CHAPTER VI
DISCUSSION

6.1 Phytochemicals of sweet peppers

6.1.1 Flavonoids and phenolic acids

Freeze-dried samples of four colored sweet peppers were extracted by 50% (v/v) aqueous methanol. To the extractant, HCl was added to hydrolyze flavonoid glycosides for removing glycosidic moieties to analyze their aglycones (Figure 6.1). tBHQ and ascorbic acid were used as antioxidants (stabilizer) for preventing oxidation of the phenolic compounds. Finally, flavonoids and phenolic acids of the extracts were determined using HPLC analysis.

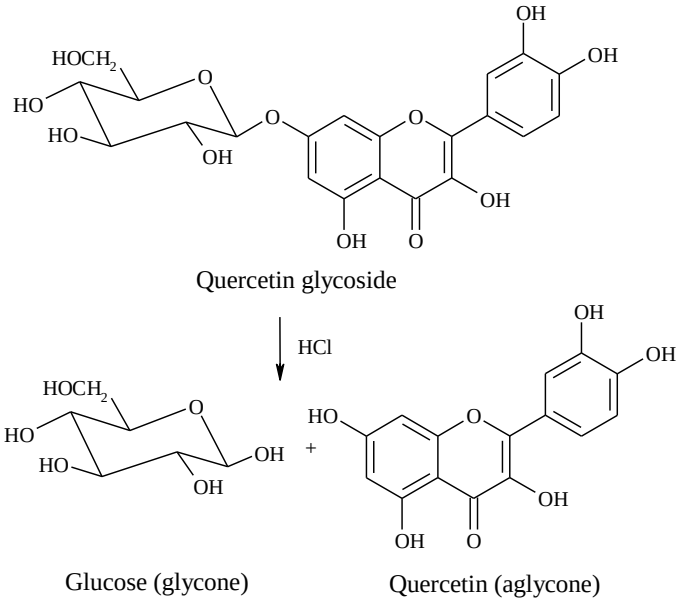


Figure 6.1 Example of hydrolysis reaction of flavonoid glycosides. Phenolic compound naturally is in form of glycoside that includes the part of glycone (sugar) and aglycone (flavonoid or phenolic acid). For detecting individual phenolic compound, HCl is used to hydrolyze it, which the example is showed in glycoside of quercetin.

As results, all four sweet peppers showed two types of flavonoids, including flavonol (quercetin) and flavone (luteolin). These compounds were also found in the previous study of four colored sweet peppers (green, red, orange and yellow) extracted with methanol [2]. However, the quantity of the flavonoids was different, in which red sweet pepper provided the highest quantity of the flavonoids, followed by yellow sweet pepper [2]. The opposite result was, however, observed in this study, in which the highest amounts of flavonoids were presented in yellow sweet pepper, followed by red sweet pepper. Besides, red sweet pepper in this study showed higher quercetin level (9-fold higher) than that of the report in 70% (v/v) aqueous methanol (91.98 μg/g dry weight in this study and 9.97 μg/g dry weight in previous study) [37]. As well, myricetin was also possessed in previous four sweet peppers [37], while it was undetected in this study. The difference in quantity might be from solvent extraction. For example, red sweet pepper extracted with 50% (v/v) aqueous methanol exhibited quercetin 9-fold higher than the previous study that use 70% (v/v) aqueous methanol. It might be because quercetin likely dissolved in higher polar solvent. In contrast, the undetected myricetin in this study might be likely dissolved in lower polar solvent. Nevertheless, other factors might also influence the quantity and quality of the compound.

According to phenolic acid results, *p*-coumaric acid and ferulic acid were found in all four colored sweet peppers. The trends of *p*-coumaric acid and ferulic acid contents between green and red sweet peppers were similar to those reported in *Capsicum annuum* cv. Padrón [85]. The content of *p*-coumaric acid was previously reported to decrease in red pepper, while the level of ferulic acid was on the opposite trend such that it could be found only in red pepper. In addition, caffeic acid and chlorogenic acid were previously found in four colored sweet peppers [37] but the compounds were not detected in this study.

The difference on type and level of flavonoids and phenolic acids of sweet pepper in each study may depend on the internal factors and environmental factors. The examples of internal factors are cultivar and maturity stage of sweet pepper. In addition, the examples of environmental factors are conditions of growth and analytical technique, such as solvent extraction or HPLC conditions.

6.1.2 Carotenoids

Carotenoids from freeze-dried sweet pepper were saponified by KOH to remove ester and R group and provide individual carotenoids (Figure 6.2). The sample was then extracted by lipophilic solvent (hexane). Hexane was the appropriate solvent for carotenoid extraction according to the report of hot pepper [68] because of the carotenoid structure that has the long chain of hydrocarbon (lipophilic). In addition, ascorbic acid was added as antioxidant for preventing oxidation of carotenoid. Finally, carotenoids were separated and detected using HPLC analysis.

