CHAPTER VI
DISCUSSION

6.1 Bioactive compounds

Phenolic acid and flavonoids are one of the largest groups of phenolics, which provide important pigments and flavors in fruits and vegetables. These compounds are effective antioxidants that can promote health benefits [117]. Flavonoids can reduce the risk of various diseases such as cancers, cardiovascular disease, obesity, diabetes and hypertension [73, 101, 118-120]. Amount of flavonoids and phenolic acids in each plant are varied.

Identification of phenolic and flavonoid contents in legumes was analyzed by HPLC using a set of standards. In this study, the only flavonoid, quercetin (Figure 6.1A), in seed coat of black bean was detected. Previous study also suggested that quercetin (1.8 ± 0.1 µM) in seed coat of black bean was found in addition to kaempferol (1.5 ± 0.1 µM), myricetin (7.4 ± 0.4 µM), and catechin (10.2 ± 0.9 µM) [121]. Phenolic acid in seed coat of black bean were found as gallic acid (3.848 ± 0.21 mg/100g), protocatechuic acid (4.136 ± 0.09 mg/100g), gentisic acid (88.20 ± 1.28 mg/100g), syringic acid (1.287 ± 0.172 mg/100g), caffeic acid (1.340 ± 0.097 mg/100g) and ferulic acid (466.2 ± 11.79 mg/100g) [83]. Although, seed coat of black bean were reported to contain anthocyanins (malvidin 3–gluocside, petunidin 3–glucoside, delphinidin 3–glucoside, and delphinidin 3,5–diglucoside), but these compounds were not reported in this study [122]. In this research, seed coat of red kidney bean was reported to contain quercetin and kaempferol (Figure 6.1A–B). Similarly, previous study found many bioactive compounds such as quercetin, kamperol, ferulic acid, flavonoid glycoside, proanthocyanidin (tannin) and anthocyanins [123]. Seed coat of mung bean only contained apigenin (Figure 6.1C) and two unknown compounds. Previous study presented two main flavonoids, vitexin (15.22 mg/g) and isovitexin (11.42 mg/g), in seed coat of mung bean [124]. It is possible that two unknown compounds detected in this study may be vitexin and

isovitexin. Seed coat of peanut was found to contain *p*–coumaric acid (Figure 6.1D). Previous research found that main bioactive compound of peanut were *p*–courmaric acid and *p*–courmaric derivatives [125]. Bioactive compounds in seed coat of white kidney bean and soybean were not found in this study. This result was corresponded to the previous study, in which no bioactive compound was detected in seed coat of white kidney bean [121]. While bioactive compound was not detected in seed coat of soybean, it is possible that these bioactive compounds might mainly locate in cotyledon and not in seed coat [126]. Nevertheless, quantity of bioactive compounds is depended on genotype, postharvest storage, maturity level, climate and geographical location [127]. Thus, bioactive compounds in legumes might be employed to control obesity, diabetes, hypertension and alzheimer through enzyme inhibition.

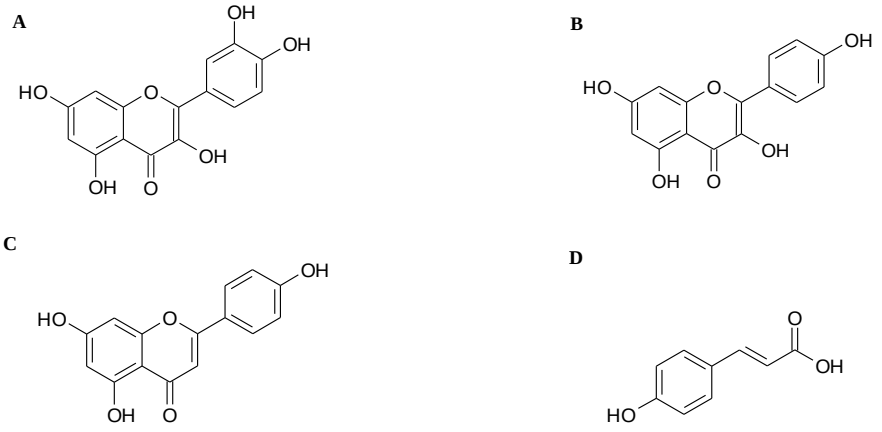


Figure 6.1 The chemical structures of (A) quercetin, (B) kaemferol, (C) apigenin, and (D) *p*–courmaric. A–C are classified as flavonoids and D is phenolic acid.

6.2 Antioxidant capacity

Black bean was found to exhibit the highest antioxidant activity. It is possible that black bean contains high quantity of phytochemicals such as anthocyanins, saponin, tannin and other flavonoids with antioxidant activities. Anthocyanin, a major color pigment in black bean, is belonged to flavonoids that can act as a powerful antioxidant. Previous researches suggests that black bean contains 3 majors anthocyanins, consisting of delphinidin 3–glucoside, petunidin 3–glucoside,

and malvidin 3–glucoside [1]. Delphinidin 3–glucoside was reported to be antagonist of the mutagenic activity of a final metabolite of benzyl–α–pyrene, a strong polycyclic aromatic hydrocarbon oxidant *in vitro* [118]. In addition, anthocyanins may be responsible for protection against DNA damage that causes by highly reactive free radicals. Cyanidin is a stronger antioxidant than cyanidin 3–0–β–D–glucoside and exhibits the same antioxidant activity as vitamin E [19, 24, 30]. As well, condensed tannins isolated from black beans (0.24–24 μM) were found to be ineffective toward the growth of normal cells but were capable of inducing cancer cell apoptosis (Caco–2 colon, MCF–7 and Hs578T breast, and DU prostatic cancer cells that induced by oxidative stress environments) [128]. All these information suggests that high quantity of anthocyanins, tannins and other flavoniods in black bean possibly contributed to high level of antioxidant activity and TPC as being observed in this study.

