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Original Research Article

Effects of (-)-epigallocatechin gallate and quercetin on the activity and structure of α-amylase

Jianhui Su, Zhe Tang*
School of Marine and Bioengineering, Yancheng Institute of Technology, Yancheng 224051, PR China

For correspondence:* ***Email:* *tangzhe1983@163.com*

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Abstract

Purpose: *To investigate the effects of (-)-epigallocatechin gallate (EGCG) and quercetin on the activity and structure of α-amylase.*
Methods: *The inhibitory effects of 7 functional factors were compared by measuring half maximal inhibitory concentration (IC_{50}) values. Lineweaver-Burk plots were used to determine the type of inhibition exerted by EGCG and quercetin against α-amylase. The effect of EGCG and quercetin on the conformation of α-amylase was investigated using fluorescence spectroscopy.*
Results: *Quercetin and EGCG inhibited α-amylase with IC_{50} values of 1.36 and 0.31 mg/mL, respectively, which were much lower than the IC_{50} values of the other compounds (puerarin, paeonol, konjac glucomannan and polygonatum odoratum polysaccharide). The Lineweaver−Burk plots indicated that EGCG and quercetin inhibited α-amylase competitively, with ki values of 0.23 and 1.28 mg/mL, respectively. Fluorescence spectroscopy revealed that treatment with EGCG and quercetin led to formation of a loosely-structured hydrophobic hydration layer.*
Conclusion: *This study has unraveled the mechanism underlying the inhibition of α-amylase activity by EGCG and quercetin in vitro. This should make for better understanding of the mechanisms that underlie the antidiabetic effects of EGCG and quercetin in vivo.*

Keywords: *α-Amylase, (-)-Epigallocatechin gallate, Quercetin; Lineweaver−Burk plots, Antidiabetic, Fluorescence spectroscopy*

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by high level of fasting blood glucose. One therapeutic approach for diabetes is to decrease postprandial hyperglycemia by the inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase [1]. α-Amylase (α-1,4-glucan-4-glucanohydrolase) catalyzes the hydrolysis of internal α-1,4-glucosidic linkage in starch, releasing glucose, maltose and maltotriose [2]. The control of carbohydrate digestion and monosaccharide absorption is beneficial for avoiding complications of diabetes. Acarbose, a fermentation product of actinoplanes species, has been shown to inhibit α-amylase competitively [3]. Studies have been carried out to identify inhibitors of α-amylase from natural sources so as to develop physiologically

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functional foods for treating diabetes [4,5]. Studies have shown that tea polyphenols and flavonoids effectively inhibit the activity of α-amylase [6,7]. Tea catechins include EGCG, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC). In recent studies, it was shown that EGCG treatment ameliorated free fatty acid-induced peripheral insulin resistance through decrease in oxidative stress, activation of the AMPK pathway and improvement of the insulin signaling pathway *in vivo* [8]. Although the prevention and treatment of type 2 diabetes mellitus have been investigated using EGCG supplementation [9], the effect of EGCG on the secondary and tertiary structures of α-amylase have not been investigated. Based on previous reports, dietary polyphenols have considerable potential for reducing the risk of diabetes. Epidemiological studies have also shown that the intake of certain types of flavonoids, including quercetin and myricetin is inversely associated with the risk of type 2 diabetes [10]. Flavonoids are beneficial for reducing the risk of metabolic syndrome. In addition to their antioxidant effects, flavonoids have been reported to prevent diabetes *in vivo* [11]. Studies on the inhibitory effects of isolated flavonoid compounds against α-glucosidase and α-amylase revealed that quercetin inhibited α-amylase with IC_{50} of 4.8mM [6,7]. However, the effect of quercetin on α-amylase conformation has not been demonstrated.

The objectives of the present study were to evaluate *in vitro* pancreatic α-amylase-inhibitory activities of 7 functional factors, and the mechanism underlying the inhibition of α-amylase by EGCG and quercetin. Furthermore, fluorescence measurements were applied to analyze changes in the tertiary structure of α-amylase due to interaction of the enzyme with EGCG and quercetin.