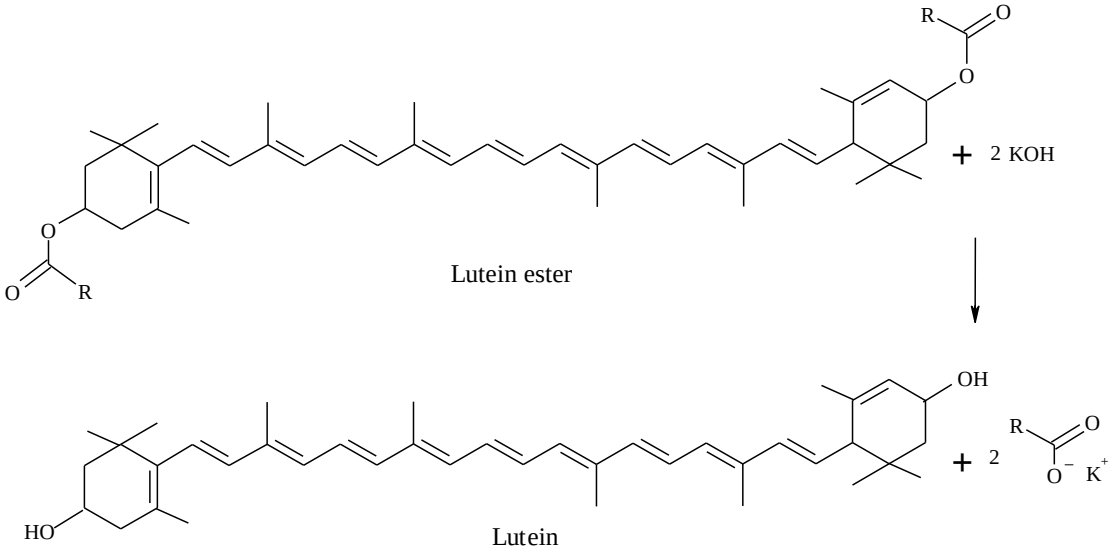


Figure 6.2 Example of saponification reaction of carotenoid ester. The samples were saponified by KOH to remove ester and R group and provide individual carotenoids, which this figure shows the reaction of lutein ester as the example.

The results indicated that sweet peppers provided capsanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, *trans*-β-carotene and *cis*-β-carotene. From previous research, capsanthin was found in three sweet peppers (red, orange and yellow fruits) extracted with methanol but not in green sweet pepper [2]. However, this study showed that it could be found in green sweet pepper but in the lowest quantity. Red sweet pepper in this result possessed the highest capsanthin content and 2-time higher amount than that reported in previous study [2]. The absent capsanthin in green sweet pepper and low amount of capsanthin in red sweet peppers as being reported in the previous study might be due to the methanol for extraction, resulting in

less soluble carotenoids. The highest capsanthin level in red color was agreed with prior studies, which indicated that capsanthin was the main pigment in red color for *Capsicum* genus [1, 36]. In addition, previous research had indicated that the highest concentration of β-carotene was present in red sweet pepper, while the lowest was detected in yellow sweet pepper [2], the results that are consistent to these observations. Yellow sweet peppers exhibited the highest level of lutein (178.20 μg/g dry weight), the compound that was responsible for yellow-orange color [1]. Zeaxanthin in red sweet pepper extracted in this experiment was 20-fold higher than red sweet peppers extracted by chloroform:methanol (1:1 v/v) in the previous research [37]. Higher concentration of zeaxanthin being detected in this experiment might result from different extraction method. The mixture of chloroform:methanol (1:1 v/v) has more polarity index than hexane from this study. The solvent with higher polarity index might not be suitable for zeaxanthin (mainly consisting of hydrophobic chain) extraction. As well, cultivar and growth condition could also affect the quantity and quality of the bioactive compounds. Beside, β-cryptoxanthin in this study was not found in green sweet pepper but presented in the highest level in red sweet pepper, the result that was in good agreement with the previous research [42].

Overall, total carotenoids in this study were significantly found to be the highest in red sweet pepper, followed by orange sweet pepper, yellow sweet pepper and green sweet pepper, respectively. These results were corresponded to the previous researches [6, 37, 40-42]. It was also related to provitamin A contents that were higher in orange and red peppers than in green pepper [1]. As well, the data were supported by the result between total carotenoids in sweet pepper and color a* parameter (using the Hunter Lab System), which were highly correlated (r^2 = 0.97 by linear regressions) [39]. The highly positive value of a* parameter is representative color that close to red color, thus the higher carotenoids in red, orange and yellow sweet peppers can be tracked using this parameter. The lowest carotenoid contents were found in green sweet pepper among all four colored sweet pepper, while chlorophyll-a and -b were exhibited to be the highest in green sweet pepper [37], resulting in green color. These compounds were then decreased in ripe pepper and replaced by other carotenoid pigments such as capsanthin, zeaxanthin and β-cryptoxanthin [36, 37, 42].

6.1.3 Volatile compounds

Volatile compounds of fresh sweet peppers were extracted using HS-SPME method. The samples were incubated to release the volatile compounds in the headspace of vial and the compounds were absorbed by SPME fiber coated with DVB/CAR/PDMS. Then, the compounds were distinguished by GC column and identified by matching the mass spectra to data base in NIST library of MS. Advantages of performing SPME method are reduced time and method for sample preparation, no solvent extraction required, easy to handle and small amount of sample used [86]. In addition, SPME method using DVB/CAR/PDMS fiber was suitable for determination of volatile compounds in equilibrium headspace of pepper [86].

