligomers formed in the absence of the PPs. The preliminary data (Figure 2.10) shows that both PPs may inhibit the caspase-3/7, resulting the reduced cytotoxic effect of Aβ42 in PC12 cells. However, more experiments are needed to confirm this observation.

In this study, we found no difference in the ThT fluorescence of Aβ42 oligomers formed in the absence or presence of PPs after incubation under oligomerization conditions for up to 48 h (Figure 2.7D). It has been shown that fibrils and oligomers with increased ThT fluorescence possess cross-β structures (that is ThT bindable structure) (Biancalana et al., 2010), whereas oligomers that do not show a change in ThT fluorescence lack these cross-β structures. An elegant study by Stroud et al. (2012) proved that the nontoxic oligomers lack cross-β structures. Furthermore, it has been reported that the toxic oligomers are more hydrophobic than the non-toxic oligomers. However, we also found that the Aβ42 oligomers formed in the absence or presence of PPs possess similar hydrophobicities, and that these oligomers were even less

hydrophobic than the nontoxic or less-toxic Aβ42 monomers as revealed by ANS fluorescence assay (Figure 2.7E). These results support the hypothesis that besides cross-β structure and hydrophobicity, there are many other factors governing the cytotoxicity of Aβ42 oligomers. One of the possible reasons of the less cytotoxic effect of Aβ42 oligomers formed in the presence of PPs may be formed by off pathway (Ladiwala *et al*., 2012) as compared to the Aβ42 oligomers formed in the absence of PPs. Similarly, the PP-bound Aβ42 oligomers may have different conformation and surface structures compared to that of Aβ42 oligomers in the absence of PPs.

Under both fibrillization and oligomerization conditions, P84 exhibited more inhibitory effect to Aβ42 cytotoxicity compared to that of P131 (Figure 2.8). Moreover, it was already explained above that P84 showed stronger Aβ42 aggregation inhibition compared to that of P131 (Figures 2.5 and 2.6). Therefore, there may be a correlation between the ability of PPs to inhibit aggregation and their efficacy in reducing Aβ42 induced cytotoxicity.

2.6 Conclusions

In this study, Aβ42 aggregation inhibitors with nM range affinity to Aβ42 were developed. The significantly improved binding affinity of PPs obtained from third library of PLM indicates the methodological effectiveness of PLM in the directed molecular evolution of novel peptides. Among the selected PPs, P131 showed the strongest binding affinity (K_d value 12 nM). However, P84 showed stronger inhibitory effect of Aβ42 aggregation compared to that of P131, indicating that peptide with stronger binding affinity may not be strong inhibitor of Aβ42 aggregation.

In this study, the decreased toxicity of Aβ42 by the action of P84 and P131 in PC12 cells was successfully demonstrated, which is a promising step to check the therapeutic potential of these novel peptides. Consistently with the results of Aβ42 aggregation inhibition by PPs, P84 showed more effective protection against the cytotoxic effect of Aβ42 compared to that of P131, indicating that there may be a strong correlation between the Aβ42 aggregation inhibition and cytotoxicity inhibition by PPs. So, in future it is planned to test the effect of P84 and P131 on toxicity of Aβ42 by delivering these peptides to the brain using in vivo mouse model. In this study, no experiments by coincubating preformed Aβ42 fibrils and oligomers with PPs were performed. However, such experiments in future are necessary in order to study the effect of PPs on the preformed cytotoxic Aβ42 fibrils and oligomers. Furthermore, another future plan will be to do detail study on the conformation of Aβ42 oligomers formed under different conditions, which is needed for the true understanding of the Aβ42 toxicity. For such study, P84 and P131 developed here must hold a great promise as a molecular tool.

