Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence

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The noncovalent binding of selected phenolic compounds (chlorogenic-, ferulic-, gallic acid, quercetin, rutin, and isoquercetin) to proteins (HSA, BSA, soy glycinin, and lysozyme) was studied by an indirect method applying the quenching of intrinsic tryptophan fluorescence. From the data obtained, the binding constants were calculated by nonlinear regression (one site binding; $y = Bx/k + x$). It has been reported that tannins inhibit human salivary amylase and that these complexes may reduce the development of cariogenic plaques. Further, amylase contains two tryptophan residues in its active site. Therefore, in a second part of the study involving 31 human subjects, evidence was sought for noncovalent interactions between the phenols of green tea and saliva proteins as measured by the fluorescence intensity. Amylase activity was determined before and after the addition of green tea to saliva of 31 subjects. Forty percent of the subjects showed an increase in amylase activity contrary to studies reporting only a decrease in activity. The interactions of tannin with amylase result in a decrease of its activity. It still remains to be elucidated why amylase does not react uniformly under conditions of applying green tea to saliva. Further, in terms of using phenols as caries inhibitors this finding should be of importance.

Keywords: Amylase activity / Green tea phenols / Protein-phenol binding / Saliva proteins / Tryptophan quenching

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1 Introduction

Dietary phenolic substances have received much attention due to their biological properties. They have been known to induce antimutagenic and anticancerogenic effects as well as being antioxidants [1], but many studies have also demonstrated their harmful effects especially when applied in high concentrations [2]. For example, inclusion of polyphenols in the diet can lead to perturbation of mineral absorption from the intestinal canal, a decrease in bodyweight gain and growth retardation, and inhibition of digestive enzymes [2, 3]. Some of these properties may be directly or indirectly induced/caused by their interactions with proteins [1]. Most of our former work was focused mainly on such reactions of the phenolic substances with proteins and enzymes that lead to covalent bonds [1]. Although both covalent and noncovalent binding is likely to take place, it seems that a larger part of these protein-phenol interactions may be governed by noncovalent binding as documented well for saliva proteins [2 – 9].

Different experimental approaches for determining binding parameters for protein-plant phenolics on the basis of noncovalent interactions may be applied [10 – 15]. With the exception of fluorescence spectroscopy, existing methods for assaying phenol binding to proteins are labour-intensive, time-consuming, or may require compound-specific assays [10, 12 – 14, 16]. Unless identification of the site specificity of phenol binding to a protein is desired, quenching of the tryptophan fluorescence of proteins by titration with a ligand may prove to be a rapid and facile method for determining the binding affinities of the phenolic compounds to proteins. Therefore, the first objective of this study was to test the applicability of such a method by applying selected phenolic compounds (chlorogenic-, ferulic-, gallic acid, quercetin, rutin, and isoquercetin) and model proteins (HSA, BSA, soy glycinin (SG), and lysozyme). In a second part, a green tea decoction (GT) representing a complex

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Abbreviations: DM, dry mass; **FI,** fluorescence intensity; **GT,** green tea decoction; **HRP,** histamin-rich proteins; **PRP,** proline-rich proteins; **SG,** soy glycinin

phenol-containing food matrix was tested in its binding affinity by quenching of the tryptophan in saliva proteins. Human whole saliva is a complex mixture of some 330 proteins and peptides (Human Salivary Proteome Project, HSPP; Wong, D., Loo, J., March 2005; http://www.hspp.ucla.edu). Green tea was chosen due to its lower content of high molecular phenolic compounds (hydrolysable and condensed tannins). In this context it has also been reported that tea consumption can be effective in reducing the cariogenic potential of starch-containing foods such as crackers and cakes by the inhibition of salivary amylase [17]. Therefore, the third objective was to test if a masking of the amylase activity in human saliva occurs by the noncovalent binding of the green tea phenolics.

2 Materials and methods

2.1 Materials

BSA (Serva, Heidelberg, Germany), α-amylase (from porcine pancreas, EC 3. 2. 1. 1, protein content 53%, 55 U/mg solid, 1 U will hydrolyze 1 μmol maltose from starch *per* min at pH 6.9 and 25°C), HSA, and lysozyme (all from Fluka, Steinheim, Germany) were applied. SG was prepared from defatted unheated soy flour as described in [18].

The following phenolic compounds were applied: Quercetin, tannin (both from Riedel-deHaën Laborchemikalien, Seelze, Germany), isoquercetin (quercetin-3-*O*-glucoside), rutin (quercetin-3-*O*-rhamnoglucoside; both from Carl Roth, Karlsruhe, Germany), (−)-epicatechin, (+)-catechin, ferulic-, chlorogenic-, and gallic acid (all from Fluka).