As results, ~20-40 peaks from chromatogram of volatile compounds in all four sweet peppers were identified. However, not all of the peaks were volatile compounds from sweet peppers but contaminants from the instruments such as injector septum, capillary column and SPME fiber [87]. Since SPME fiber was coated with PDMS, the main detected compounds were derivatives of siloxanes such as hexamethylcyclotrisiloxane, octamethylcyclotetrasiloxane, decamethylcyclopenta-siloxane, dodecamethylcyclohexasiloxane and tetradecamethylcycloheptasiloxane [84, 87]. These compounds were presented in all chromatograms of sweet peppers as contaminants. Moreover, methoxy-phenyl-oxime that found in high percent of peak area (4-26%) in all four sweet peppers was reported that it might be from the glue for attaching fiber to the syringe plunger [84]. Thus, fifteen compounds with high peak area (%) were selected as volatile compounds from sweet pepper.

The important volatile compounds of *Capsicum* species were in groups of pyrazine and other alkyl-methoxypyrazines [88]. Especially, 2-isobutyl-3-methoxypyrazine was reported as a main aroma in fresh sweet pepper fruit, generally found in green sweet pepper [89, 90]. This compound was previously reported to be decreased during maturity [91]. The results were corresponded to this study, in which the unripe green sweet pepper contained higher level of 2-isobutyl-3-methoxypyrazine than ripe stage (red, orange and yellow colors).

Copaene and 1-dodecanol were found in four sweet peppers, thus these compounds might be the main volatile compounds in sweet peppers. This result was in agreement with the previous researches, which reported the detection of copaene and

1-dodecanol in fresh sweet pepper or Brazilian chili peppers [92, 93]. Besides, other volatile compounds in sweet peppers and other peppers that also reported in both this study and the literatures were included benzophenone [92], hexadecane [88, 93, 94], heptadecane [93-95], *cis*-β-ocimene [88, 94], 2-isobutyl-3-methoxypyrazine [88] and alloaromadendrene [94]. Similar to the previous reports [86, 93, 94], unripe sweet pepper possessed higher quantity and quality of volatile compounds than those of ripe stage, leading to higher favor in unripe green pepper. In addition, some of these volatile compounds were reported to possess antioxidant activity such as β-selinene and α-copaene [50].

6.2 Anti-Alzheimer's disease of sweet pepper extracts

6.2.1 Total phenolic content and antioxidant activity

To evaluate the efficient solvents for extracting high TPC and antioxidant activity in sweet peppers, three solvents including non-polar hexane, semi-polar ethyl acetate and polar 70% (v/v) aqueous ethanol were investigated. TPC and antioxidant activity of all four colored sweet peppers extracted with 70% (v/v) aqueous ethanol were ~2-8 and ~30-80 folds higher than those with ethyl acetate and hexane, respectively. Thus, ethanol or polar (hydrophilic) solvent was an appropriate solvent for TPC and antioxidant extraction. These results were corresponded to the previous report, which suggested that higher antioxidant activity as being detected by ABTS and DPPH assays were found in the phenolic fractions (70% (v/v) aqueous methanol) than those in the oily fractions (chloroform:methanol (1:1 v/v)) [37]. As well, antioxidant activity of sweet pepper extracted with hydrophilic solvent (phosphate buffer pH 7.8) was ~10-fold higher than those in lipophilic fraction (ethyl acetate) [39].

According to high antioxidant activity as being detected in hydrophilic solvent, these anti-oxidative agents might be flavonoids and phenolic acids that are highly dissolved in polar (hydrophilic) solvents. The previous study suggested that flavonoid and total phenolic contents in peppers were presented in semi-polar and

polar solvents, namely ethyl acetate, acetone, methanol and 80% (v/v) aqueous methanol but were absent in hexane (lipophilic) extract [96]. Besides, the correlation between DPPH results and total phenolics and flavonoids exhibited positive values with r = 0.66 and r = 0.85 (by Pearson's correlation coefficients), respectively [96]. Thus, it is highly possible that these phenolics could function as antioxidants. This hypothesis was confirmed by several previous studies, which suggested that phenolic compounds could act as antioxidants [2, 17, 37, 44, 45]. As well, flavonoid structures mainly consist of hydroxyl groups, 2-3 double bond, and 4-oxo function that can promote the antioxidant activity [37]. Besides, ascorbic acid (water-soluble vitamin) that acts as antioxidant may be another compound that leads to be found antioxidant activity in the sweet peppers. Because the compound has been previously reported to be in high amounts in *Capsicum* species comparing to other fruits and vegetables such as grape, apple, banana, guava, orange, tomato, broccoli, carrots, lettuce and cucumber [62, 97]. Sweet peppers (50-100g fresh weight) contain ascorbic acid 1-2 times higher than the RDA (recommended daily administration) required (60 mg/day), depending on cultivar, maturity and growth conditions [39, 53].