Chapter 3

Improvement of binding affinity and functional activity of cathepsin E activator P109 peptide by the fourth library method of PLM

3.1 Abstract

The aspartic protease cathepsin E has been proven to show anti-cancer activities under physiological conditions. Therefore, cathepsin E-activity-enhancing peptides with enhanced functional activities in the physiological pH are potential cancer therapeutic candidates. To this end, cathepsin E activity enhancing peptide (P109) with nM affinity at neutral pH was developed by using systematic *in vitro* evolution method including paired peptide method. Although P109 showed very strong binding affinity to cathepsin E, P109 modestly enhanced the cathepsin E activity at physiological pH. In this study, it was aimed to introduce the fourth library based on the point mutation together with the DNA shuffling method to further improve the affinity and functional activity of P109. To this end, a randomly base-substituted library (fourth library) was generated by inserting point mutations in the sequence of P109. One mutant containing single point mutation of P109, here after named SK1, showed 1.6 fold improved binding affinity to cathepsin E at neutral pH compared to the affinity of non-mutated P109. SK1 also showed significantly improved functional activity (5% increased functional activity compared to that of P109). These results clearly showed the methodological effectiveness of PLM; suggesting PLM is an established *in vitro* molecular evolution method to develop potential therapeutic candidates against cancer.

3.2 Introduction

Cathepsin E was first described by Lapresle and Webb in 1962. It belongs to the third class of enzymes – hydrolases. It is an intracellular aspartic proteinase. In human, cathepsin E is encoded by the CTSE gene located in chromosome 1 (Couvreur *et al*., 1990). In human, cathepsin E occurs in erythrocytes, thymus, dendritic cells, epithelial M cells, microglia cells, Langerhans cells, lymphocytes, epithelium of gastrointestinal tract, urinary bladder, lungs, osteoclasts, spleen and lymphatic nodes (Chlabicz *et al*., 2011). Cathepsin E is similar to the cathepsin D in its structure. It differs from latter, however, in that it has broader substrate specificity, a lower optimum pH (2.5 – 2.8), it occurs in different cell organelles, and occurs only in some kinds of cells and tissues (Chlabicz *et al*., 2011). Furthermore, cathepsin E does not appear to be involved in the digestion of dietary protein and is found in highest concentration in the surface of epithelial mucus-producing cells of the stomach. Cathepsin E is the first aspartic proteinase expressed in the fetal stomach and is found in gastric cancers (Matsuo *et al*., 1996). So, inhibition of cathepsin E is a promising approach for anti-cancer therapeutics. To this end, several inhibitors of cathepsin E has been reported (Kwan *et al*., 2009, Chlabicz *et al*., 2011).

In recent years, several research studies have shown that the cathepsin E at physiological pH is associated with suppression of tumour growth and metastasis both *in vitro* and in *vivo* (Kawakubo *et al*., 2011).This means cathepsin E acts as a potent anticancer protease at physiological pH. Therefore, development of novel peptides which can activate cathepsin E at physiological pH holds a great therapeutic value for the treatment of cancer.

Although several protease inhibitors have been successfully applied to cancer therapy, very few activators have been successfully applied (Turk, 2006; Ottmann *et al*., 2010). One of the reason for a smaller number of enzyme activators designed as of now is partly because enzyme activators are difficult to identify as there are currently no rational design principles or effective screening methods that can be used (Zorn *and Wells*., 2010). Therefore, a method for obtaining enzyme activators is urgently needed. To this end, the systematic *in vitro* evolution method including progressive library method which was initially used to develop inhibitors against cathepsin E at acidic pH (Kitamura *et al*., 2009 and 2012) was adopted to develop cathepsin E activators at neutral pH (Biyani *et al*., 2011, Komatsu *et al*., 2012). The peptides selected from the third library (paired peptide library) showed a very strong affinity to cathepsin E at physiological pH and also significantly enhanced the cathepsin E activity (Komatsu *et al*., 2012). The selected peptides also strongly enhanced the apoptosis of cancer cells (HeLa cells).

These results showed that progressive library method is an effective *in vitro* molecular evolution method not only to develop peptide inhibitors but also to develop peptide activators. One peptide P109 which showed an nM affinity to cathepsin E (K_d value of 2 nM) displayed moderate functional enhancement of cathepsin E. In order to make this peptide as a potential therapeutic candidate, its binding affinity and particularly functional activity should be further enhanced.

As of now, PLM consists of three progressive libraries and each library can be assigned as module finding, module shuffling and module pairing, which are quite similar phenomena to the natural selection of protein. Furthermore,

introduction of the point mutations can further improve the characteristics of thus evolved proteins or peptides at the final stage. Therefore, the objectives of this study are i) to introduce the fourth library based on the point mutation together with the DNA shuffling method to give the completeness of PLM; and ii) to improve the binding affinity and functional activity of P109 by introducing single mutations in P109 sequences.