EXPERIMENTAL

Materials

α-Amylase was purchased from Sigma Aldrich (St. Louis, MO, USA). (-)-Epigallocatechin gallate (EGCG), quercetin, puerarin, paeonol, sulfated konjac glucomannan (SKGM) and *Polygonatum odoratum* polysaccharide (PoPs) (> 98 % purity) were purchased from Jingzhu Biotechnology Co. Ltd (Nanjing, China). Enzymatic assays were carried out using a UNIC-2100 visible spectrum.

α-Amylase inhibition assay

The inhibition of α-amylase was assayed according to the procedure of Song Liu [8]. Sample solution (50 µL) and 50 µL of 20 mM phosphate buffer (pH 6.9) containing 0.006 M sodium chloride and α-amylase solution (15 u/mL) were incubated at 37 °C for 10 min. The reaction was initiated by adding 600 µL of 1.5 % starch solution in 0.02 M sodium phosphate buffer, pH 6.9, and the mixture was incubated for 5 min at 37 °C, followed by the addition of 1 mL dinitrosalicylic acid. The reaction mixture was then placed in a boiling water bath for 5 min, and thereafter cooled to room temperature. The absorbance was measured at 540 nm in a UV–visible spectrophotometer (Shimadzu UV-1700, Japan). Acarbose was used as a positive control. Inhibition was calculated using Eq 1.

Inhibition (%) = {(Abs1- Abs2)/Abs1}100 …… (1)

where Abs1 and Abs2 represent absorbance at 540 nm without and with inhibitor, respectively.

Determination of inhibition mechanism and V_{max} and K_m values

The mechanisms of the inhibitory effect of EGCG and quercetin against α-amylase, and values of maximum velocity (V_{max}) and Michaelis constant (K_m) were determined using the Lineweaver-Burk plot [11]. Substrate solutions at concentrations of 6.0, 8.0, 10.0, 12.0, 14.0, and 16.0 mg/mL were reacted with α-amylase, with or without inhibitor. The concentrations of α-amylase and inhibitor were 0.4 and 0.2 mg/mL, respectively, while distilled water was used as control. The V_{max} and K_m values were obtained from the least-squares regression lines of the double reciprocal plots of the tested sample (inhibitor) concentration (1/[S]) against the reciprocal of reaction rate (1/v). Half –maximal inhibitory concentration (IC_{50}) was calculated from inhibition curve. V_{max} and K_m values were obtained from the least-squares regression lines of the double reciprocal plots of the tested sample (inhibitor) concentration versus the reciprocal of reaction rate.

Fluorescence measurements

All fluorescent spectra measurements on the potential interaction between α-amylase, EGCG and quercetin were carried out on an F-7000 fluorescence spectrophotometer (HITACHI, F-7000, Japan). To each of a series of 5-mL test tubes was successively added 0.3 mL buffer solution (pH 7.4), 0.2 mL α-amylase (1 mg/mL), and varying amounts of EGCG and quercetin. After equilibration for 5 min, fluorescence spectra were measured at excitation wavelength of 280 nm, and emission wavelengths of 300 - 480 nm. The slit width was set at 3 nm, and the scan speed was 12000 nm/min.

Statistical analysis

The results obtained were analyzed with SPSS version 16.0 (SPSS Inc, Chicago, IL, USA). Significant differences were determined by Student t-test. *P*- <0.05 was considered statistically significant.

RESULTS

The inhibitory effects of seven functional factors on α-amylase activities

In this study, the inhibitory effects of seven functional factors against α-amylase were evaluated, with acarbose as control. As shown in Figure 1, the IC_{50} values for α-amylase inhibition by EGCG, quercetin and acarbose (as the positive control) were 0.31, 1.36, 0.45 mg/mL, respectively. The IC_{50} value of EGCG (0.31mg/mL) was much lower than that of acarbose (0.45 mg/mL), indicating that EGCG strongly suppressed α-amylase activity, indicating that it could possibly be utilized for controlling postprandial hyperglycemia. Quercetin (IC_{50} =1.36 mg/mL) had a stronger inhibitory effect on α-amylase activity than puerarin, paeonol, SKGM, and PoPs.

It has been reported that quercetin significantly and dose-dependently decreased plasma glucose level of streptozotocin-induced diabetic rats [12]. In this study, quercetin inhibited α-amylase activity in a dose-dependent manner, indicating that quercetin inhibition may effectively reduce plasma glucose level. Puerarin and paeonol showed weaker α-amylase inhibitory activities, while PoPS and SKGM had little inhibitory activities against α-amylase.

