COMPUTATIONAL ELUCIDATION OF BINDING INTERACTIONS FOR THICAPA AND POET ON AMYLOID BETA 42 AND PRESENILIN 1 IN TREATING ALZHEIMER'S DISEASE

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by

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This thesis dedicated for UMMAH…

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LIST OF SYMBOLS

α	Alpha
β	Beta
γ	Gamma
Å	Angstroms
ε	Epsilon

LIST OF ABBREVIATIONS

A	Alanine
Aβ	Amyloid-beta
AD	Alzheimer's disease
APP	Amyloid Precursor Protein
CADD	Computer Aided Drug Design
CTF	C-Terminal Fragment
D	Aspartic acid
E	Glutamic acid
ECD	Extracellular domain
FDA	Food Drug Association
GAMD	Gaussian Accelerated Molecular Dynamic
GSI	Gamma-secretase Inhibitor
GSM	Gamma-secretase modulator
HL1	Hydrophilic Loop 1
IPS	Institut Pengajian Siswazah
LOMETS	Local Meta-Threading Server
MMGBSA	Molecular Mechanics Generalized Born Surface Area
NCT	Nicastrin
NPT	Constant number (N), constant pressure (P), and constant temperature (T)
NVT	Constant number (N), constant volume (V), and constant temperature (T)
PALP	Proline-alanine-leucine-proline
PDB	Protein Data Bank
POET	Palm Oil Extracted Tocotrienol

POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
PS1	Presenilin 1
Rg	Radius of gyration
RMSD	Root-Mean-Square Deviation
RMSF	Root-Mean-Square Fluctuation
SBDD	Structure-Based Drug Design
THICAPA	1,2,3,4-tetrahydroisoquinoline-3-carbonyl
TM	Transmembrane
USM	Universiti Sains Malaysia

LIST OF APPENDICES

PENJELASAN PENGKOMPUTERAN INTERAKSI PENGIKATAN THICAPA DAN POET KE ATAS AMYLOID BETA 42 DAN PRESENILIN 1 DALAM MERAWAT PENYAKIT ALZHEIMER

ABSTRAK

Penyakit Alzheimer (AD) bermula dengan gangguan ingatan dan kognitif dan berkembang kepada gangguan kemahiran pertuturan dan pergerakan yang mempengaruhi hampir 35% dewasa yang berusia 80 tahun. Menurut hipotesis lata amiloid, protein prekursor amiloid (APP), dan Presenilin 1 (PS1) bertanggungjawab untuk pengeluaran peptida amiloid-beta 42 (Aβ42) patogen (pesakit Alzheimer). Mutasi pada APP dan PS1 menyebabkan pembentukan Aβ42 yang kemudiannya membentuk plak Aβ42 dan terperangkap di otak. PS1 adalah komponen utama γ-secretase yang menjadi pemangkin pemotongan APP (bertanggungjawab untuk pengembangan neuron) terutama di amino asid bahagian beta di kedua-dua laluan pemprosesan amiloid. Pendekatan pendokan molekul dan dinamik molekul (MD) telah digunakan untuk mengkaji pengikatan asid 3-[[(3S)-1,2,3,4-Tetrahidroisokuinolin-3-karbonil]amino]propanoik (THICAPA) dan Tokotrienol Ekstrak Minyak Kelapa Sawit γ (POET) terhadap Aβ42 (APP), PS1. Struktur 3D Aβ42 dan PS1 asas diambil dari PDB, iaitu ID PDB: 6SZF (Aβ42(APP)), dan 7D8X (PS1) dan telah diubah suai berdasarkan mutasi variannya. I-TASSER juga digunakan untuk menghasilkan PS1 lengkap untuk kajian MD. Sebanyak 414 pengedokan yang terdiri daripada Aβ42 (APP), PS1 dan mutasi mereka telah dilakukan dengan THICAPA dan POET. Hasilnya menunjukkan semua pengedokan mempunyai Pengikat Tenaga Bebas (BFE) negatif dalam julat -5.65 kJ/mol hingga -10.94 kJ/mol. Simulasi MD THICAPA, POET, dan FTO (kawalan) dengan PS1 yang dimasukkan ke dalam dwilapisan

fosfolipid telah dijalankan. Semua ligan, termasuk kawalan, mempunyai nilai Rg yang stabil dan padat. Sisihan punca min kuasa dua (RMSD) menunjukkan protein dan ligan stabil dengan bacaan yang stabil semasa simulasi 100 ns, kecuali POET (PS1), yang mempunyai RMSD yang lebih tinggi. Hasil kajian menunjukkan bahawa THICAPA dan POET menunjukkan kestabilan asid amino dalam sistem dengan Turun naik punca min kuasa dua (RMSF) terutama di tapak pemangkin aspartik PS1 (D257 dan D385). Profil ikatan hidrogen menunjukkan THICAPA mengikat PS1 dengan lebih banyak ikatan hidrogen berbanding POET. Kedua-dua THICAPA dan POET memberikan hasil positif Luas Permukaan Ikatan Umum Mekanik Molekul (MMGBSA), dengan nilai negatif masing-masing -22.95 ± 4.88 kJ / mol dan -36.28 ± 3.0 kJ / mol. Nilai POET negatif yang lebih besar menunjukkan pengikatan PS1 yang lebih kuat berbanding dengan THICAPA. Oleh itu, THICAPA dan POET terikat dengan protein sasaran, PS1 dimana, ia boleh disasarkan yang boleh menjadi ubat yang baru untuk AD.

COMPUTATIONAL ELUCIDATION OF BINDING INTERACTIONS FOR THICAPA AND POET ON AMYLOID BETA 42 AND PRESENILIN 1 IN TREATING ALZHEIMER'S DISEASE