The results from ethanolic and ethyl acetate extractions were similar. Green and red sweet peppers exhibited the highest contents of TPC and antioxidant activity (green sweet pepper mostly higher than red sweet pepper), followed by orange sweet pepper and yellow sweet pepper, respectively. Similar results were observed in sweet pepper extracted with 70% (v/v) aqueous methanol [37]. In addition, the result was confirmed by the contents of phenolic acids, in which *p*-coumaric and ferulic acid were the highest in green sweet pepper and red sweet pepper, respectively. Besides, it was previously reported that green and red sweet peppers contain the highest ascorbic acid content [37, 39, 42]. Therefore, the TPC and antioxidant activity detecting in this experiment might the results of these compounds.

Under hexane extraction, the overall results were different from ethanolic and ethyl acetate extracts. Red and orange sweet peppers were found to exhibit the highest TPC and antioxidant activity, followed by green sweet pepper and yellow sweet pepper, respectively. Similarly, oily fraction of orange sweet peppers extracted by 70% (v/v) aqueous methanol were reported to exhibit the highest ability to quench free radicals in DPPH assay [37]. The highest TPC and antioxidant activity in red and

orange sweet peppers might be influenced from the activity of carotenoids, because the compounds were mostly extracted by non-polar (lipophilic) solvents [96]. As well, the correlation between L-TAA of sweet pepper and total carotenoids exhibited highly positive values by linear regressions (r^2 = 0.81) [39], and carotenoids also exhibited antioxidant activity [1, 39, 46, 60]. Besides, this report is associated with this carotenoids analysis, in which red and orange sweet peppers contained the highest total carotenoids and carotenoid contents, namely capsanthin, zeaxanthin, β-cryptoxanthin and *trans*- and *cis*-β-carotene. Therefore, high TPC and antioxidant activity in red and orange sweet peppers extracted with hexane may come from the presence of these compounds. In addition, *C. annuum* such as sweet peppers is also a good source of vitamin E, which provides high antioxidant activity, among other fruits and vegetables (such as grapes, orange, banana, strawberry, apple, tomato, asparagus, cabbage, broccoli and eggplant) [62]. During ripening stage of peppers, red sweet pepper contained the highest level of α-tocopherol (fat-soluble vitamin E) [37]. Thus, α-tocopherol was possibly another one compound yielding high free radical reduction in red sweet pepper extracted by hexane.

6.2.2 Cholinesterase inhibitory activities

AChE inhibitory activity was measured from kinetic reaction of yellow color (final product) production at a wavelength of 412 nm that produced during the reaction between acetylthiocholine, ATCh, (substrate) and AChE (enzyme). As results, sweet pepper extracted with ethyl acetate showed the highest inhibitory activity (IC_{50} of 6-118 g/L), followed by those extracted with hexane and 70% (v/v) aqueous ethanol, respectively. These results were in agreement with the result of *Capsicum chinense* Jacq. cv Habanero [6]. Red pepper in lipophilic fraction that extracted with hexane provided AChE inhibitory activity (IC_{50} of 0.73 g/L) [6]. However, the result was in contrast to ethanolic extract, in which only trace anti-AChE activity was observed in both green and red pepper (IC_{50}>1.00 g/L) [6]. In addition, higher anti-AChE activity was found in *Salvia* genus and *Iris suaveolens* extracts in low polar solvents (petroleum ether, dichloromethane and ethyl acetate), while it was absent in high polar solvent (methanol extracts) [98, 99]. It indicated that AChE inhibitor from those plants and sweet peppers might be likely dissolved in semi-polar

and non-polar solvents. However, this trend cannot be applied for all plant extracts [100]. The bioactive compounds of sweet peppers that likely dissolved in non-polar solvent, such as carotenoids and α-tocopherol [37], may influence on anti-AD property. It was previously shown that serum carotenoid was related to cognitive performance in healthy elderly participants [18, 101]. The participants with the lowest cognitive scores also had low plasma levels of lycopene and zeaxanthin [18]. Therefore, the reducing levels of serum lycopene, lutein and zeaxanthin in human may cause an increase in AD risk [18]. Besides, the deficiency of vitamin E and carotenoid could lead to impairments in memory and learning performances [6].

The AChE inhibitory activity of the hot and sweet pepper were very low, comparing to physostigmine (a plant alkaloid drug for AChE inhibition [16]) with an IC_{50} of 0.0002 g/L [6]. Low inhibitory activity might be due to the crude extracts of peppers being used to determine activity. These crude extracts normally contained various components that some components were unrelated to cholinesterase activity. Nevertheless, it was indicated that the inhibitions of the peppers were not too high to be unsafe for consumers (no limitation in daily diet).