Interestingly, heat treatment was found to be a significant factor that increases antioxidant capacities and TPCs of legumes. The antioxidant activities and TPCs of cooked legumes were higher than those of the raw ones. It is possible that heat treatment could promote plant cell wall disruption, leading to an increased release of bioactive compounds from legumes. In addition, heating process could breakdown glycoside moiety of flavonoids and produce aglycone, the compound that exhibited higher antioxidant capacity than flavonoids [129]. These results were also corresponded to a previous research on Beniseed (*Sesamum indicum L.*), in which the antioxidant activity and quantity of bioactive compounds, especially total flavonoid contents, were increased after boiling Beniseeds for 30 minutes [130]. Besides, it was previously shown that heat treatment could increase TPCs in seed coats of legumes [131]. It was found that darker colored seed coats of cooked legumes (such as black bean and red kidney bean) possessed higher TPCs than lighter colored seed coats of cooked legumes (such as white kidney and soybean). Since antioxidant activities were found to be correlated with TPCs, higher TPCs in darker colored seed coats of cooked legumes could lead to higher antioxidant activities than lighter colored seed coat of cooked legumes. Which results were corresponded to this research. Other factors that increase antioxidant activities was cooking process, which might activate a polymerization of polyphenols, creating a formation of new compounds [132]. For example, all cooking process of four cultivars of purple skin eggplants by boiling,

steaming and microwave showed higher TPCs and antioxidant activities than raw plant [133]. On the other hand, some previous studies showed decreased antioxidant activities and TPCs in pea, squash, leek after boiling [134]. It was found that red kidney bean (*Phaseolus vulgaris*) exhibited decreased TPCs, comparing to its raw form [135]. Thus, the antioxidant activity in cooked legumes can be described as synergistic combinations or interruption of biochemical reactions, loss of water–soluble antioxidant compositions, and formation or breakdown of bioactive compounds (such as phenolics).

Color of seed coat could affect antioxidant activity and phenolic content. The results suggest that seed coats of black bean and red kidney bean possess the highest antioxidants. Dark seed coat showed higher phenolic content than light seed coat. Previous study reported that seed coats showed higher antioxidant activities, phenolics and flavonoids than those in cotyledons [136]. Phenolics of black bean are mainly found in seed coat rather than cotyledon [137] such as ferulic acid (466.2 ± 11.79 mg/100g), gentisic acid (88.20 ± 1.28 mg/100g) and gallic acid (3.848 ± 0.21 mg/100g) [83]. In addition, seed coat of black bean was found to contain flavonol glycoside, condensed tannin (procyanidins), and anthocyanidins [137]. Moreover, other than 3 major anthocyanins (delphinidin 3–glucoside, petunidin 3–glucoside and malvidin 3–glucoside) found in whole seed of black bean, its seed coat also contains delphinidin 3,5–diglucoside [122]. Similarly, Peruvian bean cultivars with black seed coat were reported to contain mostly flavonoid glycosides such as anthocyanins, quercetin, and kaempferol derivatives [138]. While seed coat of red kidney bean presented flavonoid glycosides (3',4',5,7–tetrahydroxyflavonol 3–*O*–*β*–D–glucopyranosyl (2→1) *O*–*β*–D–xylopyranoside, quercetin 3–*O*–*β*–D–glucopyranoside and kaempferol 3–*O*–*β*–D–glucopyranoside) and proanthocyanidins (tannin) [139]. Other studies reported that anthocyanin was mainly found in seed coat of Korean cultivar kidney bean with purple and red colors [140]. No anthocyanin in brown and white seed coat of legumes were reported [140]. Various phenolics in seed coat of legume are suggested to prevent oxidative damage in the body [141]. However, quantity of TPCs and antioxidant activities are varied, depending on agronomic practices, genotype, postharvest storage, maturity level, climate and geographical location [127].

6.3 Lipase inhibitory activity

In this research, it was found that legumes were not only the rich sources of antioxidants, but also anti–lipase agents. Raw legumes exhibited anti–lipase activities, especially red kidney bean with the highest lipase inhibitory activity. It was possible that high lipase inhibitory activity of red kidney bean might be due to high quantity of its bioactive compounds such as phenolic acid and flavonoids that can act as anti–lipase agents [137, 142]. Previous studies reported that extracts from plants rich in phenolics could yield pancreatic lipase inhibitory effect, for examples, tea extract, grape extract and leaf extract from pomegranate, sage, rosemary and ginkgo. Phenolics in red kidney bean were mostly found as ferulic acid, *p*–coumaric acid and sinapic acid [143]. It was previously demonstrated that ferulic acid (IC_{50} value of 0.024 mg/mL), *p*–coumaric acid (IC_{50} value of 0.21 mg/mL) [144] sinapic acid (10% inhibition with concentration 10µM)[145] could inhibit lipase reactions *in vitro* [58]. Red kidney bean also contains high content of flavonoids, especially diglycosides of quercetin and kaempferol, and anthocyanins [123]. It was previously reported that quercetin (IC_{50} value of 3.8 µg/mL) and kaempferol (IC_{50} value of 7.2 µg/mL) could strongly inhibit lipase activities [58]. Similarly, anthocyanin in black chokeberry, cyanidin–3–glucoside, exhibited anti–lipase activity with the IC_{50} value of 1.17 mg/mL [59]. The flavonoids isolated from *Nelumbo nucifera* leaves showed anti–lipase activities with the IC_{50} value of 0.38 mg/mL [146]. These compounds could, as well, lower total cholesterol and triglyceride *in vivo* [146]. In addition, other studies had suggested that proanthocyanidins (tannin) in strawberry might be able to inhibit lipase reaction [147]. This compound was also reported in red kidney bean [139].

Interestingly, cooked legumes exhibited higher lipase inhibitory activities than raw legumes, especially black bean. The increase in anti–lipase activities could be due to the thermal process that caused elevated TPCs and antioxidants by releasing bound phenolics from food matrix, breaking down cellular constituents and cell walls, disturbing polymerization and aglycosylation, and/or promoting phenolics oxidation [127]. Moreover, it was found that the phenolics of another endogenous in the grain were possibly produced as by–products of thermal process, resulting in increased antioxidant activities [148]. Therefore, anti–lipase activities might be increased after thermal process.

