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In vitro assessment of anti-diabetic potential of four kinds of dark tea (*Camellia sinensis* L.) protein hydrolysates

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Summary

The contributions of four kinds of dark tea (*Camellia sinensis L.*) proteins and their hydrolysates to hypoglycemic activity were investigated in vitro. Four kinds of water-extracted dark tea proteins were hydrolyzed with trypsin and Alcalase, respectively. The complete proteins had α-amylase inhibitory activity with half maximal inhibitory concentration (IC_{50}) values ranging from 1.27 to 2.78 mg/mL. Most of the dark tea proteins and hydrolysates significantly inhibited α-glucosidase and dipeptidyl peptidase (DPP-IV), with IC_{50} values in the range of 0.0103-1.3114 mg/mL and 0.1000-1.3364 mg/mL, respectively. In general, Heimaojian (HMJ) and Qianliang (QL) hydrolysates displayed high α-glucosidase inhibitory activity, while HMJ, Fuzhuan (FZ), and Heizhuan (HZ) hydrolysates exhibited a strong ability to inhibit DPP-IV. This study demonstrates the potential of dark tea proteins and their hydrolysates as a source of functional food and medicine for the control of type 2 diabetes.

Key words: Dark tea; hydrolysis; DPP-IV; α-glucosidase; α-amylase

Introduction

Type 2 diabetes is one of the leading public health problems in modern society. This chronic disease increasingly exists in the aging population, especially in developed countries. By 2030, it will affect 438 million people, with 70% of cases occurring in low- to middle-income families (Yu et al., 2012). People who suffer from type 2 diabetes are strongly predisposed to atherosclerotic cardiovascular disease (CVD) (Harnedy and FitzGerald, 2013), which is a major cause of morbidity and mortality.

Some synthetic medicines are used to control type 2 diabetes. One therapeutic approach is to suppress the absorption of glucose by inhibiting carbohydrate-hydrolyzing enzymes such as α-amylase, which acts on long-chain carbohydrates, and α-glucosidase, which catalyzes the cleavage of glucose from disaccharides (Lebovitz et al., 1997). Acarbose and voglibose are widely known as inhibitors of α-amylase and α-glucosidase. Another mechanism is the inhibition of dipeptidyl peptidase-IV (DPP-IV) activity. DPP-IV rapidly metabolizes glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), two insulinotropic incretin hormones that enhance glucose-dependent insulin secretion and regulate postprandial blood glucose levels (Green et al., 2004). Many DPP-IV inhibitors are used, including vildagliptin, saxagliptin, and sitagliptin.

Tea (*Camellia sinensis* L.) is regarded as one of the most popular beverage plants consumed worldwide. It has been reported that 78% of the total amount of tea produced and consumed around the world is dark tea (full-fermented), 20% is green tea (non-fermented), and less than 2% is yellow or oolong tea (semi-fermented) (Xiao et al., 2011; Li et al., 2012). One of the most important dark tea-production areas in China is Anhua County in Hunan Province, which is famous for Fuzhuan brick tea, Qianliang tea, and Heimaojian tea.

Tea has many pharmacological and therapeutic properties, including anti-diabetic effects. Stote and Baer (2008) indicated that tea consumption may affect glucose metabolism and insulin signaling by enhancing insulin sensitivity and endothelial function. Many studies have verified that tea extracts significantly inhibit the activity of both α-amylase and α-glucosidase (Miao et al., 2015; Peng et al., 2015; Koh et al., 2010), enzymes that play key roles in carbohydrate digestion and have been recognized as therapeutic targets for modulating postprandial hyperglycemia. Gao et al. (2012) demonstrated that tea polyphenols can exert anti-oxidative and hypolipidemic effects in rats with streptozotocin-induced diabetes. Huang et al. (2013) stated that 95% ethanol precipitate from an aqueous extract of pu-erh tea exhibited a remarkable inhibitory effect against α-glucosidase in vitro, as well as a significant ($p < 0.05$) effect on postprandial hyperglycemia in diabetic mice. Recently, Chen and Guo (2017) investigated the effects of polysaccharides and polyphenolic fractions of Zijuan tea on α-glucosidase activity and blood glucose levels. The results indicated that the polysaccharide or theaflavin fractions inhibited α-glucosidase at a greater rate than acarbose (positive control). Zhou et al. (2018) reported that tea polyphenols can alter autophagy levels to improve glucose and lipid metabolism in diabetic rats with cardiomyopathy. However, the anti-diabetic effects of dark tea proteins have not yet been investigated.

This study focused on four kinds of representative dark teas from Hunan Province: Heimaojian (HMJ), Fuzhuan tea (FZ), Heizhuan tea (HZ), and Qianliang tea (QL). The objective of this work was to examine the inhibitory effects of their protein extracts and hydrolysates on the activity of α-amylase, α-glucosidase, and DPP-IV.