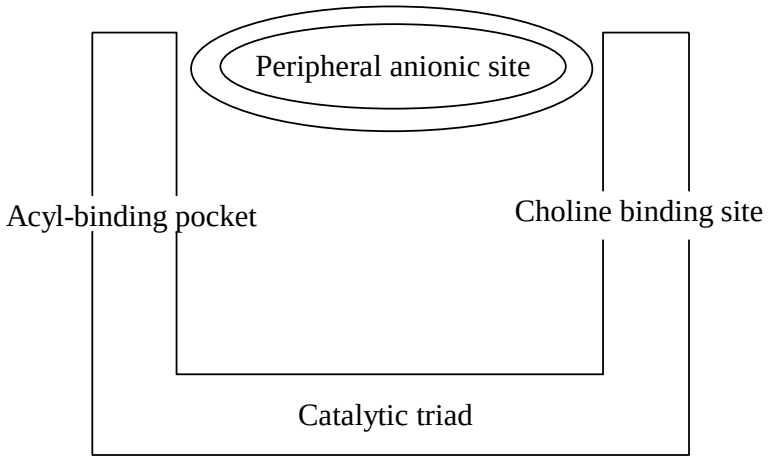
Fruit colors of sweet pepper also influenced on anti-AChE activity. The previous study reported that methanolic extract of premature green pepper (*Capsicum annuum* var. *acuminatum*) showed the highest AChE inhibitory activity (IC_{50} of 0.084 g/L), comparing to mature red pepper (IC_{50} of 0.130 g/L) [5]. These results were corresponded to this study; in which green sweet pepper exhibited twice AChE inhibitory activity of red sweet pepper. In addition, under all three solvent extractions, yellow sweet pepper exhibited the highest anti-AChE activity (IC_{50} of 5-13 g dry weight/L). This result was corresponded with the yellow sweet pepper containing the highest contents of quercetin and luteolin (102.33 and 95.89 µg/g dry weight, respectively) as well as the highest total flavonoids (198.22 µg/g dry weight). Previous research suggested that flavonoids could inhibit AChE [8, 17, 48, 49]. Quercetin and luteolin provided high AChE inhibition (IC_{50} values of 0.0047 and 0.0050 g/L, respectively), comparing to other flavonoids [49]. Moreover, high concentration of ascorbic acid in sweet pepper [1] could also inhibit AChE activity in brains of mice [73].

In addition to AChE inhibitory activity, the results suggested that polar 70% (v/v) aqueous ethanol extracts showed the highest BChE inhibitory activity, followed by semi-polar ethyl acetate extract and non-polar hexane extract, respectively. These results were corresponded to those obtained by *Capsicum chinense* Jacq. cv Habanero extracts [6]. Ethanolic extracts of unripe green and ripe red peppers could inhibit BChE reaction (IC_{50} of 0.562 and 0.806 g/L, respectively), whereas both peppers in lipophilic fractions (hexane extraction) possessed trace anti-BChE activity (IC_{50}>1.0 g/L) [6]. Therefore, it was likely that anti-BChE agents may solubilize in polar solvent rather than in non-polar solvent. As well, these results were corresponded to the TPC and antioxidant activity of the sweet pepper extracts, in which the highest TPC and antioxidant activity were obtained from 70% (v/v) ethanolic extracts, followed by ethyl acetate extract and hexane extract, respectively. Thus, anti-BChE agents in sweet peppers may be the hydrophilic compounds that could as well act as antioxidants such as flavonoids and phenolic acids.

Green sweet pepper extract showed the highest anti-BChE activity in all three solvent extractions (IC_{50} of 62.64 and 85.34 g/L in 70% (v/v) aqueous ethanol extract and ethyl acetate extract, respectively). The similar result was found in the extract of *Capsicum chinense* Jacq. cv Habanero [6], which suggested that green pepper extracted with ethanol possessed higher inhibitory activity than red pepper (IC_{50} of 0.562 and 0.806 g/L, respectively). Nevertheless, both hot peppers and sweet peppers exhibited relatively low anti-BChE activity, comparing to physostigmine with the IC_{50} of 0.0024 g/L [6]. This data was in good agreement with green sweet peppers extracted in 70% (v/v) aqueous ethanol and ethyl acetate that provides the highest TPC and antioxidant activity. It was consistent to the prior study, which *p*-coumaric acid that found the highest content in green sweet pepper could inhibit BChE activity [102]. As well, flavonoids including quercetin and luteolin that found in all found sweet peppers also provided inhibitory activity against BChE [8, 17]. Luteolin possessed ability to inhibit BChE with the IC_{50} of 0.0027 g/L [17]. This compound is a relatively strong anti-BChE agent, comparing to positive control, galantamine, with the IC_{50} of 0.00012 g/L [17]. Under the study of anti-human plasma BChE, K_i of quercetin and luteolin were 0.021 and 0.048 g/L, respectively [8]. Thus, the highest inhibitory

activity in green sweet pepper might be from the inhibitory ability of these compounds.

Binding sites in cholinesterase (AChE and BChE) that is important for accommodating and hydrolyzing ACh consist of peripheral anionic site, choline binding site, acyl-binding pocket and catalytic triad (Figure 6.3 and Table 6.1) [103, 104]. Catalytic triad at the bottom of the enzyme is the active site for breaking down ACh. It comprises of two structural binding sites including an anionic site and an esteratic site [105] (Figure 6.4). The anionic site containing Glu residue binds ACh at the positive quaternary amine of choline moiety [104]. The esteratic site containing Ser and His residues binds to the acyl group of ACh and breaks down to product acetic acid and choline [104, 106]. The hydrolysis of AChE and BChE may be similar due to enzyme structural similarity. However, acyl-binding pocket, peripheral anionic site and other residues of two enzymes were different (Table 6.1). The kinetics and substrate selectivity might also be different, leading to better binding of bulkier substrates (BTCh) and inhibitors to BChE [103].