In order to fulfil the above mentioned objectives, at first, a randomly base-substituted library (fourth library) was generated by introducing point mutations in the sequence of P109. One mutant containing a single point mutation in P109, here after named SK1, showed 1.6 fold improved binding affinity to cathepsin E at neutral pH compared to the affinity of non-mutated P109. SK1 also showed significantly improved functional activity (5% increased functional activity compared to that of P109). Bioassay tests are under study to check the effect of SK1 on apoptosis of cancer cells. Based on these results, a significantly improved binding affinity and functional activities of new mutants are expected from DNA shuffling method which is under study.

3.3 Materials and Methods

3.3.1 Preparation of protease cathepsin E and its substrate

Protease cathepsin E was prepared from rat spleens and it was kindly provided by Professor K. Yamamoto of Kyushu University. The fluorogenic substrate of cathepsin E working at the neutral pH (pH7.4) [Nma-Gly-Gly-Arg-Arg-Ser-Gly-Thy-Cys-Gly(Dnp)-D Arg-NH_2] was custom-synthesized by Peptide Institute (Japan). The

substrate was initially dissolved at 10 mM in DMSO and then used after diluted for use in the reaction buffer.

3.3.2 Generation of the fourth library (Mutated library)

cDNA construct of peptide P109 selected from the third library was used to generate the fourth library. The cDNA construct used for this purpose is given in Figure 3.1. As shown in the Figure 3.1A, P109 consists of peptide blocks 1 and 2 which were linked via a liker. In this study, only the amino acids of peptide blocks 1 and 2 were used for random mutagenesis. In this study, sequential site saturation random mutagenesis approach was used so that each amino acid of peptide blocks 1 and 2 (total amino acids 19) were substituted with 19 non-wild type amino acids at a time (Figure 3.1B) so that total size of the library will be expected as 361 of variants. This mutant-containing library was custom ordered and prepared by Life Technologies (Germany). Once the library was received, it was used for *in vitro* transcription, puromycin linker ligation and *in vitro* translation in order to make the cDNA-peptide fusion construct (Figure 3.1C) by following the same protocol as explained in Chapter Two (section 2.3.1).

3.3.3 *In vitro* selection of cathepsin E activating peptides

The overall scheme of *in vitro* selection process based on cDNA display method is shown in Figure 3.1D. First of all, negative screening was performed in order to remove any non-specifically binding cDNA-display products. For this purpose, the cDNA-peptide fusion construct was incubated in selection buffer (50 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 7.4) containing NHS magnetic sepharose beads (GE Healthcare, USA) at 25°C for 1h with gentle shaking. In this case, those cDNA display molecules which were bound to the NHS magnetic sepharose were discarded and only

the unbound DNA molecules present in the supernatant were transferred to new tube which contains 100 pmol cathepsin E