Materials and methods

Materials

Dried dark tea leaves for all four kinds of tea were provided by Aijia Biotechnology Co. (Hunan Province, China). α-glucosidase from *Saccharomyces cerevisiae* (≥10 U/mg protein), α-amylase from *Bacillus licheniformis* liquid (CAS: A4862), DPP-IV Inhibitor Screening Kit (MAK203-1KT), 4-nitrophenyl α-D-glucopyranoside (pNPG) (CAS: 3767-28-0), and soluble starch were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bradford Protein Assay Kit (P0006) was purchased from Beyotime (Haimen, China). Acarbose hydrate was purchased from Aladdin. The Alcalase enzyme (with a claimed activity of ≥200 U/mg) was purchased from Aoboxing Bio-tech Co. (Beijing, China), and trypsin (250 U/mg) was purchased from HuaQiSheng Bio-tech Co. (Guangzhou, China). 2,4,6-Trinitrobenzenesulfonic acid (TNBS, 5% in H_2O) reagent was purchased from Ark Pharm (USA). Sodium dodecyl sulphate was purchased from Biofroxx. All other reagents were of analytical grade.

Preparation and purification of dark tea protein extracts

Dark tea leaves were milled, sieved (20 mesh), accurately weighed (25.0 g), and extracted in an ultrapure water bath (500 mL) for 30 min

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at 95 °C. After filtration with gauze and quantitative filter papers, the filtrates were concentrated by rotary vaporization under reduced pressure at 50 °C to obtain the syrup extracts. Small amounts of water were then used to dilute the syrup extracts. Finally, the extracts were lyophilized, and the residues (test samples) were collected for subsequent analysis.

To purify the dark tea protein, portions of the test samples were dissolved in ultrapure water at a concentration of 5 mg/mL and placed in well-prepared dialysis membranes (500 Da). Ultrapure water (250 times the volume of the samples) at room temperature was placed on the opposite side of the membrane and replaced three times (every 12 hours). After dialysis, 5% (w/v) activated carbon was added at 45 °C for 30 min. After filtration with quantitative filter papers, saturated ammonia sulfate solution was added ($V_{ammonia\ sulfate}$:V_{sample} = 4:1), and the protein was allowed to precipitate at 4 °C for 12 hours. The subsidence was obtained by centrifugation at 6000 × g for 20 min at 4 °C, and then redissolved in ultrapure water before dialysis at room temperature for 24 hours to eliminate micromolecules.

Enzymatic hydrolysis of dark tea proteins

Dark tea proteins were enzymatically treated with trypsin and Alcalase following the method described previously (Harnedy and FitzGerald, 2013; Wang and Zhang, 2017), with some modifications. Briefly, solutions of the crude proteins were preheated to 50 °C and adjusted to pH 8.0 with 2.0 M NaOH. Either Alcalase or trypsin was added at an enzyme/substrate (E/S) ratio of 1.5% (w/w). The reaction mixtures were kept in a water bath with shaker at 50 °C for six hours, and 2.0 M NaOH was used to maintain the pH value as monitored by a pH meter (FE30, Mettler Toledo, Switzerland). The enzymes were inactivated by heating at 90 °C for 20 min. Hydrolysate samples were subsequently freeze-dried.

Determination of proteins and degree of hydrolysis

Proteins were determined using a Bradford Protein Assay Kit (P0006). Briefly, 5-μL test extract solutions (2 mg/mL) were mixed with 250 μL Coomassie Brilliant Blue (G-250) dye in a 96-well plate. Absorbance at 595 nm was measured within 2 hours using a microplate reader (Sunrise v1.05, Tecan, Switzerland). To obtain a standard curve, BSA protein standard solutions of varying concentrations (0 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, and 1.5 mg/mL) were used.

Degree of hydrolysis (DH) was monitored using the TNBS method described by Spellman et al. (2003), with some modifications. During the course of the test, 0.5 M NaOH was used to keep the reaction at pH 7.0 as monitored by a pH meter. 0.125 mL of the test samples (2 mg/mL) were added to test tubes and mixed with 1.0 mL of sodium phosphate buffer (0.2125 M, pH 8.2). TNBS reagent (1 mL, 0.1% w/v) was then added to each tube, followed by incubation at 50 °C for 60 min in a water bath (protected from light). Furthermore, the reaction was stopped by adding 2.0 mL HCl (0.1 mol/L). Samples were then allowed to cool to room temperature, and absorbance values were measured at 340 nm using a microplate reader (Sunrise v1.05, Tecan, Switzerland). L-Leucine (0-2 mM) was used to generate a standard curve. DH values were calculated using the following formula:

$$DH\% = \frac{AN_2 - AN_1}{Npb} 100$$

where AN1 is the amino nitrogen content of the protein substrate before hydrolysis (mg/g protein), AN2 is the amino nitrogen content of the protein substrate after hydrolysis (mg/g protein), and Npb is the nitrogen content of the peptide bonds in the protein substrate (mg/g protein).

Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis

The peptide compositions of dark tea protein hydrolysates were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using an Agilent 1260 HPLC system with a UV detector. Briefly, samples were diluted to 1 mg/mL in ultrapure water, filtered through a 0.22-μm syringe filter, and then injected into an analytical C18 column with a length of 250 mm, inner diameter of 4.6 mm, and particle size of 5 μm (C18-120-5 4E, Shodex, Japan). Samples were eluted at a total flow rate of 0.5 mL/min at 30 °C, with both water (solvent A) and acetonitrile (solvent B) as follows: 15% B at 0-2 min; 15%-20% B at 2-10 min; 20%-25% B at 10-20 min; 25%-80% B at 20-30 min. Elution was monitored at 215 nm with an ultraviolet-visible (UV-vis) detector.

α-Amylase inhibition assay

The α-amylase inhibitory assay was performed according to the method previously described by Yu et al. (2012), with slight modifications. A 1% starch solution was preheated at 95 °C for 5 min. 10 μL of α-amylase solution (1 U/mL in ultrapure water) was premixed with 20 μL of test sample solutions at different concentrations (0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL) in ultrapure water. Following incubation at 37 °C for 15 min, 500 μL of 1% starch solution (in 0.2 M sodium phosphate buffer, pH 6.9) was added to start the reaction. The reaction was carried out at 37 °C for 5 min and terminated by adding 600 μL of DNS reagent (1% 3,5-dinitrosalicylic acid, 12% Na-K tartrate in 0.4 mol/L Na_2CO_3). The reaction mixture was placed in a boiling water bath for 15 min. After the samples cooled down to room temperature, absorbance at 540 nm was determined by a microplate reader (Yu et al., 2011a; Yu et al., 2011b). Acarbose was used as a positive control. Sodium phosphate buffer (pH 6.9) was used in place of the test samples for the blank. For all tests, the inhibition assay was performed in quadruplicate. The inhibition of enzyme activity was calculated as follows:

$$\text{Inhibition } (\%) = [1 - (A_{sample}/A_{blank})] \times 100$$

α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was assayed according to a previous study by Lin et al. (2015), with modifications and optimization. 20 μL of test extract solutions at different concentrations (0.01 mg/mL, 0.02 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL) and of acarbose (positive control, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL) were premixed with 10 μL of α-glucosidase (0.2 U/mL) at 37 °C for 20 min. Next, 40 μL of pNPG (10 mM, in 0.2 M phosphate buffer, pH 6.8) and 50 μL of 0.2 M phosphate buffer were added and incubated at 37 °C for 30 min. The reaction was stopped by adding 100 μL of 0.2 M sodium carbonate. Phosphate buffer was used in place of the test samples for the blank. To determine sample background interference, each sample was added to the phosphate buffer during reactions without the enzyme solution. Each inhibition assay was performed in quadruplicate. Absorbance at 405 nm was measured using a microplate reader. The α-glucosidase inhibition (%) was calculated as follows:

$$\text{Inhibition } (\%) = 1 - [(A_{sample} - A_{background})/A_{blank}] \times 100$$

DPP-IV inhibition assay

The DPP-IV inhibitory activity assay was performed using a DPP-IV Inhibitor Screening Kit (MAK203). The effectiveness of the test inhibitors was compared with a known DPP-IV inhibitor, sitagliptin. Before use, reagents were allowed to come to room temperature and slightly centrifuged to maintain integrity. Assays were performed as

specified by the product technical bulletin. After all reagents were diluted to the required concentrations, 50 µL of enzyme solution and 25 µL of test sample solution were premixed and incubated at 37 °C for 10 min; then 25 µL of DPP-IV substrate was added. After waiting 15-30 min to allow all reagents to mix, Fluorescence (FLU, λ_{ex} = 360 nm, λ_{em} = 460 nm) was measured once per minute at 37 °C using a Cytation 5 imaging reader (BioTek, USA). A control assay was also prepared by using DPP-IV assay buffer in place of the inhibitor samples. To remove background interference, buffer was added instead of enzyme solution. The relative DPP-IV inhibition (%) was calculated as follows:

Relative inhibition (%) =1− [($Slope_{sample}$ − $Slope_{background}$)/$Slope_{control}$] × 100%

where Slope = (FLU_2 − FLU_1)/(T_2 − T_1) = FLU/minute

Statistical analysis

Results are presented as the mean of triplicate determinations ± SDs. IBM SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) was used to identify significant differences ($p < 0.05$) between data.