Binding sites in cholinesterase

Figure 6.3 Binding sites in cholinesterase. The binding sites consist of peripheral anionic site, choline binding site, acyl-binding pocket and catalytic triad [103, 104]. They are the important sites for substrate hydrolysis or enzyme inhibition.

Table 6.1 Residue compositions of binding sites in AChE and BChE [103]

Binding sites	Residue compositions	
	AChE	**BChE**
Catalytic triad	His447, Ser203 and Glu201	His438, Ser198 and Glu325
Choline binding site	Trp86 and Phe338	Trp82 and Phe329
Acyl-binding pocket	Phe295 and Phe297	Leu286 and Val288
Peripheral anionic site	Tyr72, Asp74, Tyr124, Trp286, Tyr337 and Tyr341	Trp231, Val288, Leu286 and Phe398

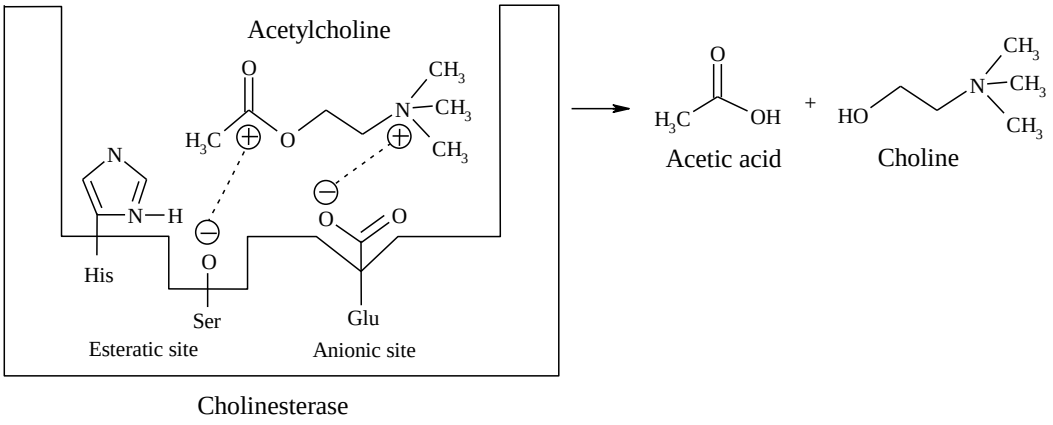
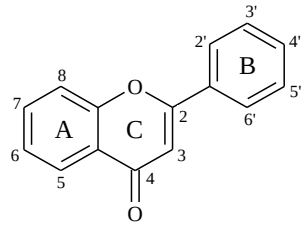


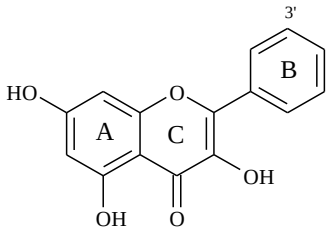
Figure 6.4 Mechanism of ACh hydrolysis by cholinesterase. ACh is hydrolyzed at catalytic triad of cholinesterase, in which the catalytic triad contains an anionic site and an esteratic site [105].

It was possible to hypothesize that cholinesterase inhibitory activity of the flavonoids may depend on their structures and interactions. Interestingly, strong anti-AChE agents (luteolin, quercetin and myricetin) possess hydroxyl groups on position 3' in ring B (Figure 6.5), whereas other flavonoids such as apigenin and kaempferol contain none [8, 49]. In addition to AChE inhibitory activity, it was likely that the decrease in BChE inhibition was related to the increased hydroxyl groups on the phenyl ring B [8]. For example, flavonoid that acted as the most efficient inhibitor, galangin, did not show any hydroxyl group on B ring. On the other hand, other flavonoids that presented the hydroxyl group (s) in B ring exhibited lower activity. Myricetin, quercetin and kaempferol, for examples, with three, two and one hydroxyl

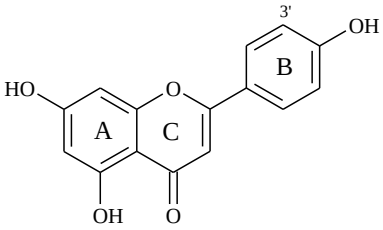
group (s) on B ring, respectively (Figure 6.5), inhibited lower BChE activities, respectively [8].