ABSTRACT

AD begins with memory and cognitive impairment and progresses to speech and movement skills which affects 35% of 80-year-olds adult. According to the amyloid cascade hypothesis, two proteins which are Amyloid Precursor Protein (APP), and Presenilin 1 (PS1) play a vital role in contributing to AD. Mutated APP and PS1 are shown to be responsible for the production of pathogenic amyloid-beta (Aβ42) peptide in AD patient, which then forms Aβ42 plaques and deposited in the brain. Presenilin 1 (PS1) is the primary component of γ-secretase which catalyses the cleavage of APP (neuron development) especially at the beta region of APP in both amyloid pathways. Molecular docking and molecular dynamic (MD) approaches were utilised to study 3-[[(3S)-1,2,3,4-tetrahydroisoquinoline-3-carbonyl] amino]propanoic acid (THICAPA) and Palm Oil Extracted Tocotrienol-γ (POET) binding to Aβ42, PS1, and their genetic variants. The basic 3D structures of Aβ42 (APP) and PS1 were retrieved from PDB, with the PDB ID: 6SZF (Aβ42(APP)), and 7D8X (PS1) and were modified accordingly to their variant mutations. A total of 414 dockings of Aβ42 (APP), and PS1 mutations were performed with THICAPA and POET. The result showed all dockings had negative values of BFE (binding free energy) ranging from -5.65 kJ/mol to -10.94 kJ/mo. I-TASSER was then used to remodel a complete PS1 for MD study. The MD simulations of THICAPA, POET, and FTO (control) with PS1 incorporated in the phospholipid bilayer were performed. Radius of Gyration (Rg) showed that the systems were stable and compact. All ligands, including the control,

had stable Rg values. Root-Mean-Square Deviation (RMSD) demonstrated proteins and ligands are stable with stable fluctuation during 100ns simulation, except for POET (PS1), which had a slightly highest. Results reveal that Root-Mean-Square Fluctuation (RMSF) of amino acid showed low fluctuation, especially at the aspartates catalytic site (D257 and D385). In hydrogen bonding profile, THICAPA bound PS1 with more hydrogen bonds than POET. Both THICAPA and POET yielded positive results for Molecular Mechanics-Generalised Bond Surface Area (MMGBSA), with a negative value of -22.95 ± 4.88 kJ/mol and -36.28 ± 3.0 kJ/mol, respectively. The greater negative POET value suggested stronger PS1 binding than THICAPA which was contributed hugely by van Der Waals interactions. Thus, this study showed that THICAPA and POET is able to form binding interactions with the targeted protein, PS1 which can be the new drug for AD.

CHAPTER 1

INTRODUCTION

1.1 Problem Statement

Alzheimer's disease (AD) is a major global public health concern which affect millions of people. It is the most common cause of dementia in older adults, accounting for 60-80% of all dementia cases (Barker et al., 2002). A survey by Nichols et al. (2019) showed that there are nearly 40-50 million people with AD world-wide, which approximately 23 million of them living in Asia and more than 123,000 cases reside in Malaysia (Nichols et al., 2019). World Health Organization (WHO) estimated that there are 8.5% of the older adults or approximately 260,000 people in Malaysia are affected by dementia (WHO, 2022). The number of AD's patient was estimated by Alzheimer's Disease International at 123,000 people in 2015 and this number was projected to be 261,000 by 2030 and further increase to 590,000 by 2050 (Alzheimer's Disease International, 2014).

AD is a progressive, fatal neurodegenerative disease that manifests first as cognitive and memory declines and eventually accompanied by changes in behaviour, speech, neuropsychiatric symptoms, and motor skills (Anand et al., 2014; De Ture and Dickson, 2019). It is a costly condition to manage and has a broader impact on society including increased healthcare costs, reduced workforce productivity and a decrease in quality of life for affected individuals and their families. The prevalence of AD is expected to triple by 2050, with the global cost estimated at >$50 billion per year (WHO, 2017). The burden on society will continue to grow due to the limited diagnostic tools and lack of efficient medical therapies for AD.

The main pathology of AD arises from the formation of the Aβ plaques which later causing the death of the neuron. Genetic studies revealed that mutations of APP and PS1 may lead to a familial form of AD (FAD). It will lead to the longer cleavage of APP product. The Aβ42 plaques is contributed by the agglomeration of the longer production of Aβ42 peptide and insoluble Aβ42 that speeds up the deposition process. Thus, the prevention or the inhibition of the Aβ42 agglomeration is very crucial for the AD treatment.

Despite being extensively researched, currently there is no specific drug to treat or reverse AD, and the available treatments mainly focus on managing symptoms. Recent findings have led to the approval of Leqembi (lecanemab-irmb) as a disease modifying therapy (DMTs) by Food Drug Association (FDA) that aims to alter the underlying pathophysiology of AD via the Accelerated Approval pathway for the treatment of Alzheimer's disease (FDA, 2023). This treatment targets the aggregation of amyloid-beta (Aβ42) plaques in the brain, which is a hallmark of AD. AD treatment needs combination therapy. Aducanumab is an immunotherapeutic considered as a human immunoglobulin gamma 1 (IgG1) monoclonal antibody. Its main mechanism of action is by crossing the blood-brain barrier and selectively targets and binds aggregated soluble oligomers and insoluble fibril conformations of Aβ plaques in the brain (Khanna et al., 2023). However, AD is seeming unlikely to be successfully treated by a single medicine or other intervention (Samanta et al., 2022).

In light of the complex nature of AD and the limited success of single-drug treatments, there is a growing interest in combination therapies and new directions for AD treatment. This study showed the drug candidates are suitable for inhibition or the blocking of APP cleavage. Two promising compounds, 3-[[(3S)-1,2,3,4-

tetrahydroisoquinoline-3-carbonyl] amino]propanoic acid (THICAPA) and palm oil extract tocotrienol (POET), have shown potential neuroprotective effects in preliminary experiments and literatures (Leow et al., 2021; Tan et al., 2023). These compounds were tested against *Drosophila melanogaster*, a model organism to study AD which showing a significant result by reducing the AD's symptoms (Tan et al., 2023; Leow et al., 2021). The result demonstrated that both THICAPA (Tan et al., 2023) and POET (Leow et al., 2021) have the ability to slow down the cellular ageing process and exhibit neuroprotective effects, making them promising candidates for AD treatment. These two compounds were simulated based on amyloid cascade hypothesis which Aβ42 (APP) and PS1 are the important proteins for production of the Aβ42 (plaque).

As the finding is preliminary, further research and rigorous clinical studies are needed to confirm the safety and efficacy of THICAPA and POET as potential therapeutics for AD. In addition, the understanding on how THICAPA and POET interact with the proteins in the molecular level during the studies remain elusive. To understand the molecular level of interaction between the compounds and the proteins, a powerful approach is used by utilising simulation studies such as molecular docking and Molecular Dynamics (MD). Computer Aided Drug Design (CADD) method is a modern computational technique used in the drug discovery process to study the ligand-receptor interactions. Molecular docking and MD simulations are the key components to understand the molecular interaction of the ligands and the targeted protein and will be used in this study to gain insights into how THICAPA and POET interact with their targeted proteins. These simulation studies allow for the examination of ligand-receptor interactions and provide valuable information for the design and optimisation of potential therapeutic agents for AD.