Results and discussion

Extraction and purification of dark tea proteins

The method of preparing dark tea proteins under ordinary pressure is not fully described in the existing scientific literature, but plant-derived proteins are usually extracted by one of three types of solvents: aqueous, alkaline, or organic (Harnedy and FitzGerald, 2013; Lin et al., 2015; Siow et al., 2016). In consideration of people's drinking habits and environmental protection concerns, aqueous solvent was used in this study. The concentration of protein in tea leaves is around 0.20 g/g dry matter. In this study, the yield ranged from 7.39% ± 0.87% (for the complete QL protein) to 20.3% ± 1.19% (for the HMJ hydrolysate treated with Alcalase).

The presence of fiber and phenolic compounds, as well as protein glycosylation, all have inhibitory effects on protein hydrolysis, which cause misleading results in enzyme inhibitory assays. Phenolic and flavone compounds also inhibit the activity of many enzymes. Purification of proteins was therefore a critical step in removing some of the inhibitory agents and increasing the accuracy of the study. After the purification treatment, the concentration of protein increased dramatically (shown in Fig. 1). The trypsin-generated QL fraction realized the largest increase at 404.8% (from 0.1080 g/g extract to 0.5452 g/g extract), followed by the complete QL protein at 321.65% (from 0.0739 g/g extract to 0.3116 g/g extract) and the complete HMJ protein at 247.16% (from 0.1767 g/g extract to 0.6134 g/g extract). The average increase in protein content for 12 protein samples was 195.11%.

Enzymatic hydrolysis of protein fractions

The primary sequence of the protein substrate and the specificity of the enzyme(s) used determined the types of bioactive peptides generated from a particular protein. Trypsin and Alcalase have been widely used on plant proteins to produce bioactive peptides (Harnedy and FitzGerald, 2013; Kalyankar et al., 2013). In this study, the TNBA assay was used to quantify the degree of hydrolysis (DH) of four kinds of dark tea protein hydrolysates. Fig. 2 illustrates that the DH of HMJ, FZ, and QL were higher when trypsin was used compared to Alcalase, with the exception that HZ reached a higher DH when treated with Alcalase. According to researchers, Alcalase is a proteolytic preparation derived from *Bacillus licheniformis* and consists mainly of subtilisin endoproteinase, with a minor glutamyl endopeptidase activity (Kalyankar et al., 2013). Subtilisin acts as a relatively nonspecific proteinase but preferentially cleaves peptide

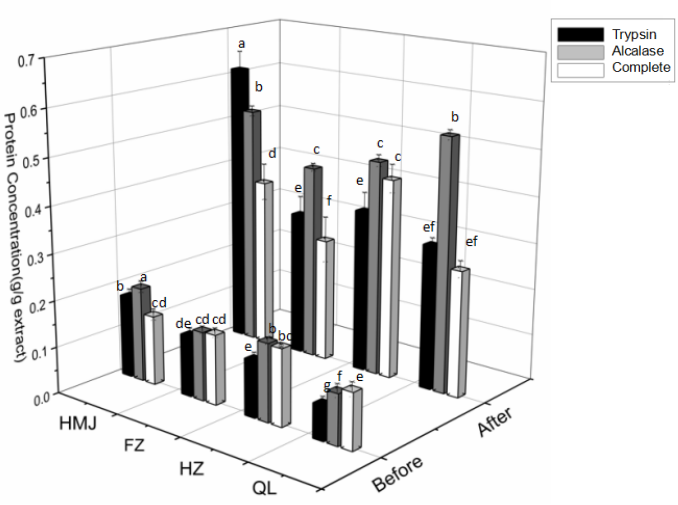

Fig. 1: Protein concentrations of four kinds of dark tea extracts and their hydrolysates before and after purification treatments. Mean ± SD (n=3). Bars with different letters are significantly different at $p < 0.05$.

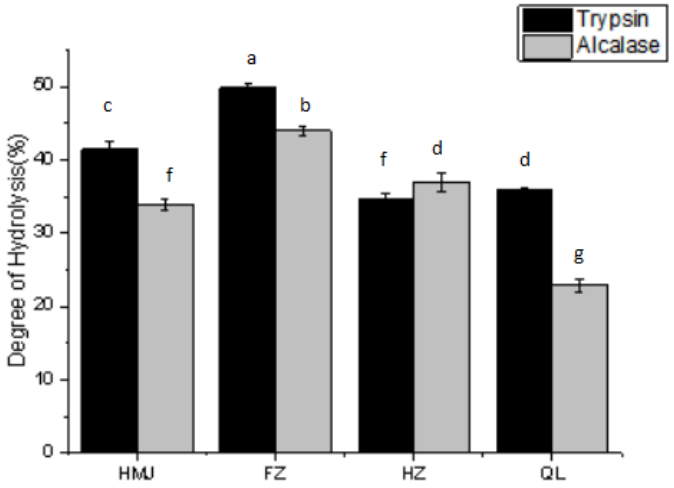

Fig. 2: Degree of hydrolysis (DH, %) for four kinds of dark tea generated by trypsin and Alcalase and their complete protein extracts. Mean ± SD (n=4). Bars with different letters are significantly different at $p < 0.05$.