2.2 Binding studies using intrinsic tryptophan fluorescence of model proteins

Quenching of tryptophan fluorescence of proteins by ligand binding was followed by adopting the method of [19]. The buffer conditions used were 0.05 M Hepes buffer at pH 7 or 4. 8. A suitable protein concentration (BSA, HSA, lysozyme, SG: 0.1–4.54 μM, see Supplementary Material) was chosen and a series of increasing phenol concentrations was added to it (quercetin, isoquercetin, rutin, ferulic-, chlorogenic-, and gallic acid: 0–500 μM, see Supplementary Material for details). Four measurements were performed for each phenol concentration step. The samples were excited with Jasco fluorescence detector FP 920 at 290 nm (slit 18 nm) and the emission recorded at 344 nm (slit 18 nm). Since the phenols are known to absorb both at the excitation wavelength (290 nm) as well as at the emission wavelength (344 nm), their absorbance was recorded for each phenol concentration step and used for the corresponding corrections during calculation of the binding parameters.

2.3 Collection of whole human saliva

Whole mouth saliva from all the 31 volunteers (14 males – 26–50 years; 17 females – 21–54 years; all being nonsmokers except one) was collected in the mornings before 12 pm. No food or drink other then water was consumed for at least 2 h before saliva collection. Fresh unstimulated saliva (whole saliva and other oral fluids) was collected using a Sarstedt Salivette collection device (Sarstedt AG, Nümbrecht, Germany) [20]. The collected samples were immediately stored on ice to prevent proteolytic degradation. Saliva was separated from the cotton roll by centrifugation at $700 \times g$ for 10 min (4°C), followed by centrifugation at $14000 \times g$ for 30 min (4°C) to remove whole cells or their fragments. The supernatants were pooled together (8–12 mL) and than divided into aliquots, transferred to new tubes and finally stored at −70°C till needed.

2.4 Characterization of the saliva proteins

The protein content of the saliva samples was determined using the assay with bicinchoninic acid according to [21]. The SDS-PAGE analysis was performed with a 14% separating gel applying the procedure of Laemmli [22].

2.5 Characterization of the green tea phenols

The amount of total phenolics in green tea was determined according to the Folin-Ciocalteu procedure [23]. Sample preparation: 200 mg of green tea leaves were extracted twice, each time with 5 mL of methanol-water mixture (70:30) for 10 min at 70°C, centrifuged at $3000 \times g$ for 5 min and the supernatants combined and filled up to 10 mL. This extract was diluted 1:100 for the determination. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams *per* gram dry material.

Determination of the composition of the green tea phenolics was conducted as follows: Green tea (0.2 g) was extracted with *ca.* 23 g of 1% *o*-phosphoric acid for 30 min in an ultrasonic bath. This extract was then diluted (1:2, with a solution containing 250 mL of 1% *o*-phosphoric acid, 225 mL of methanol and 200 mg of ascorbic acid).

Diluted samples were filtered and 10 μL were injected and analyzed by RP-HPLC on a Gromsil 120 ODS-5 column (20 mm × 2 mm, 5 μm; Alltechs Grom, Rottenburg, Germany) at 40°C column temperature. The flow rate was 0.3 mL/min and the eluents applied were A = methanol and B = 0.005 M tetra-*n*-butylammoniumhydrogensulfate. The

gradient was under the following conditions: 5% eluent A – 0 min; 30% eluent A – 35 min; 40% eluent A – 40 min; 5% eluent A – 5 min (regeneration/equilibration). Detection and identification was conducted at 205 and 275 nm using the corresponding external standards.

2.6 Preparation of GT

Commercially available green tea leaves (1 g; Grüner Tee, TEEHAUS, Dresden, Germany) were extracted (daily fresh preparation) with 60 g of boiling deionized water by stirring for 10 min, where the liquid level was then filled up to 65 g with deionized water and stirred for a further minute. Fifty milliliters of this decoction were then centrifuged at 3300 × *g* for 10 min and the supernatant was applied as is or with the corresponding dilution for the binding studies.

2.7 Incubation of the green tea with saliva samples

Effect of exposure of green tea to light and air (0–300 min): Saliva (50 μL) of three subjects (S4, S8, S9) and HSA solution (50 μL; 1 mg/mL) were mixed with 10 μL GT, diluted to 1 mL and the tryptophan fluorescence intensity (FI) of the solution was immediately measured as described above.

Incubation of all saliva samples with green tea: Saliva (X μL; corresponding to final protein concentration of 50 μg protein/mL) and HSA solution (50 μg/mL) were mixed with 5 μL GT, diluted to 1 mL and the tryptophan FI of the solution was immediately measured as described above.

Incubation of saliva and α-amylase with green tea and main representatives of the phenolic compounds in green tea ((+)-catechin, (−)-epicatechin, and tannin): Stock solutions (250 μg/mL) of (+)-catechin, (−)-epicatechin, and tannin were applied. Green tea was diluted 1 : 5 before application. Thirty-five microliters of a saliva sample (S9, 1 mg protein/mL stock solution) and amylase (35 μL; 1 mg/mL stock solution) were mixed with different amounts (3–50 μL) of the diluted green tea and the main representatives of the phenolic compounds, filled up to 1 mL and the tryptophan FI of the solution was immediately measured as described above.