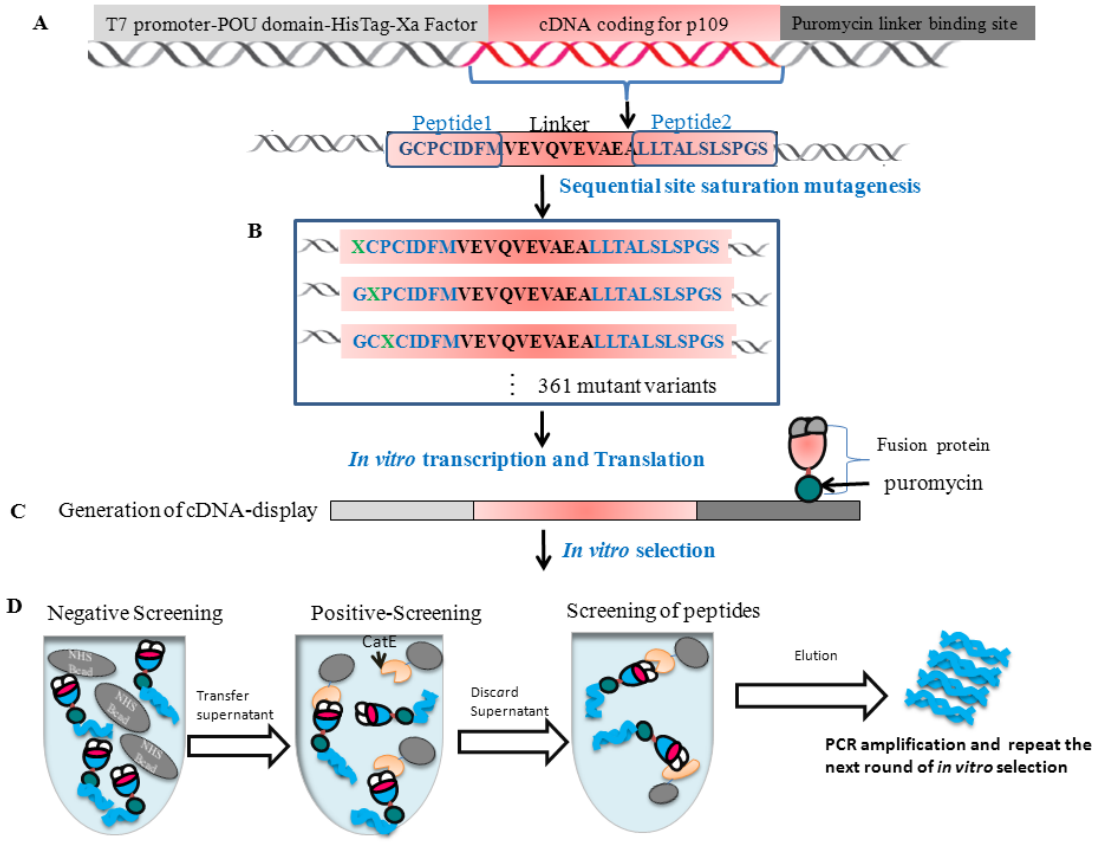

Figure 3.1. Schematic representation of generation of the fourth library and *in vitro* selection of cathepsin E binding peptides. (A) represents the complete cDNA construct containing cDNA coding for P109 which consists of two peptide blocks (Peptides 1 and 2) separated via a linker. (B) represents the sequential site saturation mutagenesis of cDNA coding for P109. (C) represents the cDNA display product obtained by *in vitro* transcription and *in vitro* translation of mutant variants of the fourth library. D shows the *in vitro* selection of cathepsin E binding peptides.

immobilized on NHS magnetic sepharose. The coupling of cathepsin E on NHS magnetic spharose was performed by following the manufacturer`s protocol. The reaction was incubated at 25^{o}C for 15 min. Then, any unbound cDNA display molecules were removed by washing with selection buffer while those DNA molecules which

bound to cathepsin E immobilized on a bead were eluted for next round of selection. This process was called as positive selection. The *in vitro* positive selection process was repeated for 4 rounds with increasing selection stringency i.e. for the second, third and fourth round of selection, incubation of cDNA-display product with cathepsin E immobilized on NHS sepharsose was performed at 37^{o}C for 15 min, 10 min and 5 min, respectively. The elution of cDNA-display product was performed by using 100 µl of 25% ammonia solution incubating at 65^{o}C for 15 min and by using 100 µl of 2% SDS incubating at 95^{o}C for 5 minutes. The eluted DNA at the fourth round was subjected to cloning and sequencing to identify cathepsin E-activating peptides.

3.3.4 Affinity measurement

The dissociation constant (K_d) of selected peptide against cathepsin E was measured by the SPR method using Biacore 2000 (GE Healthcare, UK). Cathepsin E was immobilized on a Biacore sensor chip CM5 (GE Healthcare, UK) by the general amine coupling method. Cathepsin E (150 µg/ml) dissolved in acetate buffer (50 mM sodium acetate, 100 mM NaCl, pH 4.5) was injected into a flow cell for the sample lane while the reference lane was prepared similarly without cathepsin E. In all experiments, Neutral pH buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) was used as the running buffer and a 50 mM NaOH solution for removing the cathepsin E-binding peptides on the sensor chip. Obtained sensorgrams were curve-fitted by 1:1 Langmuir binding model and K_d values were calculated using BIA evaluation software (GE Healthcare, UK).