1.2 Aims and Objectives

The aim of this study is to elucidate the binding interaction of THICAPA and POET with different genetic variants of proteins involved in AD using CADD approach. To achieve this aim, the following objectives have been defined:

1. To elucidate the binding interactions (*in silico*) of POET and THICAPA with the Aβ42 (APP) wild type and the variation mutations associated with the formation of amyloid plaque.

2. To elucidate the binding interactions (*in silico*) of POET and THICAPA on the component of γ-secretase, specifically Presenilin 1 (PS1) and its variation mutations responsible for formation of amyloid plaque.

3. To investigate the dynamical behavior of the highest affinity target of THICAPA and POET towards either Aβ42 (APP), or PS1 using MD simulation.

CHAPTER 2

LITERATURE REVIEW

2.1 Alzheimer's disease (AD)

Alzheimer's disease (AD) is recognised as the main cause of dementia (Prince et al., 2013). There are approximately 40 million people suffering from dementia throughout the world. This number is estimated to rise up to twice as much every 20 years until approximately 2050 (Prince et al., 2013). The term Alzheimer comes from a German psychiatrist, Dr Alois Alzheimer in 1907 who described the case of a 51 years old woman who presented with a rapidly deteriorating memory along with psychiatric disturbance. She suffered from a variety of progressive and fatal neurological conditions (ALZHEIMER, 1907). Four years later, the term "Alzheimer's disease" was coined by Emil Kraepelin, to honour Dr. Alzheimer's contribution to its discovery and understanding (Berrios, 1990). The disease manifests as a decline in cognition and memory and is ultimately accompanied by changes in behaviour, speech, and speech skills, as well as neuropsychiatric symptoms (DeTure and Dickson, 2019; Kumar et al., 2015). AD has led to an intensive growth in research which focussed on treatment of the disease. Despite the abundant research been caried out, there are still no effective treatment options for the disease (Cummings et al., 2019; Scheltens et al., 2016).

Alzheimer's disease is a significant public health concern that affects millions of people globally. The World Health Organization (WHO) stated that approximately 50 million people worldwide have dementia and the majority of them have AD (Alzheimer's Disease International, 2014). Moreover, AD is categorised as the most common dementia in adults which account for 60-80% of all dementia cases (Ng et

al., 2021). There is approximately 23 million of them living in Asia and more than 123, 000 cases reside in Malaysia (Nichols et al., 2019).

2.1.1 Classification of Alzheimer's disease (AD)

There are two types of AD which can be further classified into two clinical conditions following their age of onset (Castellani et al., 2010). A term presenile dementia or Early-Onset Alzheimer's Disease (EOAD) is reserved for people who suffer from AD aged below than 65 years old while those aged more than 65 years old is referred as senile dementia or Late-Onset Alzheimer's Disease (LOAD) (Roth et al., 1966, 1967). EOAD can be described as a prominent cognitive impairment in other domains besides memory, such as language problems, prominent apraxia, or executive dysfunction (Mendez, 2017; Smits et al., 2012).

Late-onset AD (LOAD) is much more common in society, accounting for 90% of all AD cases. It is a complex hereditary condition with a heritability of 60-80%. EOAD and LOAD can be differentiated by their genetic profile. EOAD is mainly contributed by the mutation of APP, and PS1 (Mendez, 2019). These two pathogenic mutations results in the APP's abnormal cleavage or aggregation. The abnormal cleavage will be causing the formation of the plaques (Jarmolowicz et al., 2015). However, the primary genetic risk factor for LOAD is Apolipoprotein E (APOE) gene (Cruchaga et al., 2012). LOAD is greatly influenced by the APOE 4 allele which encodes the leading cholesterol transporter in the brain and has three frequent alleles: ε2 (8.4% estimated population allele frequency), ε3 (77.9%), and ε4 (13.7%) (Rabinovici, 2019). The presence of the four alleles will bind to the soluble Aβ and speed up the deposition process which increase the plaque formation (Yamazaki et al., 2019). As a result, APOE ε4 is associated with a higher rate of longitudinal Aβ

accumulation in cognitively healthy, amyloid-negative individuals, whereas no differences in the deposition rates are observed in amyloid-positive individuals with various APOE genotypes (Lim et al., 2017).

2.1.2 Pathophysiology of Alzheimer's disease (AD)

2.1.2(a) Hypothesis of Alzheimer's disease

The neurodegenerative process is subject to a number of hypotheses. However, it is hard to simulate AD in people due to the absence of *in vivo* models. There is also no known treatment for the condition; instead, the medications that are currently on the market only treat the symptoms. The intricacy of the disease etiology and the ineffectiveness of the delivery of medications that are specifically aimed at AD have presented two significant obstacles in the search for the same. There are two basic theories that may be used to describe Alzheimer's disease exactly. They are cholinergic hypothesis (Bartus et al., 1982) and amyloid cascade hypothesis (Hardy and Higgins, 1992).

2.1.2(a)(i) Cholinergic Hypothesis

Cholinergic hypothesis was proposed by Peter Davies and Maloney in 1976 (Davies and Maloney, 1976). They studied the activities of the key enzymes involved in the synthesis of neurotransmitter, including acetylcholine, γ-aminobutyric acid, dopamine, noradrenaline, and 5- hydroxytryptamine, in 20 regions of AD and as well as control brain. The cholinergic hypothesis is the earliest theory of pathogenesis of AD. The cholinergic hypothesis in AD is proposed by Raymond T. Bartus et al. (1982) which linked the dysregulation of the basal forebrain cholinergic neurotransmission, alteration in the levels of cholinergic markers such as acetylcholine, choline and

choline acetyltransferase to the age-dependent cognitive functions' decline with AD (Bartus et al., 1982). In the brain, acetylcholine is involved in some physiological processes such as memory, attention, sensory information, learning and other critical functions (Behl, 2023). The deterioration of the cholinergic neurons was found to take place in AD and causing the reduction in the choline uptake and release of acetylcholine (Kunnath et al., 2023). This was shown by Francis et al. (1993) in his biochemical investigation of biopsy tissue (Francis et al., 1993) and post-mortem brain tissues from AD patients which showed reduced choline acetyltransferase activity (Wilcock et al., 1982), acetylcholine synthesis (Sims et al., 1983), choline uptake (Rylett et al., 1983) and acetylcholine release (Nilsson et al., 1986).