2.8 Determination of amylase activity

The hydrolytic activity of α-amylase was investigated by degradation of starch (soluble starch; Merck, Darmstadt, Germany) using 3,5-dinitrosalicylic acid (Sigma quality control test procedure) and maltose (Sigma, Steinheim, Germany) as external standard [24]. One unit will liberate 1 mg of maltose from starch in 3 min at pH 6.9 and 20°C.

Sample preparation: The saliva samples were diluted 1 : 300 with 20 mM sodium-phosphate buffer containing 6.7 mM NaCl (pH 6.9) before determination.

For the incubation with green tea extract: 100 μL of the green tea were mixed with 100 μL saliva and immediately 20 μL of the mixture was removed and diluted to 3000 μL with sodium-phosphate buffer for the determination of the amylase activity.

For the determination of the amylase activity as influenced by the concentration of the phenolic compounds: 35 μL of amylase solution (1 mg/mL) was mixed with different volumes of the tannin solution (0–50 μL of the stock solution containing 1 mg tannin/mL) or of the GT (0–50 μL) and filled up to 1 mL with deionized water. These were then diluted 1 : 4 with the sodium-phosphate buffer before using them for the determination of the amylase activity.

2.9 Statistical analysis

Generally, the analyses were repeated at least three times and evaluated by their means and SD. The averaged data obtained from the binding studies were used for the calculations of the binding parameters (binding constant K_D). The best-fit values for binding parameters were achieved by applying nonlinear least-squares regression using the software Microcal Origin 6.0 (Microcal Software, Northampton, USA). The statistical significance for $p < 0.05$ was determined according to the Mann und Whitney test using the software SPSS (vers. 11; SPSS Software, Munich, Germany).

3 Results and discussion

3.1 Determination of the binding parameters

It has been reported that the binding of certain ligands to proteins may lead to a quenching of tryptophan fluorescence [19]. Quenching of tryptophan fluorescence may occur directly by optically transparent ligands with low or high affinities or by chromophores whose absorption either overlaps the tryptophan emission (330–350 nm) and quenches by energy transfer or absorbs light at the tryptophan fluorescence excitation wavelength (295–305 nm) producing absorptive screening as well as fluorescence quenching [19]. The analysis of the data was performed according to [19] using the following equation:

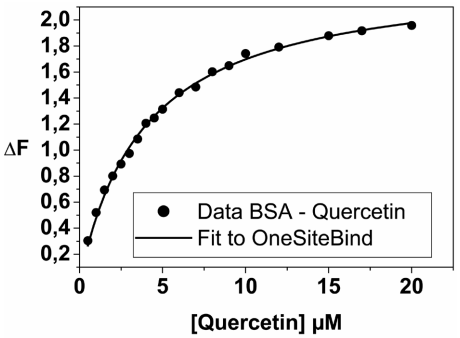

Figure 1. Determination of K_D using the quenching of the tryptophan fluorescence at pH 7. [Quercetin] = 0–20 µM; [BSA] = 1 µM; model = one site binding; Chi² = 0.00076, R^2 = 0.99, ΔF_{max} = 2.37 ± 0.03, $K_D \times 10^{-6}$ = 3.9 ± 0.1 M. ΔF is the corrected difference between F_0 and F (see Eq. (1) and (2)), where F and F_0 are the measured fluorescence emission intensity of the protein solution in presence and in absence of the ligand.

$$F_0 - F = \frac{(F_0 - F_\infty)L_0}{K_D + L_0} \quad (1)$$

where F and F_0 are the measured fluorescence emission intensity of the protein solution in the presence and absence of the ligand, respectively, F_∞ when the protein is saturated with the ligand, and L_0 the total concentration of the bound and unbound ligand. The decrease in tryptophan fluorescence in the presence of different concentrations of the ligand is measured and the dissociation constant as well as the value for maximum fluorescence difference ($\Delta F_{max} = F_0 - F_\infty$) can then be calculated as shown in Fig. 1. Since many phenolic compounds absorb both at the wavelength of excitation and emission, a correction is needed according to Lambert Beer's law as follows [19]:

$$F = F_u 10^{(\varepsilon_{\lambda ex} + \lambda_{em})} l L_0 \quad (2)$$

where F_u is the experimentally measured uncorrected and F the corresponding corrected value for the fluorescence; $\varepsilon_{\lambda ex}$ or $\varepsilon_{\lambda em}$ represents the molar extinction factor at the excitation or emission wavelength, and l is the path length in the measuring cell. The extinction factors are determined from the measured extinction and their linear correlation. The plotting of the corrected fluorescence is analyzed by means of nonlinear least-squares regression fit using Eq. (1) as shown in Fig. 1 for the BSA-quercetin model at pH 7. The values thus obtained are given in Table 1. Besides the phenolic compounds listed in Table 1, rutin, chlorogenic-, and gallic acid were also tested. But, these were found to incur no quenching of the tryptophan fluorescence in the applied protein and phenol concentrations. Rutin produced a change in the tertiary structure of BSA (measured by circular dichroism) and the application of the Hummel-Dreyer method also showed a positive binding of chlorogenic- and gallic acid to BSA [14]. Ferulic acid and quercetin, on the other hand, seem to prefer to bind near the tryptophan sites of HSA and BSA. HSA has only one tryptophan residue, which is situated in the domain IIa and is also known to be an important part of the binding site Sudlow1 [25]. In this context, recent data suggested that the association between flavonoids and BSA did not change the molecular conformation of BSA (FT-IR Spectroscopy) [26], whereas circular dichroism measurements do prove changes in protein tertiary structure accompanying ligand binding with secondary structure remaining intact [14]. Experiments with isoflavones (genistein and daizein) have also identified the binding of these flavonoids to subdomain IIa of HSA, with circular dichroism and FT-IR data indicating changes in secondary and tertiary structures [27, 28].

As documented in Table 1, quercetin has high affinity for HSA and BSA, followed by SG and lysozyme. Further, quercetin also has a better affinity compared to ferulic acid and isoquercetin. In case of isoquercetin, these results underline the importance of the C3–OH group, which is blocked here. The loss of a hydroxyl group and the corresponding decrease in the binding affinity to BSA also reflects the role of electrostatic/hydrogen bonds as already

Table 1. Dissociation constants (K_D) for binding of selected phenolic compounds to different proteins as determined by the quenching of the tryptophan fluorescence (model = one site binding) in comparison to other methods

Conditions			$K_D \times 10^{-6}$ (M) – method applied		
Protein	Phenol	pH	Tryptophan fluorescence	Quercetin fluorescence[a)]	Hummel-Dreyer method[a)]
BSA	Quercetin	7.0	3.9 ± 0.1	9.1 ± 0.5	–
Lysozyme	Quercetin	7.0	35.4 ± 4.6	2000 ± 800	–
SG	Quercetin	7.0	16.5 ± 0.7	4.1 ± 0.6	–
HSA[b)]	Quercetin	7.0	2.7 ± 0.2	1.9 ± 0.2	–
BSA	Isoquercetin	7.0	40 ± 20	–	–
BSA	Ferulic acid	4.8	200 ± 46.8	–	58 ± 5
BSA	Ferulic acid	7.0	18 ± 4.0	–	249 ± 14

a) According to [14].
b) Reference values $K_D = 5.3 \times 10^{-6}$ M (quercetin-anisotropy) [16] or $K_D = 3.7 \times 10-^6$ M (ultracentrifugation) [10].

Table 2. The standard Gibbs free energy (ΔG) for binding of selected phenolic compounds

Protein	Phenol	pH	ΔG (kJ/mol)
BSA	Quercetin	7.0	−32.3
Lysozym	Quercetin	7.0	−26.8
Sojaglycinin	Quercetin	7.0	−28.7
HSA	Quercetin	7.0	−33.2
BSA	Ferulic acid	4.8	−22.5
BSA	Ferulic acid	7.0	−28.6

reported in [14]. Rutin did not induce a quenching of tryptophan and is larger (MW = 665 Da) in size than isoquercetin (MW = 464 Da), which in turn is larger than quercetin (MW = 302 Da). This observation shows that the molecule size of the ligand may also play a role in the binding of phenolic compounds.

On the basis of the binding constants obtained, it is possible to calculate the standard Gibbs free energy (ΔG) as documented in Table 2.

$$\Delta G = -RT \ln(K_X) \quad (3)$$

The free enthalpy is negative for the studied binding constants, indicating the "freeness" of the interactions taking place between the proteins and the phenolic compounds. The relatively strong binding enthalpy obtained, *e.g.*, for the HSA- and BSA-quercetin complex, underlines the stability of these complexes from the energetics point of view.