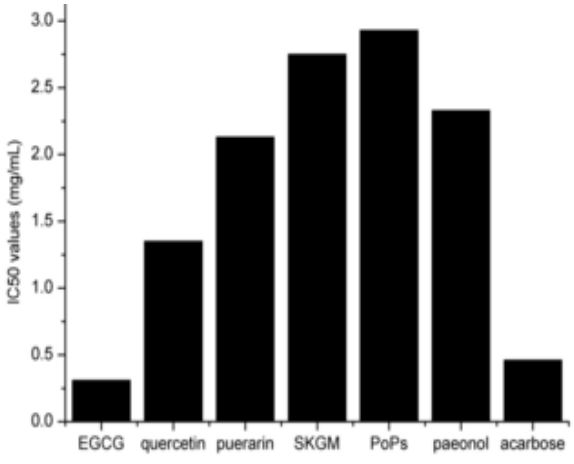

Figure 1: IC_{50} values of 7 functional factors against α-amylase activities

Determination of inhibition types and V_{max} and K_m Values

To investigate the inhibition characteristics of EGCG and quercetin against α-amylase, the kinetics of α-amylase reaction was investigated at different substrate concentrations. The Lineweaver - Burk plots for EGCG (Figure 2 A) and quercetin (Figure 2 B) showed the same intersection on Y-axis, indicating that the mode of inhibition of α-amylase by EGCG and quercetin was competitive.

As the dose of EGCG increased in Figure 2 A, the K_m value for α-amylase increased, while the value of V_{max} remained unchanged. Such results are consistent with competitive inhibition characteristics. The K_i values for EGCG and quercetin were 0.23 and 1.28 mg/mL, respectively. The smaller the K_i, the higher the affinity of the inhibitor for α-amylase and the higher is the inhibition. It appears therefore that the inhibition of starch hydrolysis was significantly higher with EGCG than with quercetin.

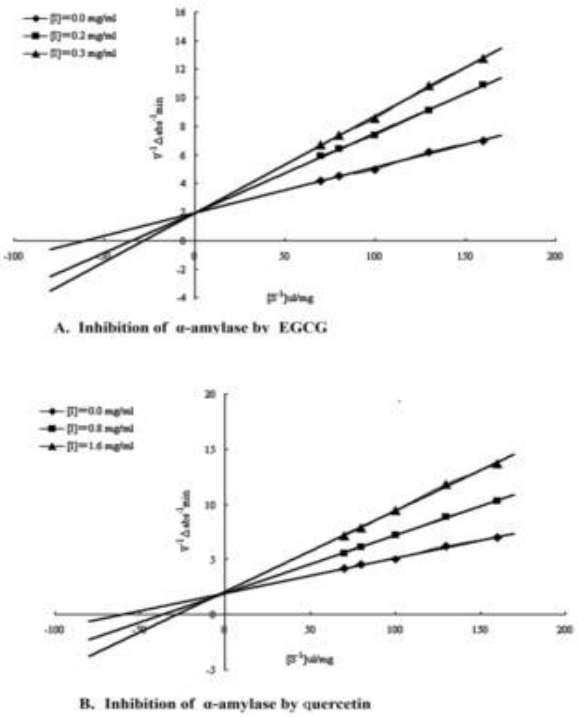

Figure 2: Lineweaver–Burk plots for inhibition of α-amylase by EGCG and quercetin. A: kinetics of inhibition by EGCG; B: kinetics of inhibition by quercetin

Effects of EGCG and quercetin on the tertiary structure of α-amylase

To monitor the changes in the microenvironment of aromatic amino acid residues of α-amylase in

response to EGCG and quercetin treatment, intrinsic fluorescence spectra of the enzyme were recorded in the range of 300 – 500 nm. As shown in Figure 3, the relative fluorescence quantum yields of EGCG- and quercetin-treated α-amylase exhibited obvious decreases. A blue shift in the maximum peak wavelength was observed with increasing concentrations of EGCG and quercetin. The intrinsic fluorescence of α-amylase was quenched by EGCG and quercetin. Compared to quercetin, the addition of increasing concentrations of EGCG caused more progressive reductions in fluorescence intensity. The reduction in fluorescence intensity indicated that EGCG and quercetin treatment induced disruption of hydrophobic bonds, thereby exposing the nonpolar amino acid residues (e.g., tryptophan) to a more polar environment. It also caused the formation of a loosely structured hydrophobic hydration layer, and the fluorescence was quenched by that environment.