There are studies demonstrated that cholinergic synaptic loss and amyloid fibril formation are related to Aβ oligomers' neurotoxicity and interactions between acetylcholinesterase and Aβ peptide (Carvajal and Inestrosa, 2011). Previous research finding into amyloid-β plaques have shown that amyloid-β plaques bind to acetylcholine receptors, which can reduce the amount of acetylcholine available to neurons (Dineley, 2007). The evidence reviewed here seems to suggest a pertinent role of cholinergic activity to the pathogenicity of AD in not only the consequences of the disease but may also contribute to its progression and severity.

2.1.2(a)(ii) Amyloid Cascade Hypothesis

Apart from cholinergic hypothesis, amyloid cascade hypothesis is the most frequently studied hypothesis in establishing AD theory (Ju and Tam, 2022). In 1992, John Hardy proposed that amyloid cascade hypothesis in the basic form states that, amyloid deposits in AD resulted from a multitude of genetic or environmental insults

and are at the origin of the neurodegeneration that subsequently leads to development of dementia (Hardy and Higgins, 1992). The amyloid cascade hypothesis is that the deposition of amyloid-β protein, which is the main component of the plaques, is the causative agent of neurodegeneration in AD's pathology (Hardy and Higgins, 1992). The deposition of amyloid-β plaques acts as pathological trigger for a cascade that includes neurofibrillary tangles, cell loss, vascular damage, and dementia (DeTure and Dickson, 2019; Hardy and Higgins, 1992). Amyloid cascade hypothesis is supported by genetics, biochemical and pathological evidence which postulates that accumulation and aggregation of amyloid-β plaques are the primary causes of AD (Chen et al., 2000; Goate et al., 1991; Kayed et al., 2003).

According to amyloid cascade hypothesis, protein mutations play a significant part in the pathogenesis of AD via amyloidogenic pathway (Wu et al., 2022). It was first discovered that mutation of APP will lead to AD (Del-Aguila et al., 2019). Later, this hypothesis is further supported by the discovery that AD could also be caused by autosomal dominant mutations in Presenilin 1 (PS1) (Greenough et al., 2022). They are both homologous proteins that forming the catalytic active site of γ-secretase. The normal cleaving pathway of APP is via non-amyloidogenic pathway. It involves the first cleaving by α-secretase and final cleavage by γ-secretase which resulted in the production of soluble APP (Rajendran and Annaert, 2012). However, the amyloidogenic pathway that leads to the development of AD is contributed by the protein mutations or malfunction of these 2 proteins, APP, and PS1. The mutated APP will undergo sequential cleavage by β-secretase enzyme (BACE) and γ-secretase enzyme and eventually produced the Aβ (Rajendran and Annaert, 2012). The Aβ which has the sticky properties due to its longer protein Aβ42/43 than the normal

cleavage at Aβ40 leads to accumulation and aggregation that later forming Aβ plaques (Scheuner et al., 1996).

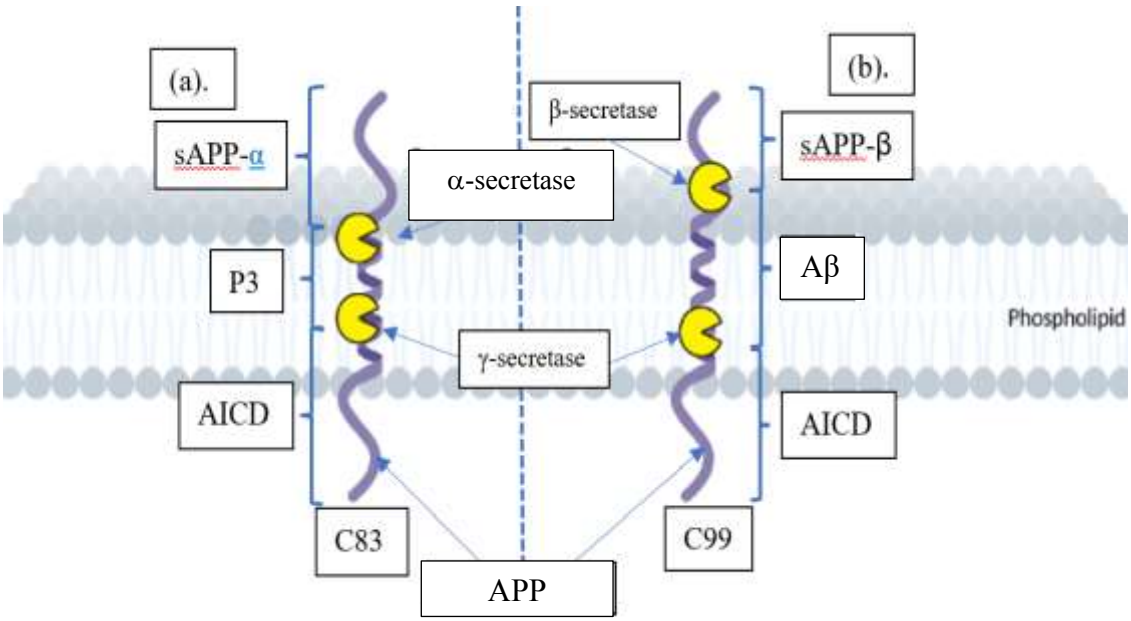

Figure 2.1 Proteolytic cleavage of APP by (a) non-amyloidogenic and (b) amyloidogenic pathways. This figure is generated using Biorender based on Hur. (2022).

Fig. 2.1 shows the proteolytic cleavage of APP by two different enzymes according to the specific pathway respectively. In the non-amyloidogenic pathway (a), APP is cleaved by the α-secretase enzyme while in amyloidogenic pathway, APP is cleaved by β-secretase enzyme. Non-amyloidogenic pathway produce C83 fragment and sAPPα while amyloidogenic pathway produce C99 and sAPPβ. C83 is further cleaved into a non-toxic *p3* fragment and AICD while C99 is further cleaved by γ-secretase and producing Aβ peptide and AICD.

The research questions in this study are mainly focused on the Amyloid Cascade Hypothesis. It is based on a study by Ju et al. (2022) which showed that Amyloid Cascade Hypothesis is the main focus hypothesis that best to describe the pathophysiology of AD (Ju and Tam, 2022). Kepp et al. (2023) showed an agreement

with the proposed idea which describe Amyloid Cascade Hypothesis plays important hypothesis to describe Alzheimer's disease (Kepp et al., 2023). The further cleavage of APP by the γ-secretase after the β-secretase showed a significant effect that produce the longer abnormal APP product which led to the formation of the sticky Aβ42.