bonds after large non-β-branched hydrophobic residues (Gron et al., 1992), while trypsin specifically targets diaminocaproic acid and arginine. This may indicate that lower concentrations of diaminocaproic acid and arginine exist in HZ. Moreover, when hydrolyzed by trypsin at a pH of 8.0 for 6 hours at 50 °C, FZ reached the highest DH at 49% ± 0.52%, followed by HMJ (41.39% ± 1.02%), QL (35.96% ± 0.145%), and HZ (34.53% ± 0.52%). These values were higher than those obtained in previous studies; for instance, *Spirulina platensis* hydrolyzed by trypsin had a DH of 18.26%-20.2% (Fan et al., 2018; Wang and Zhang, 2016). The DH for proteins treated with Alcalase followed a slightly different order than for those treated with trypsin, as follows: FZ (43.98% ± 0.6 3%) > HZ (36.93% ± 1.2%) > HMJ (33.86% ± 0.73%) > QL (22.84% ± 0.92%). These values were similar to those found in the literature; for instance, 20.8%-24.4% for *Spirulina platensis* hydrolyzed by Alcalase (Fan et al., 2018; Wang and Zhang, 2016).

The peptide compositions of dark teas were analyzed by RP-HPLC. This study displays partial profiles during min 0-10, since no signal was obtained in the last 10 min (not included in Fig. 3). In min 0-10, obvious differences were seen between the complete protein extracts