Interestingly, seed coat of red kidney bean showed the highest anti–lipase activity. It was previously reported that the phenolics and flavonoids (quercetin, kaemperol, ferulic acid, flavonoid glycoside and proanthocyanidin) were mainly located in seed coat rather than in cotyledon [136]. For example, pea seed coat contained vanilic acid and (–)–epigallocatechin in the concentrations of 11.95 and 78.82 µg/g dry matter, respectively [149]. Its cotyledon contained 3.18 µg/g dry matter of vanilic acid, while (–)–epigallocatechin was undetectable [149].

Nevertheless, legume extracts had lower effect toward anti–lipase activities than the commercial drug, orlistat, which exhibited the IC_{50} values of 0.016 mg/mL [58]. Orlistat, the competitive inhibitor of lipase, can form covalently bond with catalytic Ser residue in the active site of the enzyme (Figure 6.2 and 6.3). This interaction replaces the interaction between lipase and its natural substrate, TGs. Thus, the enzyme's catalytic pocket is unavailable, leading to a letdown in lipid digestion and absorption [150]. Quercetin and ferulic acid, non–competitive inhibitors [151, 152], can interact with lipase to form enzyme–substrate–inhibitor (ESI) complex, leading to a decrease in lipase activity. These bioactive compounds function differently from orlistat. The competitive inhibitor (i.e. orlistat) normally possesses similar chemical structure as the substrate (a substrate analogue), which binds to the enzyme at its active site. It forms interaction(s) with free enzymes, and enzyme–inhibitor complex could slow down enzyme kinetics. Non–competitive inhibitor, on the other hand, interacts with enzyme–substrate complex at non–active site to form enzyme–substrate–inhibitor (ESI) complex. This type of inhibitor can also bind to free enzyme and free substrate. All types of interactions could lead to suppression of product production. Nevertheless, absorption and bioavailability of bioactive compounds were required to be further investigated.

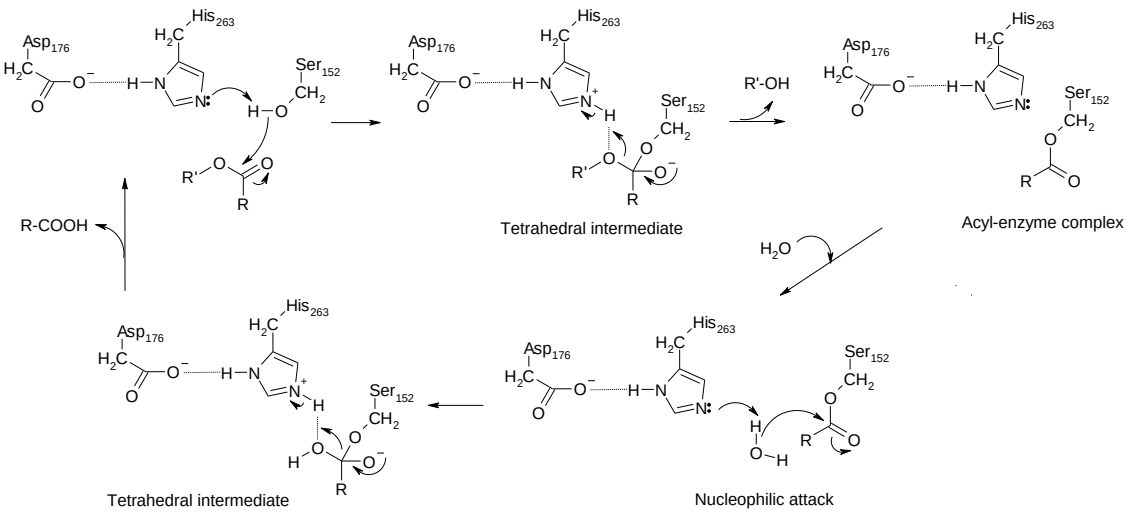


Figure 6.2 The reaction mechanism of human pancreatic lipase using the catalytic triad Ser^{152}, Asp^{176} and His^{263} in the active site of the enzyme.

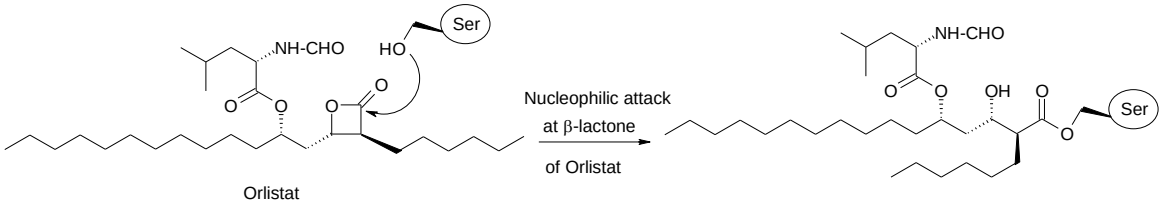


Figure 6.3 The scheme showing the reaction of orlistat that inhibits activity of pancreatic lipase by forming covalent interaction with one of active residues (Ser from catalytic triad of Ser^{152}, His^{263} and Asp^{175}) in the active site of the enzyme, thus preventing the interaction with the enzyme's actual substrate i.e., triglyceride.