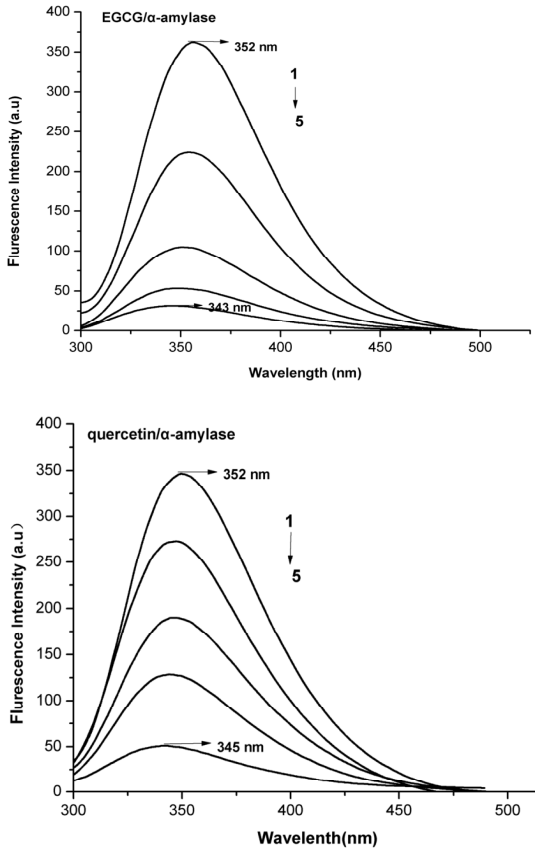

Figure 3: Fluorescence spectra of α-amylase (2.0 mg/mL) with EGCG 1-5: (0, 0.05, 0.1, 0.15, 0.20 mg/mL) and quercetin 1-5: (0, 0.05, 0.1, 0.15, 0.20 mg/mL) pH 7.4, T = 298

DISCUSSION

It has been suggested that the inhibition of α-amylase and other carbohydrate-hydrolyzing enzymes is a potential way of controlling postprandial blood glucose levels. Thus, the search for effective and non-toxic inhibitors of α-amylase has important significance for the prevention and treatment of diabetes.

Radovanović has assessed the antioxidant and antimicrobial activities of polyphenolic extracts of three wild berry fruit species from Southeast Serbia [13]. The anti-glycemic and hypolipidemic potential of polyphenols from *Zingiber officinale* in streptozotocin-induced diabetic rats have been reported [14]. Previous studies have shown that polyphenols and flavonoids inhibit or activate enzymes *in vitro* [15]. In a study by Kalita *et al*, it was reported that potato polyphenolic compounds inhibited pancreatic α-amylase *in vitro* [16].

Radovanović have assessed the antioxidant and antimicrobial activities of polyphenolic extracts of three wild berry fruit species from Southeast Serbia [13]. The antiglycation and hypolipidemic potential of polyphenols from *Zingiber officinale* in streptozotocin-induced diabetic rats have been verified [14]. Previous research have shown that polyphenol and flavonoids have the ability to inhibit or activate enzymes *in vitro* [15]; Kalita discovered that potato polyphenolic compounds have the ability to inhibit pancreatic α-amylase *in vitro* [16].

Recent findings showed that *Qingzhuan* tea extracts exerted potent inhibitory effects on α-amylase [17]. In addition, tea polyphenols composed of EGCG, EGC, ECG and EC inhibited α-amylase with an IC_{50} of 0.41 mg/mL. In the study, EGCG which appeared to be one of the main components of tea polyphenols, exhibited the most effective inhibition of α-amylase, with IC_{50} value of 0.31 mg/mL.

It has been reported that quercetin significantly and dose-dependently decreased the plasma glucose level of streptozotocin-induced diabetic rats [12]. In this study, the inhibition of α-amylase by quercetin was dose-dependent, with IC_{50} value 1.36 mg/mL, indicating that the inhibition may be an effective approach towards decreasing plasma glucose level. Overall, the findings suggest that EGCG and quercetin may limit the release of simple sugars from the gut, thereby alleviating postprandial hyperglycemia.