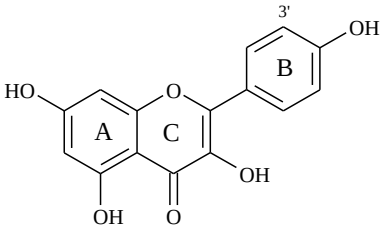
Flavonoid basic structure



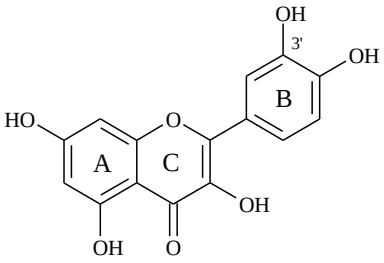
Galangin



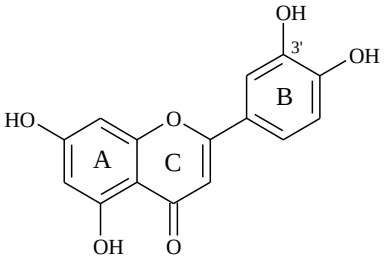
Apigenin



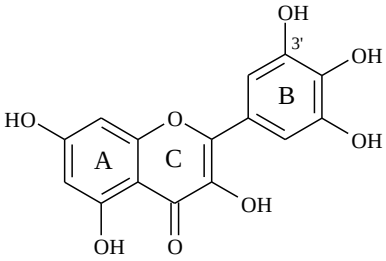
Kaempferol



Quercetin



Luteolin



Myricetin

Figure 6.5 Chemical structures of flavonoids. Galangin, apigenin, kaempferol, quercetin, luteolin and myricetin which some of all possessed AChE, BChE and BACE1 inhibitory activities. The inhibitory activities against AChE, BChE and BACE1 may be explained by the hydroxyl groups on ring B of these flavonoids [8, 9, 23, 49].

From molecular docking study, flavonoids may inhibit AChE by crossing or binding to peripheral anionic site. Peripheral anionic site is the binding site for modulating small molecules including substrates or inhibitors that enter into catalytic gorge (Figure 6.3) [49]. Therefore, flavonoids may enter and bind the catalytic triad of enzymes or block the entrance of the substrate, leading to inhibition of AChE catalytic activity [49]. For example, quercetin exhibits several hydrogen bonds with the active site residues of AChE, such as Tyr133, Trp86, Tyr72, Gln71 and Asp74 residues, as well as hydrophobic interactions with Ser125, His447 and Glu202 residues [107]. The O4 of hydroxyl moiety in quercetin could interact with choline binding site of AChE by hydrogen bonding at O atom of Trp86 residue [107]. Quercetin could also bind with peripheral anionic site by hydrogen bonding, in which the interactions were between O atoms of its phenyl side chain and O atoms of Tyr72 residue as well as its O7 and N atom of Asp74. In addition, quercetin could interact with catalytic triad (Ser125, His447 and Glu202 residues) by hydrophobic interactions [107]. In addition, the hydroxyl groups of quercetin showed predicted binding with amino acid residues of BChE, such as Asp70, Glu197, Gly115 and Tyr128 by hydrogen bonding, while the aromatic rings interacted with Trp82, Phe329 and Tyr332 by π-π interactions [8]. The interactions might prevent substrate hydrolysis by enzyme (AChE or BChE). Therefore, anti-cholinesterase activity of the sweet pepper extracts might be from these interactions.

6.2.3 β-secretase inhibitory activity

As results, the ability to inhibit BACE1 of sweet pepper extracts with all three solvents was in a range of 40-90% inhibition at 30.56 g dry weight/L. The results of BACE1 inhibitory activity were similar to anti-AChE reaction, in which hexane and ethyl acetate extracts exhibited higher activity than ethanolic extract. These results were in disagreement with the previous study of chili pepper (*C. annuum*), which indicated that methanolic extracts (0.25 g/L) could inhibit BACE1 activity (~55% inhibition) with a higher rate than hexane extract and ethyl acetate extract, respectively (~25 and ~5 % inhibition, respectively) [108].

Among all colored sweet peppers, green sweet pepper exhibited the highest BACE1 inhibitory activity, the similar results in BChE inhibitory activity. The

BACE1 inhibitors from sweet peppers may be flavonoids and phenolic acids with AChE and BChE inhibitory activities. This result was corresponded with the highest content of *p*-coumaric acid in green sweet pepper, and it exhibited BACE1 inhibitory activity (IC_{50} of 0.00147 g/L). In addition, quercetin and luteolin that found in all four sweet peppers were previously reported to possess anti-BACE1 with the IC_{50} of 0.00096 and 0.00056 g/L, respectively [17]. Quercetin possessed relatively high cell-free BACE1 inhibitory activity (IC_{50} of 0.00085 g/L), comparing to other flavonoids such as kaempferol and apigenin [9]. Its concentration at 0.0060 g/L could also reduce neural BACE1 activity in the primary neuronal culture (cell-based assays) [9]. Besides, quercetin also inhibited the formation and extension of β-amyloid fibrils [48] and exhibited the highest reduction of β-amyloid levels by ELISA analysis [9]. Thus, the anti-BACE1 activity of sweet peppers may be from the properties of these compounds.

BACE1 is a type of transmembrane aspartic protease, which its catalytic residues are included Asp32 and Asp228. Amyloid precursor protein (APP) is hydrolyzed by Asp32 and Asp228 residues at catalytic triad (Figure 6.6). Asp32 and Asp228 residues form a tightened enzyme-substrate complex, and then the peptide bond of APP is broken and form β-amyloid [109, 110]. The inhibitory activity against BACE1 of flavonoids may be resulted from the interactions between flavonoids and BACE1 binding residues through hydrogen bonding [9, 23]. The previous reports showed that the flavonoids with more hydroxyl group on B ring exhibited higher anti-BACE1 activity, for examples, kaempferol, quercetin and myricetin with one, two and three hydroxyl group (s), respectively [9, 23] (Figure 6.5). From previous molecular docking studies, C3-OH on C ring of quercetin formed a single hydrogen bond with Asp32 (the BACE1 catalytic residue) (Figure 6.7) [9]. This interaction is more important for BACE1 inhibition than bonding with other residues, since it could block substrate from interacting with the enzyme [23]. In addition, hydroxyl groups on A ring and B ring of quercetin also interacted with Gln73 and Trp198 residues of BACE1, respectively, by hydrogen bonding (Figure 6.7) [9]. These interactions are important for stabilization of the docking poses between flavonoids and BACE1 [23]. The higher inhibitory activity of quercetin as being compared with that of kaempferol may also be from interaction between hydroxyl group on A ring of quercetin with