6.4 α–Amylase and α–glucosidase inhibitory activities

6.4.1 α–Amylase inhibitory activity

Legumes with abundant micronutrients such as anthocyanins, lecithin, and trypsin inhibitors have been suggested to have protective and therapeutic effects on

many diseases. In this study, raw legumes exhibited higher anti–α–amylase activities than processed legumes. Raw mung bean showed the highest α–amylase inhibitory activity. It was previously reported that mung bean contains four main phenolic acids, including salicylic acid, *p*–coumaric acid,3–hydroxycinnamic acid and ferulic acid [9]. Ferulic acid and *p*–coumaric acid from green coffee bean exhibited anti–α amylase activity with IC_{50} value of 5.45 and 4.86 mM, respectively [153]. Salicylic acid, competitive inhibitor, can inhibit *H. armigera* gut α–amylase activity with *Ki* value of 0.05 mM [154]. The study in peppermint also suggested that it contained the highest *p*–coumaric acid and showed the correlation between *p*–coumaric acid and α–amylase inhibitory activity, which was moderate and negative correlation ($r = -0.25$, $P<0.05$) [155]. These results were corresponded to our study, which suggested that raw mung bean showed the highest anti–α–amylase activity might be due to main phenolic acid, which could act as α–amylase inhibitors.

From this results, mung bean contained apigenin, which was previously reported to inhibit α–amylase activity (31.4 ± 3.1% inhibition with final concentration 1.5 mg/ml) [156]. Previous study reported that mung bean also possesses two main flavonoids, including vitexin (1.5 mg/g) and isovitexin (1.0 mg/g) (Figure 6.4) [9, 26], which are flavone C–glucosides [157]. These compounds were mainly located in seed coat (15.22 , 11.42 mg/g, respectively) [124]. The n–butanolic extract from *F. deltoidea* with the highest isovitexin and vitexin contents (24.63 and 8.3 mg/g, respectively) exhibited high α–amylase inhibition activity with the IC_{50} value 39.42 µg/mL [158]. Orientin, isoorientin, vitexin and isovitexin from bamboo leaf extract were proven to be competitive inhibitors of α–amylase (Ki values of 152.6, 11.5, 569.6, and 75.8 µg/mL, respectively) [159]. Vitexin and isovitexin could, as well, reduce blood glucose *in vivo* [160]. Other flavonoids such as luteolin, myricetin and quercetin were also found in mung bean. Previous study reported that luteolin (IC_{50} value of 0.103 mg/mL), myricetin (IC_{50} value of 0.121 mg/mL) and quercetin (IC_{50} value of 0.169 mg/mL) could, as well, inhibit α–amylase reaction [77]. As well, isolation quercetin extract from *Vaccinium arctostaphylos* leaves provided a dose dependent α–amylase inhibitory activity with the IC_{50} value of 0.17 mM [161]. Furthermore, phytic acid, which was found mainly in mung bean, had been previously

reported that phytic acid binding protein could inhibit α–amylase activity, whereas free phytic acid could not [162].

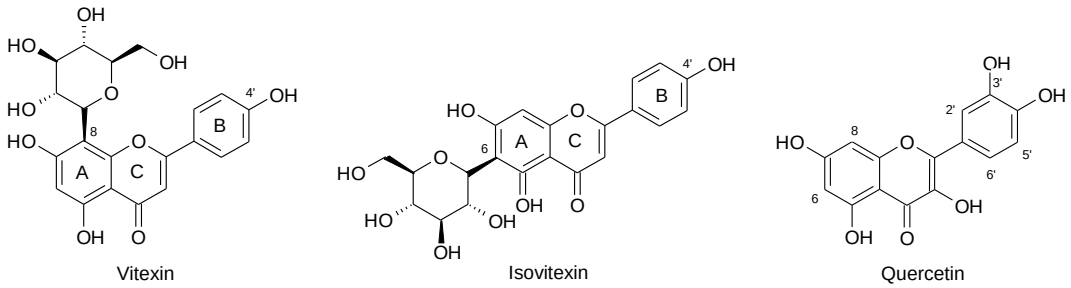


Figure 6.4 The chemical structures of flavonoids including vitexin, isovitexin and quercetin, which exhibited anti α–amylase activities [159, 161].

Cooked legumes exhibited lower anti–α–amylase activities than raw legumes. Cooked red kidney bean provided the highest α–amylase inhibitory activity. Red kidney bean was previously reported to be able to decrease rate of carbohydrate digestion through inhibition of α–amylase by protein that acts as α–amylase inhibitor. It is thought that, by possibly preventing the digestion of complex carbohydrate, this inhibitor may reduce calorie intake, thereby promoting weight loss. Red kidney bean contains five major anthocyanins, including cyanidin–3,5–diglucoside, delphinidin–3–glucoside, cyanidin–3–glucoside, petunidin–3–glucoside and pelargonidin–3–glucoside, as well as quercetin and kaemferol [139, 140]. Anthocyanins that were extracted from China violet flower (*Asystasia gangetica*) exhibited α–amylase inhibitory activity with 71.46% inhibition [163]. All anthocyanins are sensitive toward heat treatment, in which their existences were decreased during thermal process [164].

Likewise, seed coat of red kidney showed the highest anti–α–amylase activity. It might be that the concentration of phenolics and flavonoids were mainly located in seed coat rather than in cotyledon [136]. From molecular docking studies, vitexin and isovitexin, flavone C–glycosides, can interact with the active site of α–amylase. The active site of *Bacillus licheniformis* α–amylase consists of catalytic Asp231, Glu261, and Asp328, which are key acidic residues for hydrolysis of starch chain [159]. The active site of enzyme is built by the number of subsites (Figure 6.5). The hydrolysis occurs between subsites –1 and +1. The reaction is initiated by the