3.3.5 Cathepsin E protease activity assay

The selected peptides were first prepared by *in vitro* translation and some of them were chemically synthesized. Cathepsin E (20 nM) was preincubated with various concentrations of selected peptides (10 μM, 100 μM, 1 mM, and 5 mM) in selection buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 25°C for 10 min. The fluorogenic substrate (5μM) was then added to the solution and incubated at 37°C for 1 h for the enzyme reaction. Then, the fluorescence of the reaction product was detected at 440 nm (excitation at 340 nm) with a microplate reader, Infinite 200 (TECAN, Japan). The percent of cathepsin E activation (A) was calculated by the following equitation:

$$A = 100 \times \frac{S_f - B_f}{C_f - B_f} \text{ [\% Activation],}$$

Where S_f represents the fluorescence intensity of cathepsin E protease reaction product in the presence of a selected peptide; C_f is that of control reaction in the absence of a selected peptide; B_f is the background fluorescence of the solution containing only the fluorogenic substrate.

3.4 Results

3.4.1 *In vitro* selection of cathepsin E binding peptides from the fourth library

The fourth library was obtained by sequential site saturation mutagenesis of P109 which was selected from the third library. The fourth library was then subjected to *in vitro* selection by affinity to cathepsin E and after four rounds of selection, the eluted DNA was used for cloning and sequence analysis in order to find out the most frequently occurred mutants which have the improved binding affinity to cathepsin E at

physiological pH. As shown in Table 3.1, the single point mutation was found either in peptides 1 or 2 against P109. Two mutants were found to be occurred multiple times in the library as shown in Table 3.1. Although more than 60 clones were used for the sequencing, only the colonies which have the expected sequence derived from P109 with mutations are listed in Table 3.1. Based on the frequency of occurrence of the clones, clone 1, here after called as SK1 which has mutation of phenylalanine to lysine at position 7 of peptide 1, was selected for chemical synthesis in order to determine the binding affinity and functional activity of this mutant against cathepsin E at physiological pH.

Table 3.1. Cathepsin E binding peptides obtained from *in vitro* selection of the fourth library

Clone number	Amino acid sequence	Occurrence	Mutations
1	**GCPCIDKMVEVQVEVAEALLTALSLSPGS**	**2**	F7K
3	**GCPCIDFMVEVQVEVAEALLTALSLSEGS**	**2**	P27E
5	**GCPCKDFMVEVQVEVAEALLTALSLSPGS**	**1**	I5K
6	**GCPCIDFMVEVQVEVAEALLTALSLSPGR**	**1**	S29R
7	**GCPCIDFMVEVQVEVAEALLTALWLSPGS**	**1**	S24W
8	**GCPCEDFMVEVQVEVAEALLTALSLSPGS**	**1**	I5E
9	**GCPCIDFMVEVQVEVAEALLHALSLSPGS**	**1**	T21H

The amino acids written in red color are mutated. The amino acids of linker part are indicated by green color whereas the amino acids of peptide blocks 1 and 2 are written in black color.

3.4.2 Improved binding affinity and functional activity of SK1 to cathepsin E

The chemically synthesized SK1 was used for SPR experiments to determine its binding affinity to cathepsin E at physiological pH. As shown in Figure 3.2 below, SK1 showed strong binding affinity to cathepsin E. The binding affinity of SK1 in terms of dissociation constant (K_d) was calculated by using 1:1 Langmuir equation. The K_d of

SK1 was found to be 1.2 nM which was about 1.6 fold higher than the K_d value of its parent peptide P109 (K_d value of 2 nM) which was selected from the third library (Komatsu et al., 2012).