2.1.2(b) Amyloid Precursor Protein (APP)

Around the late 1990s, research by Glenner and Wong (1984) was first to identify APP as a precursor protein of amyloid-β which accumulates in the brains of patients with AD (Glenner et al., 1984). It is a type-1 transmembrane protein that is evolutionarily conserved from *Caenorhabditis elegans* to humans (Cho et al., 2022). It is the major component of amyloid plaques in AD's brain. Kang et. al (1987) was the first group to isolate and sequenced an apparently full-length complementary DNA clone coding of APP (Kang et al., 1987). Moreover, over the years of studies, the regulation of APP expression, the mechanisms of APP trafficking, post-translational modification and proteolytic cleavage of APP are now well understood (Dawkins and Small, 2014).

2.1.2(b)(i) Structure of APP

The mammalian APP family is widely expressed and distributed across diverse tissues and organs (Puig and Combs, 2013). APP is notably responsible for the major roles in the regulation of several important human cellular functions, especially in the nervous system, where it is involved in synaptic plasticity and synaptogenesis (Gralle and Ferreira, 2007). APP has been discovered to be produced in significant quantities in neurons and to be rapidly metabolised (Lee et al., 2008; Uddin and Amran, 2019). APP processing is regulated by the secretase enzymes such as α-, β-, and γ-secretases

(Yuksel and Tacal, 2019). There are specific catalytic regions of APP that will cleave by specific secretase as shown in **Fig. 2.2** while **Fig. 2.3** shows the cleavage site of the APP by specific pathway. The processing of APP via non-amyloidogenic pathway which include the cleaving of APP by α-secretase at the α region as shown in **Fig. 2.2** leads to production of sAPPα, and α-CTF83. Whereas in amyloidogenic pathway, the cleavage of APP by β-secretases at the β region as shown in **Fig. 2.2** will subsequently produce sAPPβ, and β-CTF99, respectively (Uddin and Kabir, 2019). Later, both pathways will be subjected to the γ-secretase cleavage to produce either the soluble APP peptide (*p3* fragment) or the amyloidogenic Aβ peptide accordingly to their pathways (Uddin et al., 2020). Importantly, the amyloidogenic pathway generates amyloid-β peptides (Aβ) with 38 to 43 amino acids which is a hallmark of AD while non-amyloidogenic pathway produce the soluble *p3* fragment which later discarded in the normal pathway (Fujimoto et al., 2019).

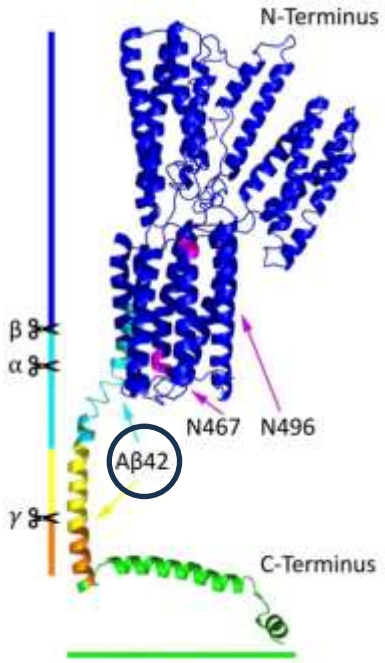

Figure 2.2 Diagram showing the catalytic region of APP by α-, β-, and γ-secretase. Taken from Lin, and Tjernberg, et al., (2021).

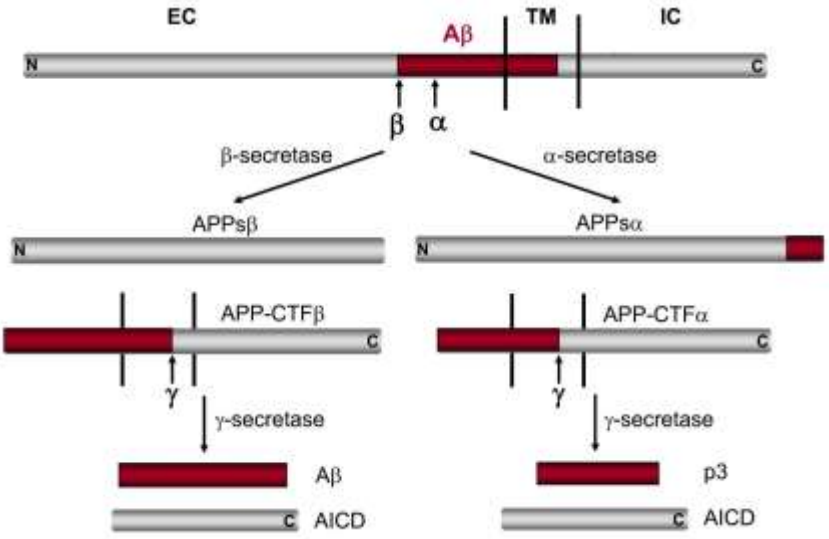

Figure 2.3 The cleavage site of the APP by specific pathway. Taken from Zheng, H., & Koo, E. H. (2011).

2.1.2(b)(ii) Variation mutations of APP

The discovery of the dominant inherited mutations in the amyloid precursor protein gene (APP) that causing disease are a foundation of the amyloid cascade hypothesis of Alzheimer's disease (AD) (Hardy and Higgins, 1992). The first serious discussions and analyses of the mutation of APP emerged during 2014 is that there are four cluster of familial Alzheimer's disease (AD) mutations that occurred in APP (Tang et al., 2014). Previous study of mutation in APP showed that three out of four cluster mutations were shown to be located close to α-, β-, and γ- cleavage sites respectively (Kaden et al., 2012). Specific mutations within these clusters (α- cluster) influence the proteolytic cleavage of APP which leads to increase Aβ40: Aβ42 ratio which was caused by increasing Aβ42 production over the shorter Aβ peptides (Aβ40) (Tang et al., 2014). Lastly, the fourth cluster of FAD's mutations is located below the α-secretase cleavage site (Suzuki et al., 2023). They are comprised of A21G (Flemish), E22Q (Dutch), E22G (Arctic), E22K (Italian), and D23N (Iowa) mutations (Grabowski et al., 2001; Hendriks et al., 1992; Inouye et al., 2010; Schilling et al., 2023).