protonation of glycosidic oxygen on starch chain by catalytic Glu261. This matter leads to unstable molecule due to extra bonding on the glycosidic oxygen and causes a nucleophilic attack by catalytic Asp231 on C1 of the sugar molecule at the subsite–1. After sugar aglycon part of the starch chain leaves, Asp261 will try to resume its initial (rest) state by attacking hydrogen atom from water molecule. This activated water molecule subsequently cleaves the covalent bond between the nucleophile oxygen of Asp231 and the C1 of the sugar molecule. As a result, sugar molecule is released from the active site, and the enzyme is resumed its initial state. Another active site residue, Asp328, plays no direct role in the catalytic mechanism, but maybe important for stabilizing other catalytic residues. It was previously reported that bulky vitexin can form hydrogen bond with non–catalytic Asp in the active site of α–amylase, thus blocking the substrate from entering the active site of the enzyme. On the other hand, extended isovitexin could form hydrogen bond with catalytic Asp in the α–amylase catalytic pocket, thus forming a linear structure of enzyme–inhibitor complex that resembles the interaction of the enzyme and its natural substrate. These interactions lead to a stronger inhibitor of isovitexin (Ki of 75.8 μg/mL) than vitexin (Ki value of 569.6 μg/mL). Comparing to acarbose (IC_{50} of 0.08 mg/mL), this anti–diabetic drug can completely replace substrate in the substrate binding site [159]. Acarbose is a mixed non–competitive inhibitor, which can directly binds with free enzyme at the active site or enzyme–substrate complex at a secondary carbohydrate binding site [165]. Since this inhibitor fits perfectly in the active site of the enzyme, the enzyme–substrate complex cannot be formed. Thus, inhibition of α–amylase would prevent carbohydrate degradation and glucose absorption.

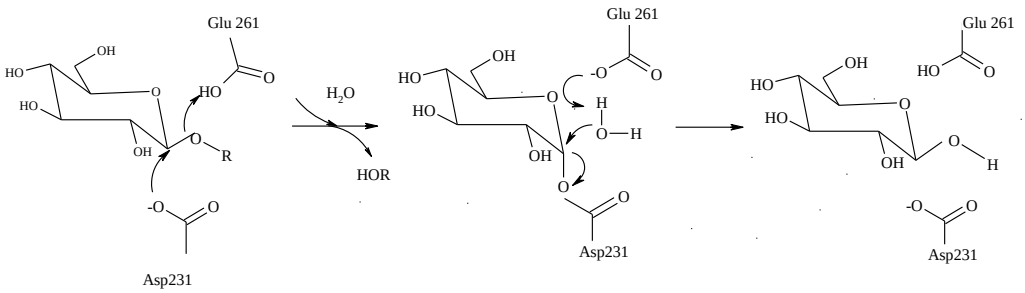


Figure 6.5 The reaction mechanism of *Bacillus licheniformis* amylase. The reaction is initiated by the protonation of glycosidic oxygen on starch chain by catalytic

Glu261, followed by a nucleophilic attack by catalytic Asp231 on C1 of the sugar molecule and a hydrolysis of nucleophile oxygen of Asp231 and the C1 of the sugar molecule by water molecule.

6.4.2 α–Glucosidase inhibitory activity

Peanut exhibited the highest α–glucosidase inhibitory activity in our study. Peanut has been reported to contain many phytochemicals such as trans–resveratrol, phytosterols, isoflavones genistein and daidzein [119]. Some of these phytochemicals possess potential antioxidant capacities including polyphenolics [166], tocopherols and proteins [167]. A predominant polyphenolic found in peanut is *p*–coumaric acid, which accounted for 40–68% of total phenolics present [168-170]. This bioactive compound is a free radical inhibitor with chain–breaking activity [171]. Previous research also suggested that *p*–coumaric acid content was correlated with potential α–glucosidase inhibition. This compound possesses the IC_{50} of 0.004 mM against α–glucosidase [155].

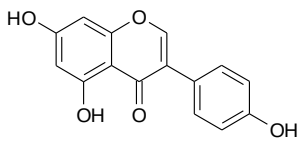
Comparing to the IC_{50} of acarbose (0.42 mM) under the same studied condition, *p*–coumaric acid is considered as a very effective α–glucosidase agent. Besides, resveratrol (3,5,4'–trihydroxy–trans–stilbene) was abundantly found in peanut (84 μg/100 g) [172]. This *trans*– resveratrol exhibited IC_{50} <0.1 mM against α–glucosidase [173]. Comparing to acarbose, which can inhibit 35% of α–glucosidase activity at the concentration of 0.5 mM under the same studied condition [173], *trans*–resveratrol is also considered as an effective agent against α–glucosidase. Thus, high anti–α–glucosidase activity as being observed in peanut might be a result of abundant *p*–coumaric acid and *trans*–resveratrol. Interestingly, the fact that the later was only found in peanut and pistachios and has not been found in other nuts [172] might contribute to the highest α–glucosidase inhibitory activity among investigated legumes.

However, in cooked legumes, it was showed that red kidney bean exhibited the highest α–glucosidase inhibitory activity, followed by white kidney bean, mung bean, peanut, soybean and black bean, respectively. The inhibition of α–glucosidase that functions similarly to α–amylase in term of carbohydrate digestion has not been investigated yet. Red kidney bean has been reported to contain

anthocyanins, including cyanidin–3,5–diglucoside, delphinidin–3–glucoside, cyanidin–3–glucoside, petunidin–3–glucoside and pelargonidin–3–glucoside with delphinidin–3–glucoside being the most abundantly found [140]. It was previously found that some acylated anthocyanin derivatives extracted from red flowers of the morning glory, *Pharbitis nil* cv. Scarlett O'Hara, exhibited anti–α–glucosidase activity [174]. Besides, cyanidin–3,5–diglucoside with the IC_{50} of 6.01 mg/mL [60] was reported, while delphinidin–3–glucoside had been reported as a main anthocyanins in *Vaccinum floribundum* and *Aristotelia chilensis*, in which their crude extracts exhibited α–glucosidase inhibitory activity [175]. All anthocyanins are sensitive toward heat treatment such that their existences were decreased during thermal process [164]. In addition, other researches had found phytohaemagglutinin from red kidney bean (lectin), which inhibited α–glucosidase [26]. Besides, raw legumes exhibited higher α–glucosidase inhibitory activity than cooked legumes in all cases. While inhibition activity was decreased in cooked legumes, it was possible that phenolics contained seed coat of legumes are destroyed during heat treatment [73]. For example, the decrease in anti–α–glucosidase activity in peanut might be due to stability of its bioactive compounds, *p*–coumaric acid and *trans*–resveratrol. The former was undergone thermo degradation, in which it was continuing destroyed under 75 °C, while the later was found to be heat stable. Likewise, anthocyanins in red kidney bean has been report to be sensitive toward heat treatment [26]. Nevertheless, the drastic decrease in α–glucosidase inhibitory activity was observed in black bean (approx. 2.5–time reduction). Black bean contains anthocyanins such as delphinidin–3–glucoside (56%), petunidin–3–glucoside (26%), and malvidin–3–glucoside (18%) [176]. All boiling and soaking processes cause the decrease in anthocyanin content and total phenolics by oxidative degradation, release of free phenolic acids from conjugate forms, and formation of complex structure of phenolic substances [26].