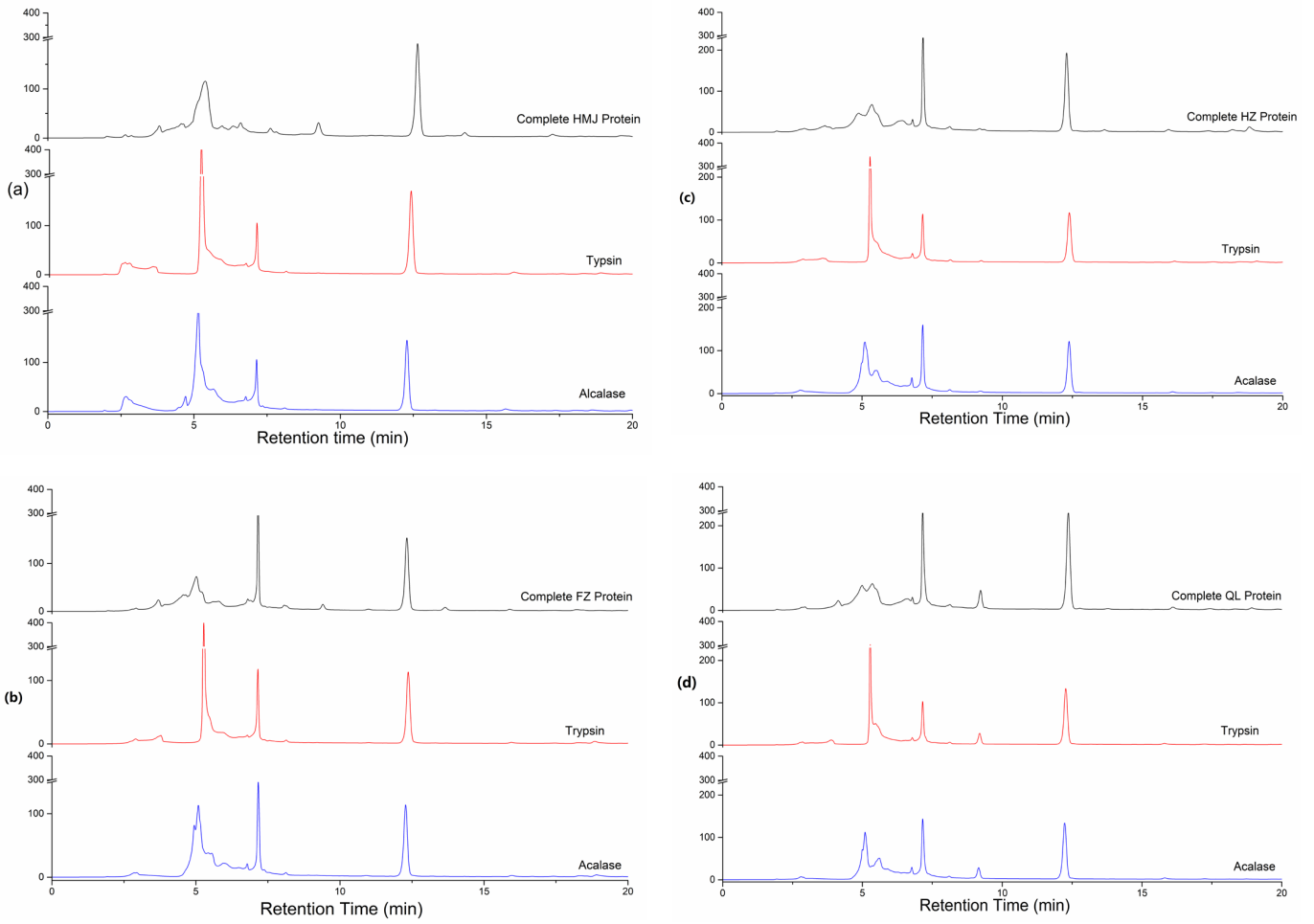

Fig. 3: RP-HPLC absorbance profiles at 215 nm for complete proteins and hydrolysates of HMJ (a), FZ (b), HZ (c), and QL (d).

and the trypsin- and Alcalase-generated hydrolysates. For example, the HMJ protein mainly had one wide peak at around 6 min, whereas after hydrolysis, there were two high peptide peaks. The trypsin hydrolysates displayed narrower and higher peaks than the Alcalase hydrolysates. All four kinds of dark tea hydrolysates generated by trypsin displayed similar, moderate peptide profiles in which the first peak was eluted out earlier, at around 5 min. The order of elution time was FZ < HMJ < HZ ≈ QL (Fig. 3), which was also consistent with the order of their DH values. This suggests that trypsin released peptides from the various dark teas at different rates.

In addition, for both complete proteins and hydrolysates, a narrow high peak appeared at a retention time of 12.5 min, with a nearly undetectable signal at 280 nm (not included in Fig. 3), the absorbance of peptide bound. The strongest signal was at 215 nm, suggesting the presence of a flavanoid. Although for an individual protease, higher DH might cause more bioactive peptides to be released from proteins, the ultimate and maximal inhibition activity of the hydrolysates were determined by the enzyme's structure, especially the nature of the peptides released in different hydrolysates. This explains the lack of correlation between the DH and the inhibitory activity of the samples.

In vitro assessment of biological activity

α-Amylase inhibition activity

Several plant proteins and peptides have been found to inhibit α-amylase activity in vitro (Siow et al., 2017; Sintsova et al., 2018; Natashya et al., 2018). However, to date, no reports are available on the α-amylase inhibitory activity of dark tea protein hydrolysates. The IC_{50} values of the four kinds of dark tea hydrolysates generated by trypsin and Alcalase were as follows: 2.78 ± 0.04 mg/mL for HMJ, 1.38 ± 0.03 mg/mL for FZ, 1.95 ± 0.06 mg/mL for HZ, and 1.27 ± 0.03 mg/mL for QL. The IC_{50} value of acarbose was 0.56 ± 0.06 mg/mL. It is possible that compounds with α-amylase inhibitory activity in the complete protein may be degraded during hydrolysis by trypsin and Alcalase. A similar phenomenon was observed by Harnedy and FitzGerald (2013), in which the *Palmaria Palmata* protein exhibited higher renin inhibitory activity than its hydrolysate generated by Alcalase.

Fig. 4 indicates the dose-response curves of four kinds of complete proteins. Inhibitory activity reached nearly its highest stage at a sample concentration of 4 mg/mL, in the following order: QL (64.94% ± 1.25%) > FZ (61.08% ± 0.76%) > HZ (59.09% ± 1.10%) > HMJ (56.24% ± 0.90%).

Interestingly, the α-amylase inhibitory activity of all four kinds of dark tea was largely diminished after hydrolysis, and none of the hydrolysate fractions demonstrated significant inhibitory activity at a concentration range of 1-4 mg/mL (indicated in Tab. 1). Most of the protein hydrolysates of HMJ, HZ, and QL negatively inhibited α-amylase. Low inhibitory activity was found in the hydrolysates of FZ; the highest value occurred at a concentration of 1 mg/mL, (21.91% ± 0.02% for the trypsin-generated hydrolysate and 29.48% ± 0.04% for the Alcalase-generated hydrolysate). No regular dose-response relationship was found (see Tab. 1). The reason for this is unknown; perhaps α-amylase's acting sites are related to the cleavage sites of trypsin and Alcalase, whereas FZ has a different protein composition than HMJ, HZ, and QL.