The fluorescence spectrum was associated with polarity of the environment of the tryptophan and tyrosine residues. The decreases in fluorescence quantum yield may be due to the interaction of chromophores with quenching agents. Changes in intrinsic fluorescence emission have been attributed to the changes in protein tertiary

structure [18]. Molecular interactions between pancreatic lipase and EGCG have been studied [19]. It has been shown that the α-helix content of pancreatic lipase secondary structure decreased as a function of EGCG concentration, and that static fluorescence quenching occurred as a result of EGCG treatment [20].

Tryptophan fluorescence is considered a very reliable index of conformational changes in proteins [21]. Thus, it was used to investigate the effect of EGCG and quercetin on the tertiary structure of α-amylase in this study. The fluorescence intensity of α-amylase decreased with increasing concentrations of EGCG and quercetin. This implies that the binding of EGCG and quercetin to α-amylase caused microenvironment changes in α-amylase.

Inhibitors of α-amylase may directly interact with the side chains of Asp197, Glu233, and Asp300: substitution of these residues lead to a considerable drop in catalytic activity of the enzyme [22]. The inhibitory activity of EGCG on α-amylase led to the formation of soluble or insoluble complexes. The hydrogen bonds between the hydroxyl groups of EGCG and the catalytic residues of the binding site stabilized the interaction with active site [23].

Some researchers have used molecular docking to study the structure-activity relationship in the binding of flavonols to α-amylase and the possible mechanisms involved. Molecular modeling studies revealed that salivary α-amylase inhibitors occupied a docking mode that allowed for H-bonds between the enzyme Asp197 side chain carboxyl oxygen atom and the hydroxyl groups in ring B of the flavonoid skeleton [24]. Thus, the hydrogen bond formed between the quercetin hydroxyl groups and the binding site of the catalytic residue accounts for the inhibition of α-amylase by quercetin.

CONCLUSION

The results of this study indicate that EGCG and quercetin inhibit α-amylase activity in a dose-dependent manner. Lineweaver−Burk plots demonstrate that inhibition of α-amylase by EGCG and quercetin are competitive. Furthermore quenching of fluorescence ofα-amylase induced by EGCG and quercetin suggest possible changes in the conformation of α-amylase which decreased enzyme catalytic activity. If this antidiabetic function is confirmed after clinical studies in type 2 diabetic patients, EGCG and quercetin should be beneficial in the treatment of hyperglycemia.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. All authors read and approved the manuscript for publication.

REFERENCES

1. *Souza PM, Sales PM, Simeoni LA, Silva EC, Silveira D, Magalhães PO. Inhibitory activity of α-amylase and α-glucosidase by plant extracts from the Brazilian cerrado. Planta Med 2012; 78: 393.*
2. *Mj VDM, Van DVB, Uitdehaag JC, Leemhuis H, Dijkhuizen L. Properties and applications of starch-converting enzymes of the alpha-amylase family. J Biotechnol 2002; 94: 137-155.*
3. *Al KM, Desseaux V, Marchismouren G, Prodanov E, Santimone M. The mechanism of porcine pancreatic alpha-amylase. Inhibition of maltopentaose hydrolysis by acarbose, maltose and maltotriose. Febs Journal 2010; 252: 100-107.*
4. *Hansawasdi C, Kawabata J, Kasai T. Î±-Amylase Inhibitors from Roselle (Hibiscus sabdariffa Linn.) Tea. J Agricul Chem Soc Japan. 2000; 64: 1041-1043.*
5. *Kusano R, Ogawa S, Matsuo Y, Tanaka T, Yazaki Y, Kouno I. α-Amylase and Lipase Inhibitory Activity and Structural Characterization of Acacia Bark. Proanthocyanidins. J Nat Prod 2011; 74: 119-128.*
6. *He Q, Lv Y, Yao K. Effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin and lipase. Food Chem 2007; 101: 1178-1182.*
7. *Nickavar B, Abolhasani L. Bioactivity-Guided Separation of an α-Amylase Inhibitor Flavonoid from Salvia virgata. Iran J Phanm Res 2013; 12: 57.*
8. *Li Y, Zhao S, Zhang W, Zhao P, He B, Wu N, Han P. Epigallocatechin-3-O-gallate (EGCG) attenuates FFAs-induced peripheral insulin resistance through AMPK pathway and insulin signaling pathway in vivo. Diabetes Res Clin Pr 2011; 93: 205-214.*
9. *Wolfram S, Raederstorff D, Preller M, Wang Y, Teixeira SR, Riegger C, Weber P. Epigallocatechin Gallate*