Hu et al. (2017) reported that the first mutation identified in APP were at A21, E22, and D23 (Hu et al., 2017). They are situated within a cluster in the extracellular region (above β cluster) of the APP protein which is focussed in this study due to their big differences on APP processing Aβ peptide aggregation. The production of the pathogenic Aβ42 counted from this cluster. Thus, the amyloid structure from the beta site was taken for the docking process known as Aβ42 (APP). The numbering use for the amino acid to address the mutation as mentioned in previous paragraph are from the β-CTF numbering, which also coincides with the numbering of the Aβ peptides, example: Asp1 is the first residue of the β-CTF and the Aβ peptide (Kienlen-Campard

et al., 2019). The numbering of the mutation's position is based on the amyloid-β (Aβ) fragment (Yang et al., 2023).

In the amyloidogenic pathway, the cleaving of APP by β-secretase occurred at the β-site near the boundary between ectodomain and extracellular juxtamembrane of APP (Hussain et al., 2003; Papadopoulos et al., 2022). It resulted in the shedding of the ectodomain and is required for the second cleavage (Urban et al., 2021). The second cleavage of both non-amyloidogenic and amyloidogenic pathway is controlled by γ-secretase cleavage which produce the pathogenic Aβ42.

The fourth cluster which situated at α-secretase cleavage site (Suzuki et al., 2023). Over the past few years, the mechanisms which suggested these mutations influence the proteolysis of APP or the conversion of Aβ monomers to fibrils have begun to come to light (Gorman et al., 2008; Hu et al., 2017). The mutations of APP which directly affect the cleavage this cluster might raise the effect to the doubling the Aβ42 production up to four-fold and increasing the rate of fibril formation (Jonghe et al., 1998; Tang et al., 2019; Tian et al., 2010).

2.1.2(c) Gamma-secretase (γ-secretase)

γ-secretase is a membrane-bound enzyme complex that play a crucial role in the processing of various transmembrane proteins such as APP and also other transmembrane proteins that play an important role in cell signalling such as Notch receptors (Liu et al., 2023). It consists of a large complex containing Presenilin 1 (PS1), Nicastrin (NCT), anterior pharynx deficient 1 (APH-1), and presenilin enhancer 2 (PEN-2) (Figure 2.4) (Lazarov et al., 2006).

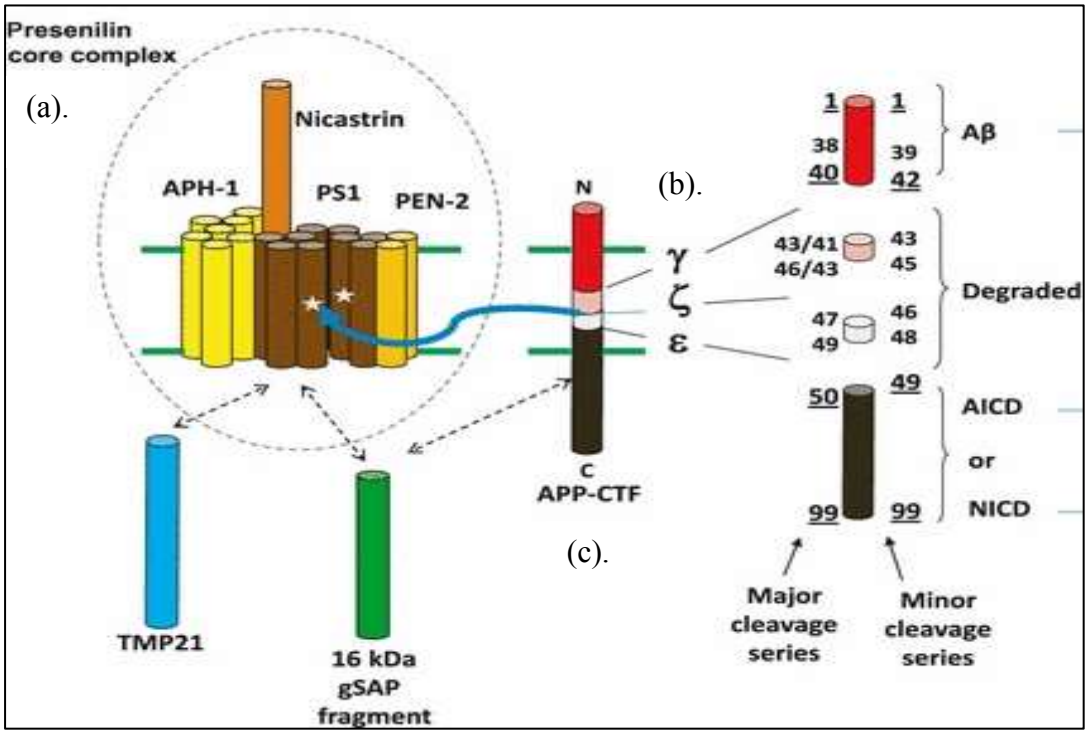

Figure 2.4 (a) The complete structure of the γ-secretase inside the lipid bilayer. (b)The APP will be cleaved by the γ-, ζ-, and ε- secretases and producing three types of products. (c) The catalytic site of the PS1 which can be classed into γ-, ζ-, and ε-secretases taken from St George-Hyslop P, Fraser PE (2012).

The entire structure of γ-secretase is shown in **Fig. 2.4(a)**, along with an approximation of where it is located in the lipid bilayer. **Fig. 2.4(b)** also shows the locations where certain enzymes, such as γ-secretase which cleave the APP (George-Hyslop and Fraser, 2012). **Fig. 2.4(c)** indicates the cleavage sites of PS1 are located

within the membrane. It is presented with the site of cleavage of the γ-, ζ-, and ε-secretases accordingly. Genetic studies revealed that mutations of APP, and PS1 may lead to a familial form of AD (FAD). Mutation of these proteins resulted in elevated levels of Aβ42/43, a proteolytic processing fragment of APP that is responsible for AD (DeTure and Dickson, 2019; Lendon et al., 1997).

Alterations in the genes encoding presenilin 1 (PS1) is frequently the cause of familial AD (FAD). Numerous PS mutations have been shown to have elevated Aβ42 (Aβ42/Aβ40) ratio and decreased γ-secretase activity, both of which cause FAD and early amyloid deposition (Islam et al., 2022). The alterations affected multiple downstream signalling pathways and caused a partial loss of function in the γ-secretase complex. Presenilin insufficiency brought on by imperfect amyloid-peptide digestion may enhance brain susceptibility and explain the early start of the hereditary type of Alzheimer's disease (De Strooper, 2007).