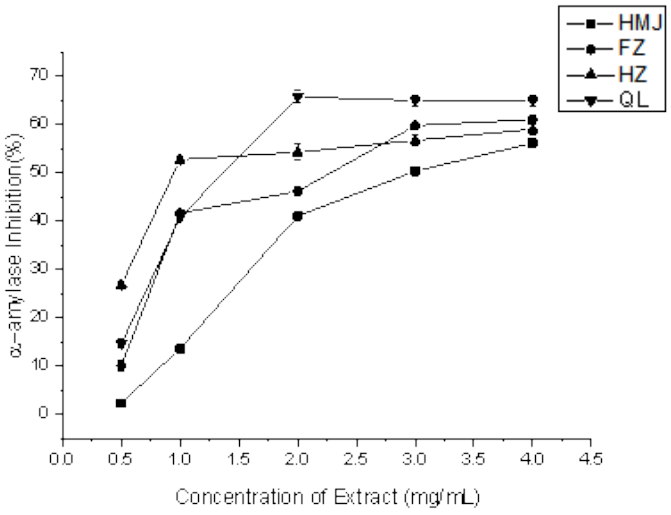

Fig. 4: Inhibitory activity against α-amylase of different concentrations of four kinds of complete black tea proteins. Mean ± SD (n=4).

Tab. 2: IC_{50} (concentration that inhibits enzyme activity by 50%) of four kinds of dark tea and their hydrolysates generated by trypsin and Alcalase against α-glucosidase and DPP-IV. Mean ± SD (n=3). IC_{50} with different letters for each of the activities are significantly different at $p < 0.05$.

Sample	Hydrolysate IC_{50} (mg/mL) for α-glucosidase		
	Complete	Trypsin	Alcalase
HMJ	0.0942 ± 0.0023^{g}	0.2161 ± 0.0036^{f}	0.2036 ± 0.0022^{f}
FZ	0.5401 ± 0.0042^{d}	1.3114 ± 0.0174^{a}	0.6588 ± 0.0045^{b}
HZ	0.5478 ± 0.0128^{d}	0.6231 ± 0.0355^{c}	0.5069 ± 0.0011^{e}
QL	0.0103 ± 0.0025^{h}	0.0349 ± 0.0026^{h}	0.0233 ± 0.0024^{h}
Sample	Hydrolysate IC_{50} (mg/mL) for DPP-Ⅳ		
	Complete	Trypsin	Alcalase
HMJ	0.1794 ± 0.0204^{ghi}	0.1315 ± 0.0017^{hi}	0.1000 ± 0.0266^{i}
FZ	0.9124 ± 0.0216^{d}	0.1011 ± 0.0190^{i}	0.1998 ± 0.0176^{gh}
HZ	0.8467 ± 0.0035^{e}	0.3993 ± 0.0178^{f}	0.2477 ± 0.0278^{g}
QL	1.0900 ± 0.0270^{b}	1.3364 ± 0.0056^{a}	1.0281 ± 0.0160^{c}

α-Glucosidase inhibition activity

As an important carbohydrate-hydrolyzing enzyme, α-glucosidase plays a key role in carbohydrate digestion. The inhibition of α-glucosidase helps to delay carbohydrate digestion and prolongs overall carbohydrate digestion time, reducing the glucose absorption rate and consequently blunting the postprandial plasma glucose rise (Bhandari et al., 2008; Kim et al., 2011). Several previous studies have reported the inhibitory activities of plants. For example, Zhou et al. (2017) reported that flavanone compounds in dark tea had an IC_{50} value of 10.2 μM. Liu et al. (2013) reported that aqueous extracts prepared from *Nelumbo nucifera* leaves achieved an IC_{50} value of 1.86 ± 0.018 mg/mL. As indicated in Tab. 2, nearly all the proteins and their hydrolysates exhibited higher α-glucosidase inhibitory activity than acarbose (IC_{50} = 0.7265 ± 0.058 mg/mL). QL exhibited high activity, with an IC_{50} value of 0.0103 ± 0.025 mg/mL before hydrolysis, and 0.0349 ± 0.0025 mg/mL and 0.0233 ± 0.0024 mg/mL after treatment with trypsin and Alcalase, respectively. HMJ showed lower inhibitory capacity after being hydrolyzed, suggesting that some proteins with α-glucosidase inhibition activity may have been cleaved. Moreover, no significant differences were detected among the complete HZ proteins and the two enzymatic hydrolysates. The lowest inhibitory activity was observed in the FZ hydrolysate digested by trypsin, with an IC_{50} value of 1.3114 ± 0.0174 mg/mL.