In general, different methods may be suitable to explore the interactions between proteins and plant phenolic compounds [10, 13, 14, 16]. Each method does not necessarily lead to a useable result. So, for example, no protein-bound quercetin, rutin, and isoquercetin could be determined by the Hummel-Dreyer method [14]. Moreover, rutin, chlorogenic acid, and gallic acid did not quench the tryptophan fluorescence of BSA as shown above. The extent of binding is determined by parameters like equilibrium binding constants, which in turn depend on the method applied as shown in Table 1. The best agreement between the indirect methods was obtained for the binding parameters of quercetin to HSA (Table 1), also in agreement with those reported elsewhere [10, 14, 16] using other methods (Hummel-Dreyer method, quercetin-anisotropie and analytical ultracentrifugation). The values obtained for BSA and SG differ slightly, whereas for lysozyme, a strong deviation is noted (Table 1). The physicochemical basis for the indirect methods lies in the spectroscopic properties of tryptophan. The content of tryptophan in the proteins applied differs, where HSA has 1, BSA 2, lysozyme 6, and SG may have 15 – 20 tryptophan residues *per* molecule (NCBI Protein data bank). It appears that with increasing amount of tryptophan residues present in the molecule, the difference in the binding parameters obtained from the indirect methods also sway from one another. Not only, the molecular size and structure of the proteins, but also the evaluation of the raw data obtained plays an important role. A comparison of the direct and indirect studies is given for the binding of ferulic acid to BSA (Table 1). The interpretation of these results would mean that the binding affinity of ferulic acid to BSA increases with the decrease of pH. At the same time the number of binding sites decreased as documented in [14] using the Hummel-Dreyer method. One (or more) of these binding sites lies in the near vicinity of the tryptophan residue in BSA. The affinity for this particular site decreases with the decrease in the pH. From this example, it can be seen that further information on the nature of the binding can be obtained by applying both indirect and direct binding studies.

3.2 Interactions of human saliva proteins with green tea phenols

3.2.1 Characterization of saliva proteins

The protein concentration in saliva of the 31 subjects was determined to be between 0.8 and 2.6 mg/mL. In literature, the contents of 1 – 3.5 mg/mL protein in saliva have been reported [29]. In general, a high fluctuation was found within the whole subject group, and no significant differences between men and women were observed.

SDS-PAGE of the samples confirmed that the composition of the saliva in each case differed, especially with regard to its content of the individual proteins (results not shown). Some 330 proteins and peptides have been identified by combined application of different methods (*e.g.*, 2-D PAGE, MS, peptide analysis, *etc.*) as reported in the Human Salivary Proteome Project (HSPP; Wong, D., Loo, J., March 2005; http://www.hspp.ucla.edu).

One of the most important group (more than 22 proteins [7]) known to bind phenolic compounds includes the salivary proline-rich proteins (PRP; proline content 28 – 40% [5]), which constitute 70% of the proteins in saliva. A further interesting group also known to play a key role in phenol binding, comprises structurally related, small histamin-rich proteins (HRP, approx. 12 HRPs known) found only in saliva of humans and monkeys [2].

The major enzyme which can be identified in the SDS-PAGE protocols is α-amylase (approx. 58 – 62 kDa). A mixture of more than five isoenzymes has been reported to be present in saliva [30]. In the context of this study, the interest was directed toward interactions of phenolic compounds with α-amylase. Therefore, the content of this fraction was estimated from SDS-PAGE protocols of all 31 saliva samples and is given in Table 3. The concentration varies between 0.2 and 0.42 mg/mL. No significant differences

Table 3. Amylase content (mg/mL) and change in amylase activity of the saliva samples (U/mg protein)

Subject No.	Amylase content[a)]	Amylase activity	
		Normal (control)	With green tea[b)]
1	0.36	23.84 ± 1.86	6.55
2	0.35	21.45 ± 0.97	−38.32[c)]
3	0.30	6.59 ± 0.31	3.97
4	0.39	38.24 ± 2.06	−44.83[c)]
5	0.30	18.20 ± 0.52	−35.64[c)]
6	0.42	43.16 ± 1.99	−38.10[c)]
7	0.32	36.44 ± 0.81	−10.82[c)]
8	0.28	34.87 ± 0.66	−21.37[c)]
9	0.30	35.11 ± 2.40	13.15[c)]
10	0.23	23.13 ± 1.25	81.20[c)]
11	0.26	35.68 ± 0.72	−46.14[c)]
12	0.20	15.12 ± 1.49	47.45[c)]
13	0.32	33.51 ± 0.90	65.24[c)]
14	0.27	36.40 ± 0.66	−32.41[c)]
15	0.26	25.64 ± 1.11	−30.44[c)]
16	0.30	59.28 ± 0.84	−17.44[c)]
17	0.27	45.81 ± 2.66	−37.55[c)]
18	0.36	59.17 ± 0.65	−4.73
19	0.25	30.07 ± 1.19	−0.61
20	0.36	18.15 ± 0.47	−13.15
21	0.33	15.04 ± 1.83	68.93[c)]
22	0.35	6.48 ± 1.08	14.64
23	0.36	7.05 ± 1.49	45.72[c)]
24	0.34	10.17 ± 2.72	19.34
25	0.38	39.34 ± 3.76	−39.93[c)]
26	0.42	18.28 ± 4.12	122.88[c)]
27	0.35	39.86 ± 1.41	−55.96[c)]
28	0.38	41.93 ± 0.70	−64.17[c)]
29	0.32	18.31 ± 3.42	11.78
30	0.40	28.09 ± 0.26	−20.42[c)]
31	0.29	8.52 ± 0.14	−9.28

a) Estimated from SDS-PAGE on the basis of band intensity with MW of *ca.* 58–62 kDa.