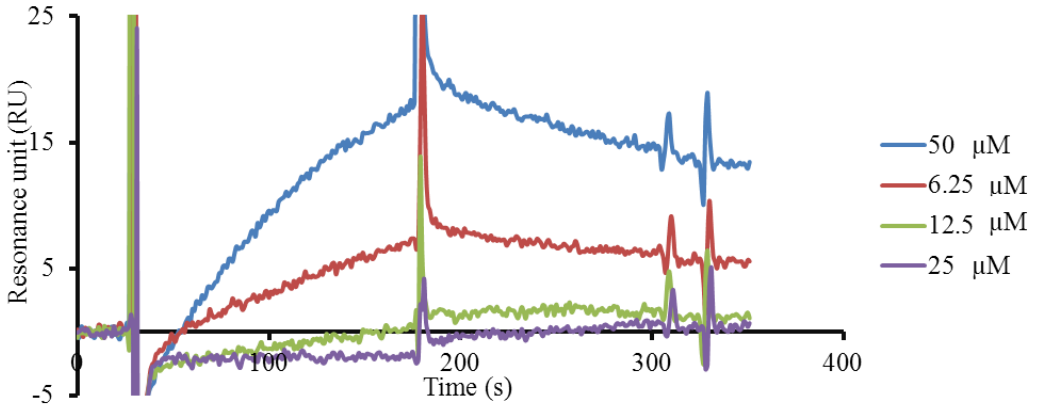

Figure 3.2. Determination of binding affinity of SK1 to cathepsin E at physiological pH. Representative binding curves were obtained by running different concentrations (50 µM, 25 µM, 12.5 µM and 6.25 µM) of SK1 over 150 µg/ml cathepsin E immobilized on the CM5 sensor chip.

In order to investigate the effect of SK1 on the enzymatic activity of cathepsin E at neutral pH, the chemically synthesized SK1 was used for enzymatic reaction (details are given in Materials and Method section of this chapter). It was found that SK1 significantly improved the enzymatic activity of cathepsin E at physiological pH as shown in Figure 3.3. Furthermore, SK1 showed improved enhancement of cathepsin E activity than that of P109 (5% improvement).

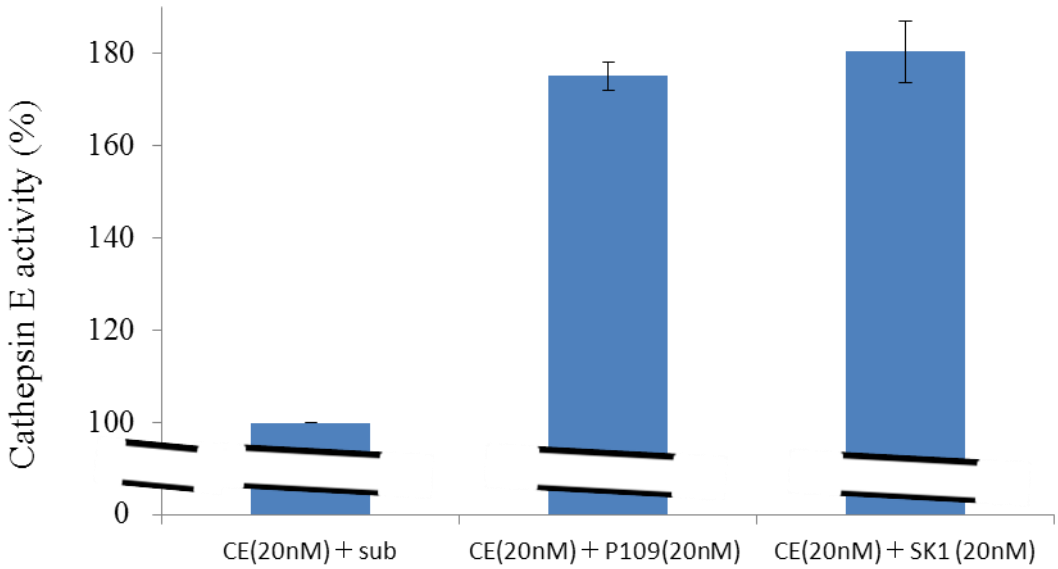

Figure 3.3. **Comparison of cathepsin E (CE) activity enhancement by chemically synthesized P109 and its mutant SK1.** Cathepsin E and peptide concentrations were 20 nM. Error bars represent the SD of triplicate experiments.

3.5 Discussion

In this study, it was attempted to establish and confirm the potency of PLM approach for identifying improved protease-activating peptides. It is likely that inhibition of protease activity, which leads to the accumulation of unprocessed proteins or a shortage of necessary processed proteins, plays a causative role in various diseases. To treat these diseases, either the amount of the protease needs to be increased or the protease activity needs to be enhanced. The latter is much easier to carry out because of the small size and high stability of proteases. Therefore, in this study, identification of peptides which can highly enhance the cathepsin E activity was reasonable.