Gln73, while kaempferol exhibited no such interaction [9]. Therefore, anti-BACE1 activity of the sweet pepper extracts might be explained from these interactions.

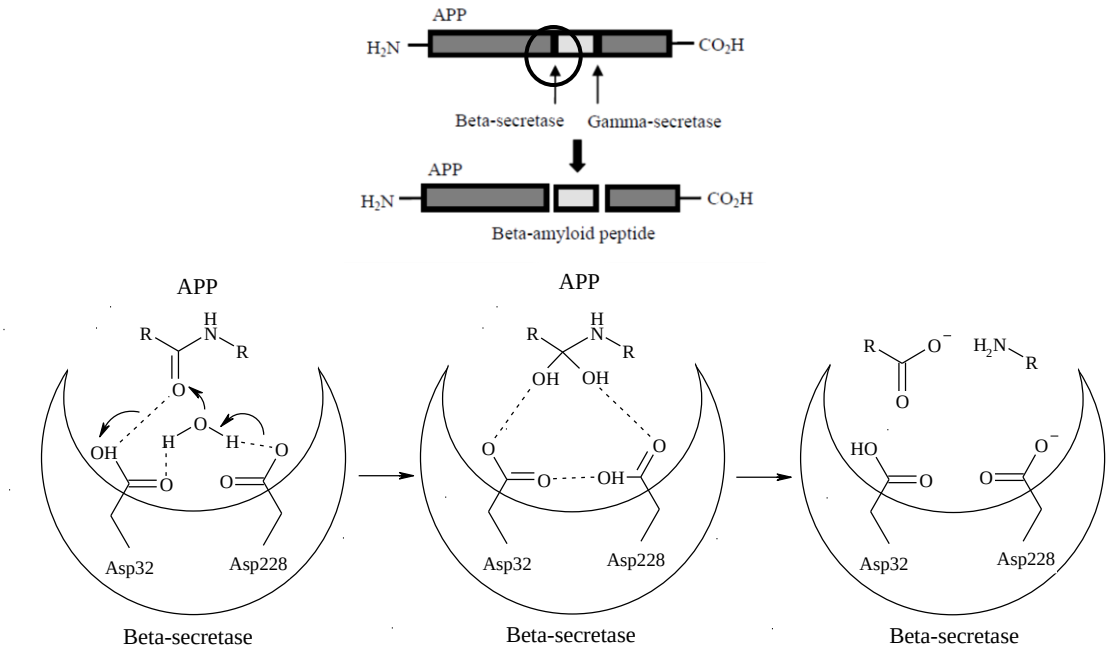
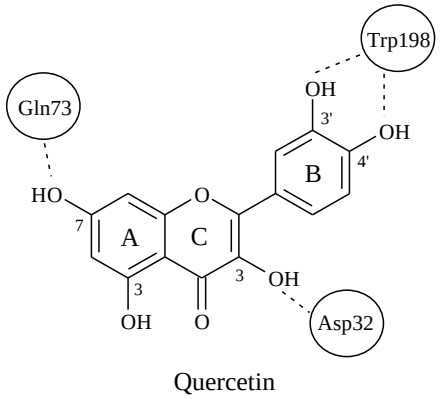


Figure 6.6 Mechanism of APP hydrolysis by BACE1. The catalytic residues of BACE1 consist of Asp32 and Asp228. Two residues cleavage amyloid precursor protein (APP) to form β-amyloid peptide [109, 110].



Quercetin

Figure 6.7 Interaction between quercetin and active site residues of BACE1. Hydroxyl group on C ring of quercetin interacted with Asp32 residue of BACE1, and hydroxyl group on A ring and B ring also interacted with Gln73 and Trp198 residues, respectively [9]. Hydrogen bonds are presented as dots.

However, the potential of AD inhibitor in human brain depends on ability to pass blood brain barrier (BBB) that is a layer between the CNS and blood circulation for allowing the pass of small molecules (solutes) into the CNS [111]. The compounds that are suitable for crossing blood-brain barrier should contain high lipophilicity, such as alkylated flavones [23]. According to general enzyme inhibitors for therapeutic treatment, drug preferably exhibits a molecular weight less than 600 Da. Flavonoids may be suitable for drug candidates due to their small molecular weights (<400 Da), leading to high viability than the general inhibitors that are therapeutic peptide-based drugs [9, 23]. In addition, both AChE and BACE1 inhibitors from sweet pepper extracts are likely dissolved in low polar solvents, which may be the potential inhibitors to cross BBB and present high viability. However, the further studies are required for more information such as cell culture study or animal study.