Supplementation Alleviates Diabetes in Rodents. J Nutr 2006; 136: 2512.

10. *Rigelsky JM, Sweet BV. Hawthorn: pharmacology and therapeutic uses. American journal of health-system pharmacy: AJHP 2002; 59: 417.*
11. *Li B, Zhou B, Lu H, Ma L, Peng AY. Phosphaisocoumarins as a new class of potent inhibitors for pancreatic cholesterol esterase. Eur J Med Chem 2010; 45: 1955*
12. *Kim JH, Kang MJ, Choi HN, Jeong SM, Lee YM, Kim JI. Quercetin attenuates fasting and postprandial hyperglycemia in animal models of diabetes mellitus. Nutr Res Pract 2011; 5: 107.*
13. *Radovanović BC, Milenković Anđelković1 AS, Radovanović1 AB and Anđelković MZ. Antioxidant and Antimicrobial Activity of Polyphenol Extracts from Wild Berry Fruits Grown in Southeast Serbia. Trop J Pharm Res 2013; 12: 813-819*
14. *Kazeem MI, Akanji MA, Yakubu MT and Ashafa AO. Antiglycation and Hypolipidemic Effects of Polyphenols from Zingiber officinale Roscoe (Zingiberaceae) in Streptozotocin-Induced Diabetic Rats. Trop J Pharm Res 2015; 14 : 55-61*
15. *Prochã ZD, Bouå OI, Wilhelmovã N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 2011; 82: 513-523.*
16. *Kalita D, Holm DG, LaBarbera DV, Petrash JM, Jayanty SS. Inhibition of α-glucosidase, α-amylase, and aldose reductase by potato polyphenolic compounds. PlosOne 2018; 13: e191025.*
17. *Cheng Q, Cai S, Ni D, Wang R. In vitro antioxid ant and pancreatic α-amyla se inhibitory activity of isolated fractions from water extract of Qingzhuan tea. J Food Sci Technol 2015; 52: 928–935*
18. *Vol. N. Effects of High-Hydrostatic-Pressure Processes on Food Safety and Quality. Food Technol-Chicago. 1993; 47(6).*
19. *Wu X, He W, Yao L, Zhang H, Liu Z, Wang W, Ye Y, Cao J. Characterization of binding interactions of (-)-epigallocatechin-3-gallate from green tea and lipase. J AGR Food Chem 2013; 61: 8829.*
20. *Wang S, Sun Z, Dong S, Liu Y, Liu Y. Molecular Interactions between (−)-Epigallocatechin Gallate Analogs and Pancreatic Lipase. PlosOne 2014; 9: e111143.*
21. *Yi J, Jiang B, Zhang Z, Liao X, Zhang Y, Hu X. Effect of Ultrahigh Hydrostatic Pressure on the Activity and Structure of Mushroom (Agaricus bisporus). Polyphenoloxidase. J Agr Food Chem 2012; 60: 593.*
22. *Brayer GD, Sidhu G, Maurus R, Rydberg EH, Braun C, Wang Y, Nguyen NT, Overall CM, Withers SG. Subsite mapping of the human pancreatic alpha-amylase active site through structural, kinetic, and mutagenesis techniques. Biochemistry-Us. 2000; 39: 4778-4791.*
23. *Bandyopadhyay P, Ghosh AK, Ghosh C. Recent developments on polyphenol–protein interactions. effects on tea and coffee taste, antioxidant properties and the digestive system. Food Func 2012; 3: 592.*
24. *Lo PE, Scheib H, Frei N, Williamson G, Grigorov M, Chou CJ. Flavonoids for controlling starch digestion: structural requirements for inhibiting human alpha-amylase. J Med Chem 2008; 51: 3555-3561.*