The peptide P109 with a very strong binding affinity to cathepsin E showed moderate functional activity. In order to enhance the cathepsin E activity at neutral pH, a new peptide or mutant of P109 was essential. For this purpose, a

mutant library of P109 was generated based on the point mutation approach as a preliminary test. As described in Results that a single amino acid substitution of P109, i.e. SK1, significantly improved both binding affinity and functional activity of P109 against cathepsin E. These results are based on the *in vitro* selection of fourth library based on selection-by-affinity approach. However, the *in vitro* selection of the same library based on selection-by-function approach is under study and based on the evidence from previous studies (Figure 3.4); new mutants with highly improved functional activities are expected from the upcoming experiments. Furthermore, these results indicate that presence of such type of beneficiary multiple mutations can further improve the binding affinity of P109 to cathepsin E. Such a novel mutant with highly improved binding affinity is expected by *in vitro* selection of mutagenic library generated based on DNA shuffling (Stemmer, W.P., 1994) approach which is under study.

As of now, the cathepsin E has been very extensively used to develop its inhibitors and activators by using systematic *in vitro* evolution method, that is, progressive library method. The novelty of this study was the generation of the fourth library based on mutagenesis approach (point mutation approach and DNA shuffling approach). Taken in account of already published work based on PLM (Kitamura et al., 2009, 2012; Biyani et al., 2011; Tsuji-Ueno et al., 2011 and Komatsu et al., 2012) and the data obtained from the fourth library, it can be stated that PLM is now completed as a strategy for proven evolution of proteins beginning from a chaotic state up to the highest functional state by following the four steps of libraries. This statement can be realized from the Figure 3.4 in which each library can be assigned as module finding, module shuffling, module pairing and final adjustment (through the mutagenesis of thus

evolved module to further improve its characteristics like binding affinity and functional activity towards its corresponding target protein). This phenomenon is quite similar to the natural evolution of proteins and peptides.