Over 90 type-1 transmembrane proteins are broken down by γ-secretase within their transmembrane region (Escamilla-Ayala et al., 2020; Su and Orange, 2020). In addition to continuing to be the primary neuropathophysiology of AD, γ-secretase is essential for a number of physiological processes, including calcium homoeostasis, innate immunity, and notch signalling (Jurisch-Yaksi et al., 2013; Lichtenthaler et al., 2011). RIP, or regulated intramembrane proteolysis, is a mechanism by which γ-secretase brought on AD. A shedder initially removes the ectodomains from the substrates during RIP, leaving membrane-embedded C-terminal fragments (CTFs) as the enzyme's direct substrates. Cleavage takes place within the transmembrane domain, resulting in the release of a smaller peptide in the extracellular space and an intracellular cytosolic fragment. The substrates of γ-secretase that have been studied the most intensively include APP and Notch receptors. As mentioned previously,

amyloid-β 42 peptides (Aβ42) serve as the primary component of amyloid plaques in the brains of AD patients, which have been generated by the shedding of APP by β-secretase 1 (BACE1) and subsequent cleavage by γ-secretase (Escamilla-Ayala et al., 2020).

Nicastrin (NCT) comprises up to approximately 45% of the total computed protein molecular mass of γ-secretase. The type-1 membrane protein NCT has a massive ectodomain and merely a single TM at its C-terminus. In comparisaon to the other γ-secretase proteins, extensive glycosylation significantly increases the NCT ectodomain's size. Although, γ-secretase activity is not dependent on presenilin-dependent NCT-hyperglycosylation, it is crucial for γ-secretase maturation and trafficking to the cell surface (Herreman et al., 2003; Shah et al., 2005). It is thought that the NCT-ECD is necessary for luring γ-secretase substrate.

NCT and APH-1 form a stable subcomplex in the endoplasmic reticulum (ER) (DeTure and Dickson, 2019). APH-1 is a seven-transmembrane protein having a mass of around 25 kDa. Apparently, human encompass two isoforms of APH-1 which are APH-1a and APH-1b. In comparison to APH-1b, APH-1a gets produced in larger quantities. There are two splice variants of the APH-1a gene: APH-1aS, which has 247 residues, and APH-1aL, which has 265 residues (Francis et al., 2002). This is due to differential splicing in their C-terminal regions (Kimberly et al., 2003). Rodents are also producing APH-1c, a similar protein to APH-1b. All these variants can be taken in into the γ-secretase complex, however the expression ratio will vary in accordance with the body part (Hébert et al., 2004; Shirotani et al., 2007). The overall proportion of Aβ42 production is larger in γ-secretase with APH-1b than γ-secretase with APH-1a (Serneels et al., 2009). APH-1 was found to resemble a seven-transmembrane domain receptor, with the C-terminus and odd-numbered loops located in the cytosol

and the N-terminus and even-numbered loops facing the endoplasmic reticulum lumen (Fortna et al., 2004). Studies have demonstrated that the amyloidogenic pathways of γ-secretase cleavage of APP ultimately resulted in the production of β-APP and Aβ peptides, subsequently contributing to accumulation in *Caenorhabditis elegans* with AD condition (Francis et al., 2002). Additionally, APH-1 interacts with presenilin enhancer 2 (PEN-2) to create an active version of the γ-secretase complex, which is in responsible for cleaving β-APP and depositing Aβ42 (Strooper, 2003).

NCT, on the other hand, incorporates a total of 709 amino acids and a glycosylation pattern that ranges from 30 to 70 kDa (Xie et al., 2014). The smallest component of γ-secretase is presenilin enhancer-2 (PEN-2). The 101 amino acids that make up PEN-2 can be separated into two membrane-spanning domains, with the NH2- and carboxyl-terminal domains facing the lumen (Francis et al., 2002). It is the final piece to come together in the γ-secretase complex and it starts the PS endoproteolysis (Kim et al., 2003; Kimberly et al., 2003). Studies using PEN-2 knockdown have demonstrated that PEN-2 is essential for complex stability and the endoproteolytic fragmentation of PS1 into its N- and C-termini (Holmes et al., 2014). Prokop et al. (2004) and Steiner et al. (2002) claimed that PEN2 may be necessary for the PS endoproteolytic cleavage (Prokop et al., 2005; Steiner et al., 2002). In the absence of PEN-2, the proteasome breaks down the γ-secretase complex. The successful rate of PEN-2 stabilises PS-subunit depends on the length and general sequence of its C-terminal domain, as well as the proximal two-thirds of its transmembrane domain 1 (Kim and Sisodia, 2005; Prokop et al., 2005). Recent research discovered that endoproteolysis seems to use the same ragged cleavages as those found with γ- and ε -cleavage to cleave the TM6-TM7 loop domain of PS1,

which is introduced into the transmembrane channel following the binding of PEN-2 (Dehury, et al., 2019).

Most studies into the pathophysiology of AD have been performed centred around the amyloid hypothesis (Selkoe and Hardy, 2016). This theory states that the build-up of Aβ42 in the brain is a major contributor to the onset of AD (Chen et al., 2017). According to the notion, as Aβ42 concentrations increase, sticky plaques develop between neurons and causing it to function incorrectly. It is thought that this mechanism sets off a series of circumstances that result in the emergence of further AD-specific traits (Kepp et al., 2023), as well as the initiation of inflammatory processes that harm brain tissue and the development of neurofibrillary tangles.

2.1.2(d) Presenilin (PS)

The first homologous of presenilin were found in *Caenorhabditis elegans* (Tandon and Fraser, 2002). Presenilin 1 (PSEN1) is the presenilin gene found in humans and encode the protein Presenilin1 (PS1) (Barazzuol et al., 2023). Through distinct interactions with other ligands, the extremely diverse hydrophilic sections at the central "loop" and N-terminal "head" domains are probably responsible for mediating cell- or PS-specific activities (Zhang et al., 2013). Studies utilising antibodies identify the protein's cytosolic or luminal regions, alterations to the N-glycosylation acceptor sites, protease digestion, and gene fusions with marker (Bagaria et al., 2022). The N-terminal segment and the large hydrophilic loop are both located in the cytosol of the protein, but the C-terminal portion is found in the extracellular space. The large hydrophilic loop also includes a membrane-related region (Bagaria et al., 2022).