b) Effect of green tea addition to saliva on its α-amylase activity given as % increase/decrease in comparison to the control values.

c) Statistically significant difference for $p < 0.5$.

depending on sex were found. The activity of amylase in saliva varies between 6.5 and 59.3 U/mg protein as documented in Table 3. The statistical evaluation by applying the Mann-Whitney test for comparison between male and female subjects showed significant difference ($p = 0.05$), men having in trend higher amylase activity in their saliva.

3.2.2 Characterization of the green tea

Green tea leaves contained a rest moisture of 4.6% and a raw protein content of 25.8% (based on dry mass (DM) as determined by the Kjeldahl method), whereby the corresponding SDS-PAGE of the tea sample did not show any protein band. The total amount of phenolic compounds (GAE) as determined according to Folin-Ciocalteu method was 17.3% (DM). The major phenolic compounds (DM basis) identified by HPLC were 6.8% epigallocatechin gallate (EGCG), 2.2% epigallocatechin (EGC), 1.6% epicatechingallate (ECG), 0.72% epicatechin, and 0.08% catechin, amounting to a total of 11.9%. Around 5.4% of the phenolic compounds were not identified, belonging most likely to the class of high M_r polyphenols (hydrolysable and condensed tannins) [17].

3.2.3 Tryptophan quenching experiments

Preliminary experiments showed that the exposure of GT to light and air for maximum of 300 min did not have any effect on tryptophan quenching (results not shown). Normally (especially under alkaline conditions), low M_r phenolic compounds may undergo spontaneous oxidation and thereby polymerize to tannin-like structures [1]. High M_r tannins show a comparatively higher protein binding capacity as reported in [17]. The pH of the GT was found to be 5.9, thus most likely limiting such reactions.

The determination of tryptophan fluorescence showed individual differences although same amount of protein was present in all the tested samples (Table 4). These results thus reflect not only differences in tryptophan content but also in protein composition of the individual saliva samples as documented by the SDS-PAGE analysis (results not shown). The addition of green tea leads in all cases to a substantial decrease in tryptophan FI as shown in Table 4, thus confirming the immediate binding of green tea components to saliva proteins. The strongest quenching of tryptophan was witnessed in HSA leading to about 81% decrease of the FI as compared to the untreated solution (Table 4). The average decrease in the FI of all the 31 saliva samples amounted to 58 ± 7%.

Several studies report the role of salivary PRPs and HRPs in binding phenolic compounds [2, 3, 5, 7, 9, 29], therefore the focus here is directed toward the binding to α-amylase. Saliva amylase contains 22 proline and 16 tryptophan amino acid residues in its sequence (NCBI Protein data bank). The active site of human saliva amylase consists of seven subsites (−4, −3, −2, −1, +1, +2, +3) and the catalytic site is located between subsites (−1) and (+1) [31]. The nonreducing end of the substrate-binding site of human salivary α-amylase contains two residues Trp58 and Trp59, which belong to β2-α2 loop of the catalytic (β/α)(8) barrel [32]. Trp58 and Trp59 are both located near the subsites (−3)/(−2) [31]. The residue Trp59 is known to stack on to the substrate [32]. Recent results suggest that also the residue Trp58 plays a critical role in substrate binding and hydrolytic activity of human salivary α-amylase [32].

As seen in Table 4, there seems to be a strong binding in the vicinity of tryptophan residues of the saliva proteins, therefore we also studied the effect on the α-amylase activity. Table 3 shows the change caused by the binding of green

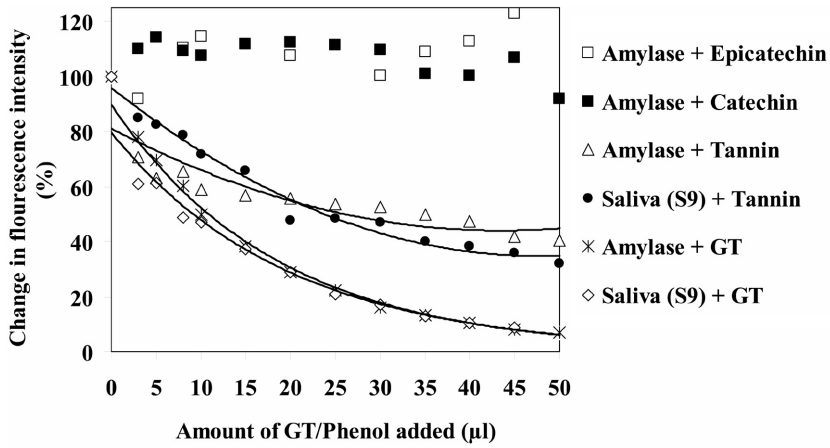

Figure 2. Effect of the incubation of saliva and α-amylase with green tea and main representatives of the phenolic compounds in green tea on the tryptophan fluorescence.