Almost all legumes showed higher anti–α–glucosidase in seed coats than raw and cooked legumes, in exception of soybean. It was possible that the concentration of phenolics and flavonoids were mainly located in seed coat rather than cotyledon. The amount of these compounds depends on growing conditions and genetic factors [136]. Interestingly, raw soybean exhibited higher α–glucosidase inhibitory activity than seed coat and cook soybean. Soybean is a rich source of

isoflavone glycoside such as malonyl–genistin, malonyl–daidzin, genistin, daidzin, genistein and daidzein [177]. These compounds were located in soybean cotyledon and were not found in the seed coat [126]. Previous research demonstrated that genistein (Figure 6.6) was the isoflavone of important in soy protein [178], a non–competitive inhibitor of α–glucosidase, could inhibit enzyme activity in a dose–responsive manner with the IC_{50} of 50 µM [179]. Thus, raw soybean exhibited the highest anti–α–glucosidase activity might be due to genistein, which could act as α–glucosidase inhibitors.



Genistein

Figure 6.6 The chemical structure of isoflavone glycoside, genistein, that provided anti–α–glucosidase activity.

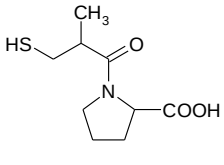
6.5 Angiotensin–converting enzyme inhibitory activity

Most researches are focused on natural product that can act as ACE inhibitors, especially plant extracts which a good source of bioactive compounds. This study suggested that anti–ACE activity was only found in cooked mung bean (80% inhibition). It might be that extraction concentration used in this experiment was low such that the ACE inhibitory activity might be measurable if applying higher concentration of the extract. Besides, anti–ACE agents might not be at its optimum level using extraction conditions in this study. For example, ethanolic extraction showed higher ACE inhibition than water extraction from the same plant [180]. Other studies suggested that plants such as coconut, choko skin, cinnamon, cocoa bean, green tea and seed extract from white grape can strongly exhibited ACE–inhibitory activities with IC_{50} of <1mg/mL. Phenolic acids in mung bean were found to be salicylic acid, *p*–coumaric acid and ferulic acid. It was previously found that syringic acid, ferulic acid and *p*–coumaric acid could act against ACE with IC_{50} value of 9.30,

4.40, 2.80 mM, respectively[181]. Main flavonoids in mung bean were vitexin and isovitexin [26]. These compounds possessed ACE inhibitory activity with 21 and 46 %inhibition [95]. Thus, anti–ACE activity as being observed in mung bean might be due to bioactive compounds that could act as ACE inhibitors.

Seed coats of black bean, mung bean and red kidney bean exhibited ACE inhibitory activities with 51, 42 and 14% inhibition, respectively. Legumes with dark seed coats showed higher bioactive compounds, especially phenolics and flavonoids, than legumes with light seed coats [138]. Seed coats of black bean contain mainly flavonol glycoside, condensed tannin (proanthocyanidins) and anthocyanidin. Previous study also reported major anthocyanins including malvidin 3–gluocside, petunidin 3–glucoside, delphinidin 3–glucoside, and delphinidin 3,5–diglucoside in seed coats of black bean [122]. Moreover, anthocyanins extracted from *Hibiscus sabdariffa,* delphinidin–3–Osambubioside and cyanidin–3–O–sambubioside, could act as competitive inhibitors against ACE with the IC_{50} values of 84.5 and 68.4 µg/mL, respectively [182]. Anthocyanin derivatives (delphinidin and cyanin (cyanidin–3,5–di–O–glucoside)) and flavonoid derivatives (quercetin) inhibited ACE reactions in dose dependent manner and reduced ACE mRNA production in the rennin–angiotensin system (RAS) [183]. Proanthocyanidins extract from seed and skin of Chilean black grapes (*Vitis vinífera* L. cv. País) showed ACE inhibition activity and suggested that the degree of inhibition was depended on availability of hydroxyl (–OH) moieties [93]. Thus, high ACE inhibition as being observed in black bean might be the function of these bioactive compounds.

Captopril (Figure 6.7), the anti–hypertensive drug (competitive inhibitor) with the IC_{50} of 0.02 µM [96], could maintain blood pressure [184]. The sulfhydryl moiety in captopril can bind with catalytic zinc atom in the active site of ACE leading to the prevention of enzyme–substrate binding complex or prevents angiotensin I from forming a complex with enzyme (Figure 6.8). Nevertheless, the information on absorption and bioavailability of these bioactive compounds in legumes are needed for further studied



Captopril

Figure 6.7 The chemical structures of anti–hypertensive drugs, captopril, that inhibit ACE for treatment of hypertension.