DPP-IV inhibition activity

The DPP-IV inhibitory activities of HMJ, FZ, HZ, and QL protein hydrolysates obtained by trypsin and Alcalase, respectively, were investigated. In general, fairly high DPP-IV relative inhibitory activity was observed in the protein fractions, with HMJ having the largest DPP-IV relative inhibitory ability, followed by FZ and HZ; QL had the lowest DPP-IV relative inhibitory activity. In a study by Harnedy and FitzGerald (2013), an IC_{50} value of 2.52 ± 0.05 mg/mL was obtained for *Palmaria palmata* aqueous extracts generated by Alcalase, which is much higher than the value in the present study. The most effective protein fraction was HMJ hydrolyzed with Alcalase, which recorded an IC_{50} value of 0.1000 ± 0.0266 mg/mL. IC_{50} values of 0.1794 ± 0.0204 mg/mL and 0.1315 ± 0.0017 mg/mL were found in the complete HMJ protein and the trypsin-treated HMJ fraction. The relative inhibitory ability of FZ and HZ increased dramatically after hydrolysis (Tab. 2), suggesting that some effective bioactive peptides were obtained. QL appeared to have the least effective relative inhibitory activity but still had IC_{50} values of 1.0900 ± 0.027 mg/mL (control), 1.3364 ± 0.0056 mg/mL (trypsin-generated hydrolysate), and 1.0281 ± 0.016 mg/mL (Alcalase-generated hydrolysate).

The results of this in vitro study clearly suggest that dark tea proteins and their hydrolysates have the potential to control hyperglycemia. The α-amylase, α-glucosidase, and DPP-IV inhibitory activities ob-

Tab. 1: Inhibitory activity (%) of four kinds of dark tea hydrolysates generated by trypsin(a) and Alcalase(b) against α-amylase at concentrations of 1 mg/mL, 2 mg/mL, and 4 mg/mL. Concentrations of 4–8 mg/mL were also investigated in the study but not included in this paper. Mean ± SD (n=3). n.a. = no inhibition detected. Values with different letters are significantly different at $p < 0.05$.

Enzyme	Sample	Inhibitory activity (%)		
		4 mg/mL	2 mg/mL	1 mg/mL
Trypsin	HMJ	$-30.97 \pm 0.06^{n.a.}$	$-29.20 \pm 0.04^{n.a.}$	$-34.16 \pm 0.06^{n.a.}$
	FZ	7.88 ± 0.01^{c}	18.81 ± 0.02^{b}	21.91 ± 0.02^{a}
	HZ	$-26.44 \pm 0.11^{n.a.}$	$-4.66 \pm 0.10^{n.a.}$	2.36 ± 0.05^{d}
	QL	$-5.91 \pm 0.02^{n.a.}$	$-10.45 \pm 0.03^{n.a.}$	$-2.65 \pm 0.05^{n.a.}$
Alcalase	HMJ	$-30.51 \pm 0.04^{n.a.}$	$-28.81 \pm 0.03^{n.a.}$	$-20.49 \pm 0.08^{n.a.}$
	FZ	1.68 ± 0.03^{c}	14.84 ± 0.13^{b}	29.48 ± 0.04^{a}
	HZ	$-54.44 \pm 0.08^{n.a.}$	$-51.27 \pm 0.07^{n.a.}$	$-24.21 \pm 0.02^{n.a.}$
	QL	$-7.96 \pm 0.05^{n.a.}$	$-13.30 \pm 0.01^{n.a.}$	$-3.10 \pm 0.01^{n.a.}$

served were different for HMJ, FZ, HZ, and QL proteins and their hydrolysates generated by trypsin and Alcalase. Generally, all four kinds of dark tea protein extracts demonstrated moderate inhibitory activity on α-amylase (IC_{50} values ranged from 1.27 to 2.78 mg/mL), but inhibitory activity was nearly undetectable after hydrolysis. All of the proteins and hydrolysates displayed good inhibitory activity on α-glucosidase (IC_{50} values ranged from 0.0103 to 1.3114 mg/mL) and DPP-IV (IC_{50} values ranged from 0.1000 to 1.3364 mg/mL). The HMJ and QL extracts were better α-glucosidase inhibitors, while the HMJ extracts, FZ hydrolysates generated with trypsin and Alcalase, and HZ hydrolysates generated with trypsin and Alcalase recorded better inhibitory activity on DPP-IV than other fractions.

It is noted that the protein concentrations in dark tea under typical drinking conditions (steeped at 85 °C for 10 min) detected by Bradford assay were as follows: 0.03595 mg/mL (HMJ), 0.04758 mg/mL (FZ), 0.01493 mg/mL (HZ), and 0.08977 mg/mL (QL). Thus, theoretically, the protein concentrations in tea as it is typically drunk is close to the IC_{50} value for α-glucosidase, but 10- and 100-fold concentrations of the tea, respectively, would be required to achieve the IC_{50} values for DPP-IV and α-amylase. In addition, due to the degradation of protein or peptides in the gastrointestinal tract, doses higher than the theoretical values are needed; hence, more effective protein-extraction techniques deserve further study in the future.

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