Table 4. Decrease in tryptophan fluorescence by the interactions of saliva proteins and HSA with green tea constituents

Sample/subject No.	Fluorescence intensity (control)[a)]	Fluorescence intensity (after incubation with green tea)[a, b)]
HSA	24 820 ± 1 851	4 763 ± 358
1	36 883 ± 6 678	14 565 ± 551
2	25 289 ± 820	10 661 ± 1 046
3	12 516 ± 1 621	3 636 ± 311
4	35 402 ± 2 786	9 796 ± 488
5	28 739 ± 4 436	11 356 ± 766
6	46 830 ± 4 883	19 140 ± 1 183
7	44 916 ± 5 166	18 236 ± 1 248
8	46 997 ± 4 382	16 882 ± 1 467
9	54 010 ± 2 335	18 602 ± 331
10	40 379 ± 2 477	14 927 ± 505
11	21 458 ± 755	7 586 ± 299
12	28 747 ± 2 331	10 815 ± 462
13	40 221 ± 4 448	19 321 ± 1 090
14	37 311 ± 1 617	13 296 ± 570
15	33 095 ± 2 874	12 626 ± 382
16	63 133 ± 1 104	24 969 ± 1 083
17	53 258 ± 10 472	20 277 ± 1 269
18	64 382 ± 4 752	24 494 ± 674
19	24 959 ± 1 183	10 978 ± 321
20	47 700 ± 1 320	22 269 ± 602
21	30 949 ± 1 548	14 469 ± 1 858
22	27 594 ± 3 841	14 155 ± 2 322
23	14 108 ± 6 126	6 477 ± 518
24	30 610 ± 1 642	13 552 ± 1 355
25	44 269 ± 1 502	21 531 ± 2 510
26	48 636 ± 2 293	26 966 ± 332
27	32 703 ± 5 735	18 569 ± 2 632
28	31 339 ± 1 373	13 667 ± 1 833
29	27 794 ± 442	13 285 ± 2 095
30	66 333 ± 7 050	31 104 ± 1 247
31	20 963 ± 281	9 324 ± 816

a) Measured as area under curve for FI between 300 and 372 nm.
b) Statistically significant decrease for all samples ($p < 0.05$).

tea components on the amylase activity. In most of the cases (60% of the subjects) there is a decrease in the activity, whereas in the saliva of some subjects an increase of up to a maximum of 120% is noted (S26, Table 3).

When individuals were allocated according to corresponding changes in amylase activity after their incubation with green tea, significant lower starting activities ($p < 0.001$) were observed in the group in which the incubation with green tea caused a decrease of the amylase activity. A negative correlation ($R^2 = 0.460$; $p < 0.01$) between the starting amylase activity and the decrease in activity after the green tea incubation was noted.

This is the first time that such an effect has been observed. Both black and green teas have been reported to exclusively inhibit the intraoral hydrolysis of starch by salivary amylase [17], and the effect of black tea has been found to be much stronger than that of green tea. Further, the inhibition has been attributed to the amount of the high M_r polyphenols (hydrolysable and condensed tannins) present in the tea decoctions [17, 31]. So, in the first step, to explain the observed increase in amylase activity (Table 3), we tested the binding behavior of selected individual green tea phenols. Figure 2 shows the effect of the amount of catechin, epicatechin, green tea, and tannic acid (termed here as tannin; representing the group of hydrolysable tannins) on the tryptophan quenching. Both catechin and epicatechin led to an increase in FI. Similarly, an increase in FI was also noted by interactions of both these phenolic compounds with saliva proteins of the subject 9 (results not shown). This increase in tryptophan fluorescence could be an indication of a structural change in the amylase molecule, leading in its turn to an exposure of formally buried tryptophan residues. Low molecular phenolic compounds have been reported to affect both the secondary and tertiary structure of the proteins as determined by circular dichroism and FT-IR [14, 27, 28]. On the other hand, both tannin and green tea led to a significant quenching of tryptophan (green tea being more effective) for both amylase and saliva proteins (S9) as illustrated in Fig. 2. This experiment thus visualizes that different binding affinities/binding sites are possible when applying a complex food matrix like a tea beverage. Reference is made to the phenol composition of green tea