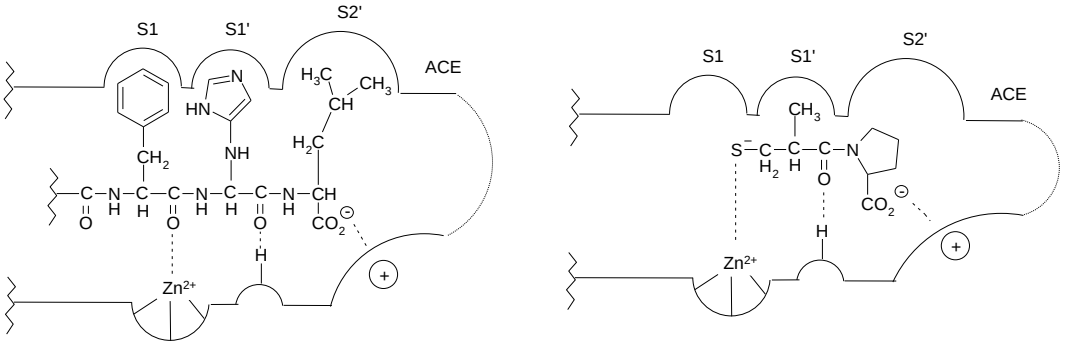


Figure 6.8 The active site of angiotensin–converting enzyme with (A) angiotensin I and (B) captopril in its catalytic pocket.

6.6 Cholinesterase inhibitory activity

Medicinal treatments of AD have been focused on cholinergic hypothesis and β–amyloid formation. The former is emphasized on cholinesterase enzymes including AChE and BChE [99]. Both enzymes can degrade neurotransmitters, causing a decrease in cognitive function. Most studies focuses on plant extracts, which are abundant sources of bioactive compounds and anti–cholinesterase agents. Legumes contain many bioactive compounds such as phenolic acids, flavonoids, anthocyanins, saponins and tannins. Some of which might act as anti–ACE agents. For example, it was previously found that flavonoid derivatives with a diethylamine group conjugated with flavonoid scaffold by a four–carbon spacer showed strong AChE inhibitory activity [185]. In this study, only raw soybean inhibited AChE activity among all raw and cooked legumes. Previous research indicated that commercial fermented soybean from the South of China exhibited the highest AChE inhibitory activity with the IC_{50} of 0.191 mg/mL [186]. Soybean from Iran also displayed anti–AChE inhibitory

activity with the IC_{50} value of 4.69 mg/mL and 68.4% inhibition with the concentration of 40 mg/mL [187].

It was previously reported that isoflavone in raw soybean is presented in four chemical forms, including malonylglycosides (70–80%), acetylglycosides (5%), glycosides (25%) and aglycones (2%) [188]. The major isoflavones in soybeans were presented in different forms, including glycosides (daidzin and genistin), aglycone (daidzein and genistein) [189], malonylglycoside (genistin, daidzin) and glycoside derivatives (genistin, daidzin, genistein and daidzein) [177]. These compounds were located in soybean cotyledon and were not located in the seed coat [126]. Antioxidant activities of aglycones genistein and daidzein in soybean were higher than that of glycoside [190]. Since these compounds are abundantly found in soybean, they might, as well, possess anti–AChE activities, leading to observed AChE inhibitory activity in soybean, while others were not detectable. However, there is no scientific evidence if these compounds could act as anti–AChE agents. Additionally, phenolic acids found in soybean are ferulic acid, syringic acid, hesperidin, *p*–coumaric acid, sinapic acid, rutin, hydroxybenzoic acid, protocatechuic acid, caffeic acid, gallic acid and quercetin [117]. Quercetin and gallic acid demonstrated AChE inhibitory activity with 76.2 and 15.7% inhibition [101].

Similarly, soybean exhibited the highest anti–BChE activity among all investigated legumes. Quercetin and gallic acid substances that can inhibit AChE also exhibited ant–BChE activity with 46.8 and 48.8 % inhibition, respectively. Moreover, genistein, a flavonoid found in soybean, presented BChE inhibitory activity with 65.7% inhibition [101]. Interestingly, anti–BChE activity was higher than anti–AChE activity, which might be due to less substrate specificity of BChE [120].

Thermal process, especially cooking process, is responsible for loss of phenolic compounds [191]. All processes of soy milk production by soaking, grinding and heating were found to cause a decrease in total isoflavone content [192]. Thus, AChE–inhibition activities in all cooked legumes were not detected.

Seed coat of red kidney bean exhibited the highest AChE and BChE inhibitory activities. It might be due to phenolic compounds and flavonoids that were mainly located in seed coat more than cotyledon [136]. Seed coat of red kidney had many bioactive compounds such as quercetin, kamperol, ferulic acid, flavonoid

glycoside, proanthocyanidin (tannin) and anthocyanins [123]. Previous study reported that flavonoid derivatives that powerfully inhibited AChE and BChE were macluraxanthone and quercetin [193]. Moreover, it was found that sugar moieties in the flavonoid derivatives are necessary for AChE inhibition [193]. For example, rutin and kaempferol 3–*O*–β–d–galactoside with glucose molecules exhibited ineffective with both enzymes [193]. Proanthrocyanidin extracted from different location of *C. schoenanthus* showed anti–AChE activity with IC_{50} values of 0.64 ± 0.07 from mountain, 0.44 ± 0.09 from desert and 0.75 ± 0.09 mg/mL from experiment plot [194]. Flavones C–glycosides, vitexin and isovitexin, that are mainly found in mung bean exhibited anti–AChE activity with the IC_{50} values of 12.16 and 6.24 µM, respectively [195]. Vitexin and isovitexin also possessed ani–BChE activities with the IC_{50} values of 6.73 and 6.48 µM, respectively [195]. Nevertheless, the information on absorption and bioavailability of these phytochemicals in legumes are essential for further studied