The transmembrane aspartyl protease, also known as presenilin (PS), is recognised as the catalytic component of the γ-secretase complex (Güner and Lichtenthaler, 2020; Wolfe et al., 1999). It is a polytopic membrane protein which undergoes endoproteolysis inside its transmembrane domains, resulting in the creation of N- terminal fragments (NTF) and C-terminal fragments (CTF) that continue to be linked together as a heterodimer. The development and activation of the γ-secretase complex, which enable it to cleave a variety of transmembrane proteins, including the amyloid precursor protein (APP) and Notch, depend on the endoproteolysis of presenilin. It has been suggested that additional members of the γ-secretase complex, namely NCT, APH-1, and PEN-2, are involved in the exact process of endoproteolysis of presenilin (Strooper, 2003).

Mammalian PS1 is produced as a 50 kDa polypeptides, and it is expected that they will pass through the membrane 6–10 times. The amino and carboxyl termini are pointed in the direction of the cytoplasm (Tandon and Fraser, 2002). A novel 467 amino acid protein with seven possible transmembrane domains is encoded by PS1. The most recent finding, however, demonstrated that each presenilin has nine helical transmembrane domains and two residues that function as catalytic parts. For each, they are present on transmembrane 6 and transmembrane 7 (Nadendla and Mohiuddin, 2020). The existence of ten hydrophobic regions (HR) in the amino acid sequence of PS that can act as TM domains has led to the development of many models for PS with 6 to 9 TM segments (Vetrivel et al., 2006).

As it is involved in the breakdown of the amyloid precursor protein (APP) into smaller pieces including Aβ, Presenilin 1 (PS1) plays a vital role in the amyloid hypothesis (Laudon et al., 2005). Aβ42 are produced after γ-secretase cleaves the C99-fragment (Zhang et al., 2017).

The notion that PS1 is the catalytic centre of γ-secretase is strongly supported by data from two research avenues. Genetic studies provide the basis of the initial line of the research. S182 gene was involved in PS1 mutations at the time of its discovery (Sherrington et al., 1995), it has been discovered in some uncommon, hereditary types of Alzheimer's disease (AD) that advance more quickly and with an earlier onset than the more prevalent sporadic types of the illness. Due to mutations which is close to PS1's transmembrane domains, these alterations raise the possibility that they may affect the protein's structure or functionality. Studies on these mutations have revealed that they enhance the generation of Aβ, indicating that PS1 is involved in the processing of APP and the generation of Aβ (Herreman et al., 2003).

Additionally, research has demonstrated that two highly conserved aspartate residues in TM6 (D257) and TM7 (D385) are crucial for the catalytic activity of γ-secretase (Vetrivel et al., 2006). Mutations in PS1 affect these aspartate residues and causes AD and inactivation of γ-secretase (Knappenberger et al., 2004). The pathogenic PS1 proteolysis and γ-secretase activity is induced by mutations in PS which later affecting D257 or D385. Furthermore, it has been demonstrated that D345 (PS1) which is situated in the loop bridging TM6 and TM7, essential for γ-secretase activity. D345 was discovered to be close to a protease cleavage site and to play a crucial role in the interaction between PS1 and its binding partners (Brunkan et al., 2005). It is believed to have a role in the coordination of a water molecule necessary for the cleavage of the protein's substrate (Brunkan et al., 2005).

Biochemical studies provide the basis of the second research avenue. To determine the enzymes involved for cleaving APP and producing Aβ42, researchers employed cell-based tests and biochemical methods. Esler and colleagues showed in the year 2000 that an affinity reagent particularly created (as a transition state

analogue) to interact with the active site of γ-secretase binds to presenilin in a direct manner (Esler et al., 2000). Similar findings were made in research by Li et al., (2000) who revealed that powerful transition state analogue inhibitors that serve to target the active site of an aspartyl protease photoaffinity labelled PS1 in their findings. These inhibitors of γ-secretase transition state bind specifically to PS1 NTF/CTF heterodimer (Li et al., 2000). These findings demonstrate that presenilin is the γ-secretase active site.

The N-terminal region of PS1 (TM1, HL1, and TM2) plays a significant role in the catalysis of substrates by the γ-secretase complex. The interaction between TM2 and TM3 may have an impact on the active site's conformation. The number of the active site accessible may depend on the interaction between γ-secretase (either "semi-open" or "completely open") (Bagaria et al., 2022). Short amyloid production may benefit from the enzyme's "semi-open" conformation. In contrast, the alterations could cause the enzyme to adopt an "completely-open" conformation and produce normal amyloid peptides over a longer period of time (Somavarapu and Kepp, 2017). For the creation of longer amyloid peptides, the enzyme's "open" shape would be preferable. It is possible that TM5 and TM6 are in charge of both endoproteolysis and the maturation of the PS1 protein through their function as "gate-plug mechanism" controllers (Duncan et al., 2018). The endoproteolysis of PS1 and the impact of γ-secretase activation are both increased as a result of the direct interaction between the TM4 domain of PS1 and PEN2, according to research. The large loop between TM6 and TM7 and the N-terminal portion of PS1 might not be necessary for the cleavage of amyloid. In order for hydrolysis to take place within the lipid bilayer, it was revealed that there is a cavity between TM6 and TM7 that is filled with water. The two catalytic

aspartates may be present in each of the TM6 and TM7 domains (De Strooper et al., 1998; M. S. Wolfe, Xia, Ostaszewski, et al., 1999).

2.1.2(d)(i) Structure of Presenilin

Presenilin 1 (PS1) is a type 1 integral membrane protein which comprises of nine transmembrane domains (TMDs) and a key hydrophilic loop region (HL) between TMDs 6 and 7 (**Fig. 2.5 (b)**). The length of the TMDs varies significantly, with TM9 being the longest at 30 residues and TM7 being the smallest at only 18 residues. The location of autocatalytic cleavage is located in the expansive HL region between TMD6 and TMD7, which contains hydrophilic and mostly disordered sequences. Within the TMDs of PS1, the two catalytic residues, D257 and D385, are located in different places. While D385 is positioned on the cytoplasmic side of TMD7, D257 is positioned in the centre of TMD6 and leans slightly to the extracellular side (**Fig 2.5 (b)).** There is a sizable spatial gap between D257 and D385, as seen by the 10.6 Å separation (**Fig. 2.5 (c)**) between D257 and D385. The catalytic residues are closer together in certain active aspartate proteases, such as pepsin, than they are in this separation, which is significantly greater (Cooper et al., 1990). However, these catalytic residues between TM6 and TM7 are situated close to the PAL (Proline-Alanine-Leucine) signature motif, which is assumed to be involved in substrate recognition (Bai, et al., 2015) (**Fig. 2.5 (c)**).