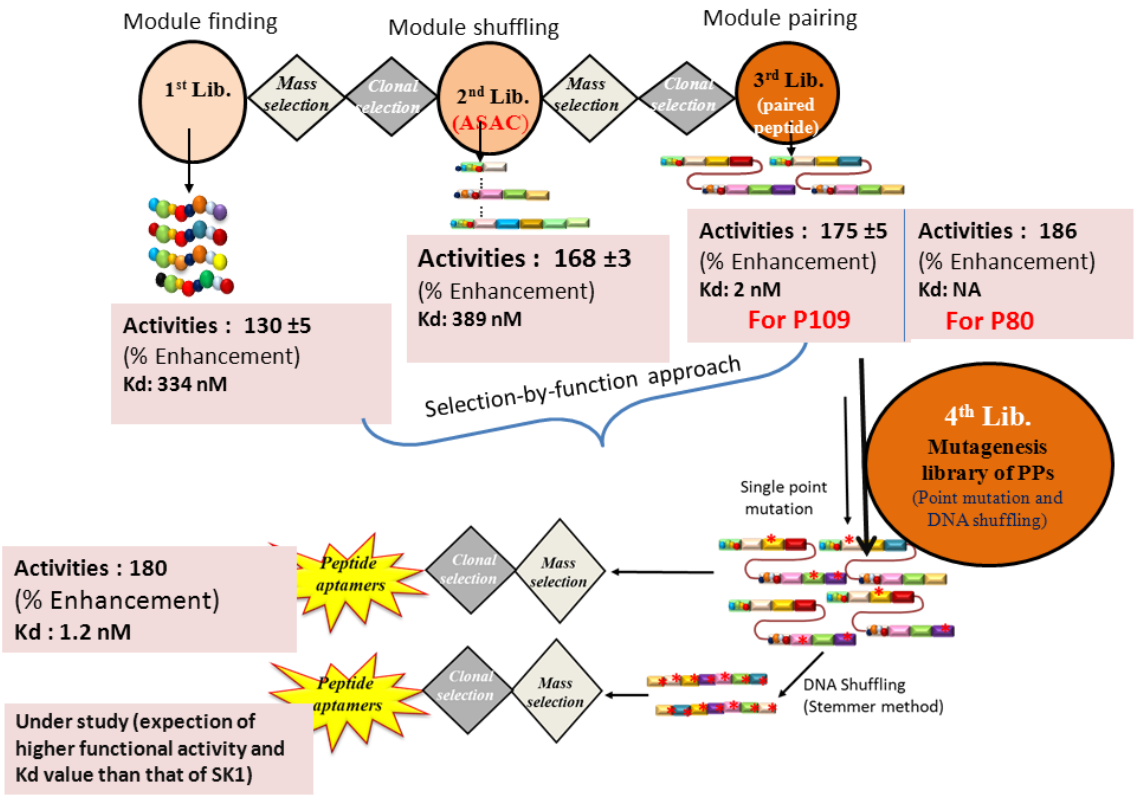

Figure 3.4. **Demonstration of methodological effectiveness of complete progressive library method (PLM).** Note that the results of first three successive libraries are based on *in vitro* selection by function approach where as that of the fourth library is based on *in vitro* selection-by affinity approach. *In vitro* selection of the fourth library based on selection-by-function approach is under study.

3.6 Conclusions

In this study, a new mutant peptide with an improved binding affinity to cathepsin E was developed. The mutant could enhance the cathepsin E activity at physiological pH. These data demonstrated that the fourth library based on mutagenesis of PLM has

potency to further improve the function of peptides (and proteins). The sophistication of the fourth library by the DNA shuffling method or utilization of the pre-exhaustively generated hamming distance library (that is, not single but double, triple, quadruple and more mutations-containing library construction) will reinforce the products of the fourth library and this will lead to truly complete the PLM method.

Chapter 4

Overall conclusions and prospects to future study

4.1 Overall conclusions

In this study, by adding two cases of library evolution strategy, it was successfully shown that PLM is an effective strategy to evolve novel peptides with a strong binding affinity and functional activity against different target proteins. By employing PLM, novel PPs with nM affinity peptide inhibitors against the Aβ42 aggregation and cytotoxicity were evolved. To the best of our knowledge, this is the first report showing the development of randomly evolved peptide aptamers with the highest binding affinity to Aβ42. Therefore such peptide aptamers can be valuable seeds for the therapy and diagnosis of AD. The exact mechanism of how these PPs inhibited the Aβ42 aggregation is not known at the moment. However, the data obtained in this study suggest that these PPs interfere the Aβ42 monomers to undergo the oligomerization and fibrillization processes. It is believed that these two PPs developed here hold a great promise as a molecular tool to understand the mechanism of Aβ42 aggregation and toxicity.

Similarly, the successful development of SK1, which is a mutant of P109, with a better binding affinity and functional activity to cathepsin E than that of the parent peptide P109 provided the two fruits. One is of-course, the knowledge on the precious molecule which can be a seed for cathepsin E-related therapy and the other is the final scheme

with which the PLM strategy can be completed. This has a special meaning in the PLM strategy since the protein evolution should contain the point mutation process and it could be included very naturally as expected.

Eventually, the PLM strategy can be applied to the evolution of proteins/peptides for various functions. It is quite parallel to the natural selection of proteins (from chaos to highly ordered state) by climbing up the steps of hierarchy (simple to complex) through the mechanisms of `recombination (or block shuffling)` and `point mutations`. Therefore, our systemic *in vitro* selection is a kind of complete solution for the artificial evolution of proteins.

Furthermore, this study strongly suggests that PLM can be used to design therapeutic peptides against plethora of target proteins/peptides, contributing in the peptide based drug discovery industry.

4.2 Prospects to the future study

In this study, cytotoxicity inhibition of Aβ42 by PPs was shown using PC12 cells as a model experiment, which is a good first step, however these PPs should be tested *in vivo* animal model in order to test whether these PPs can inhibit the Aβ42 aggregation after crossing the blood brain barrier (BBB), which is assumed to be the main hurdle to most of the AD therapeutic candidates. So, testing the effect of P84 and P131 on the toxicity of Aβ42 by delivering these peptides to the brain using *in vivo* mouse model is the next step to clear. Furthermore, in future, detailed studies on the conformation of Aβ42 oligomers formed under different conditions are needed for the true understanding of the Aβ42 toxicity.

On the other hand, to obtain further evolved peptides from the fourth library is left for the future work.

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