6.7 β–secretase inhibitory activity

Several researches were focused on β–secretase (BACE1) to be the main protease to produce β–amyloid peptides [196]. The results in this experiment suggested that all raw legumes could inhibit β–secretase activity, especially raw soybean, which showed the highest anti–β–secretase activity. Bioactive compounds in soybean are isoflavone glycoside and derivatives (malonyl–genistin, malonyl–daidzin, genistin, daidzin, genistein and daidzein) [177], phenolic acids (ferulic acid, syringgic acid, hesperidin, *p*–coumaric acid, sinapic acid, rutin, hydroxybenzoic acid, protocatechuic acid, caffeic acid, gallic acid) and flavonols (quercetin) [117]. Previous reports suggested that four flavonols and a flavone could strongly inhibit BACE1 activity [197, 198]. These bioactive compounds are myricetin (IC_{50} value of 2.8 µM), guercetin (IC_{50} value of 5.4 µM), kaempherol (IC_{50} value of 14.7 µM), morin (IC_{50} value of 21.7 µM) and apigenin (IC_{50} value of 38.5µM) [197, 198]. Besides, bioactive compounds from dried rhizome of *Smilax China L.* including trans/cis–resveratrol mixture, oxyresveratrol, veraphenol and *cis*–scirpusin could act as non–competitive inhibitors of BACE1 [36]. Vitexin (IC_{50} of 51.07 µM) showed higher anti–BACE activity than isovitexin (IC_{50} >100 µM) [195]. Phytochemicals such as caffeic acid,

ferulic acid, gallic acid, genistein, proanthocyanidins and quercetin showed amyloid–β–aggregation inhibitory activity [199]. Saponin was mainly found in legumes, especially soybean, mung bean and peanut. Soybean contained approximately 0.2–0.3% of saponin in cotyledon, while none was found in seed coat [200]. It was previously found that saponin extract from *Panax ginseng*, could inhibit BACE1 activity by forming hydrogen bond with catalytic residues of BACE1 (Asp32 and Asp228) (Figure 6.9) [201]. Interestingly, cook legumes provided higher anti–BACE1 activity than raw legumes. Cooked soybean showed the highest BACE1 inhibition. Previous report suggested that processing soybeans contained less typsin inhibitor than isoflavone and saponin, in which the contents were elevated after heat treatment [200].

In seed coat, only seed coats of mung bean and black bean could inhibit BACE1 reactions. Mung bean exhibited higher anti–BACE1 activity than black bean. It was previously found that seed coat of mung bean contained phytic acid, myo–inositol hexakisphosphate and anti–nutrition factors [202]. Epidemiologic study reported that phytic acid from rice grain extract could act against BACE1 activity (IC_{50} value of 0.18 µg/mL) in a dose dependent manner [203]. Phytic acid could, as well, terminate Aβ production in cell culture studies [203].

From molecular docking studies, flavonoids could inhibit BACE1 through hydrogen bonding interactions between flavonoids and BACE1 catalytic core. Quercetin could form single hydrogen bond interaction with Asp32 residue of BACE1. Flavonoid, which strongly interacts with catalytic Asp32, might increase anti– BACE1 activity [198]. Moreover, the potency of anti–BACE1 activity depends on hydroxyl group on B ring of flavonoid, which contains more hydroxyl groups on B ring. For example, kaempferol (IC_{50} value of 14.7 µM), quercetin (IC_{50} value of 5.4 µM) and myricetin (IC_{50} value of 2.8 µM) have one, two and three hydroxyl group(s) in B ring, respectively (Figure 6.10) [204]. However, further studies are required for more information such as bioavailability or animal study.

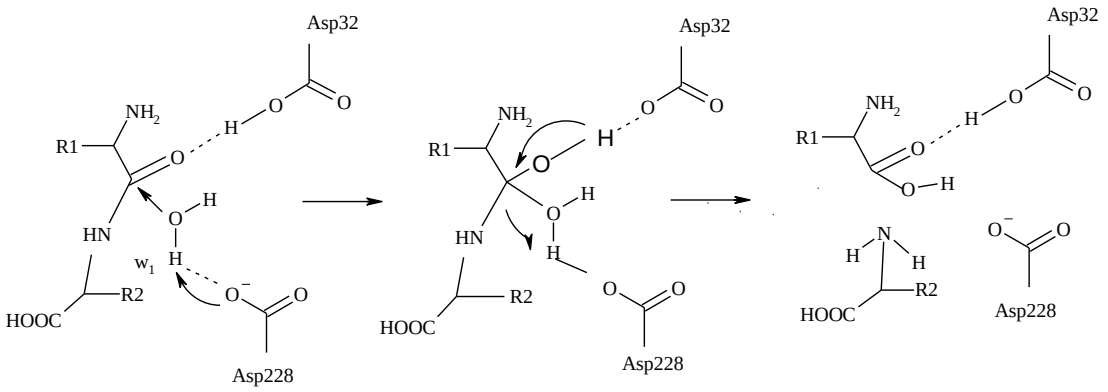


Figure 6.9 The reaction mechanism of BACE1. Catalytic Asp228 is protonated and abstracts a proton from catalytic water molecule, thus generating a hydroxyl ion, which subsequently forms a nucleophilic attack on the carbonyl carbon of the scissile peptide. Then, the carbonyl oxygen of the peptide snatches hydrogen atom from Asp32 to produce the tetrahedral gem–diol intermediate. Both catalytic residues then try to resume the initiate state, thus resulting in the cleavage of the peptide bond that generates the amino (–NH2) and carboxy (–COOH) terminals.

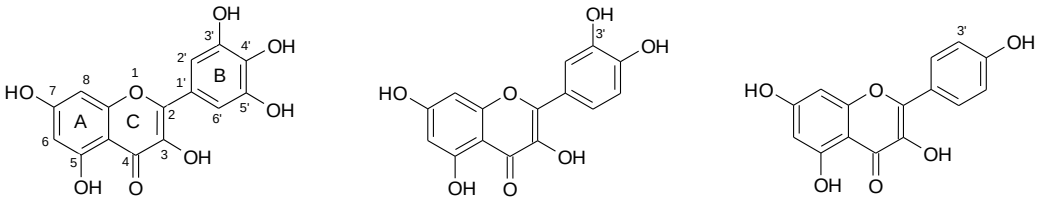


Figure 6.10 The chemical structures of flavonoids including kaempferol, quercetin, and myricetin, which provided anti–BACE1 activities. The inhibitory activities BACE1 may be explained by the hydroxyl groups on ring B of these flavonoids [198, 204].