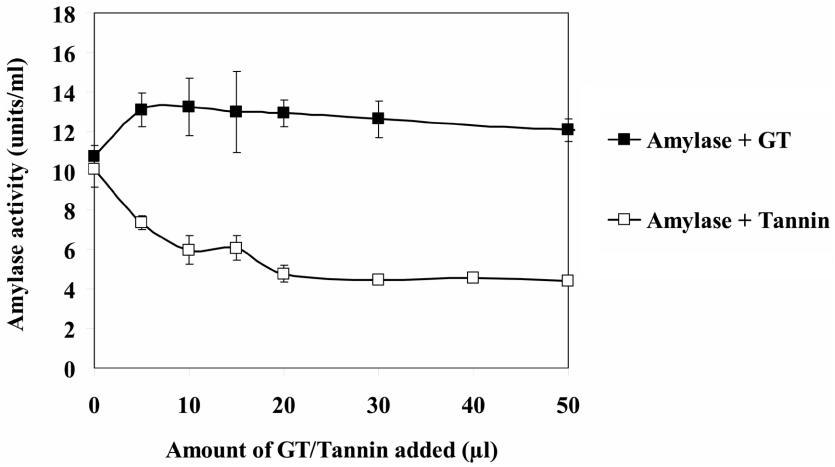

Figure 3. Effect of the amount of GT and tannin on the porcine pancreatic α-amylase activity.

described above. This effect of different phenolic compounds present in green tea is further reflected in the change caused in the activity of porcine pancreatic amylase (Fig. 3). Initially there is a slight increase in amylase activity (reflecting perhaps structural changes caused by the binding), which tends to decrease with increasing amount of green tea added (Fig. 3). The addition of tannin leads solely to a diminishing amylase activity, in agreement with [17, 31]. It has been shown that catechin was inhibitory at a concentration greater than 2 mg/mL [17]. Furthermore, the same authors [17] reported that the green tea inhibited the activity of saliva amylase by *ca.* 25% and that of a streptococcal amylase by 12%. Thus it is clear that with increasing amounts of tea added, the amounts of hydrolysable and condensed tannins increase, these in turn are responsible for the observed decrease in amylase activity [17, 31]. In comparison, we applied a much lower ratio of saliva to tea (1 : 1 v/v; see Section 2) and considering the fact that 69% of the phenolic compounds present in the green tea applied have low M_rs, it is possible that one of the reasons for the observed increase in amylase activity as shown in Table 3 may be due to structural changes induced by their binding.

A further explanation can also be sought regarding the effect of the addition of green tea on the existing protein-protein interactions in saliva. As already mentioned, some 330 proteins and peptides are present in saliva, and micelles representing macromolecular structures in saliva may be formed [33]. Micelles exhibit a complex pattern consisting of individual particles or clusters of particles with different sizes and shapes. Micelles-containing proteins with high (MG2 and secretory IgA), intermediate (lactoferrin, amylase, and glycosylated PRP) and low (lysozyme) M_r have been identified [33]. Thus the amylase activity may also increase as a consequence of dissolution of such micelles initiated by the addition of green tea in low concentration. So, for example, procyanidin C1 monomers, dimers, and trimers were found to have higher affinities for PRPs than for α-amylase [5].

Finally, five amylase isoenzymes have been isolated from human saliva [30], which in turn may have different binding capacities for different phenolic compounds and their effect on activity also needs to be investigated further.

4 Concluding remarks

This study describes the eligibility of investigating the protein-phenol interactions on the basis of tryptophan quenching. The method does have some short backs (*e.g.*, the binding must occur in the vicinity of tryptophan, presence of tryptophan in the protein sequence, accessibility of these tryptophan residues, *etc.*), but may also deliver valuable information on binding sites as a complementary analysis to direct binding studies. It can also be applied to study interactions of complex food matrices (represented by green tea) with complex protein mixtures in the biological matrix (represented by human saliva). These preliminary experiments partly showing an increase in the activity of amylase as caused by GT need to be investigated further by acquiring a larger group of participants and applying complementary methods for determining the amylase activity (*e.g.*, with synthetic substrates besides starch). In this context, the role of protein-protein interactions in saliva and their effect on amylase activity also needs to be tested as well.

Some salivary proteins, such as PRPs, HRPs, secretory IgA, *etc.*, have been demonstrated to be associated with dental caries [30]. Amylase can not only catalyze starch hydrolysis but can also play an important role in composing dental acquired enamel pellicle and binding specifically to several species of oral streptococci. These functions of amylase can lead to the formation of dental plaque and the occurrence of dental caries [30]. It has been further shown that the composition of salivary isoenzymes is also related to the occurrence of dental caries. Therefore, food matrices containing

high M_r polyphenols (hydrolysable and condensed tannins), *e. g.*, tea beverages, red wine, *etc.* may be suggested due to their high binding affinity for saliva proteins and inhibition of amylase activity for prevention of dental caries.

5 References

[1] Kroll, J., Rawel, H., Rohn, S., *Food Sci. Technol. Res.* 2003, *9*, 205 – 218.

[2] Bennick, A., *Crit. Rev. Oral Biol. Med.* 2002, *13*, 184 – 196.

[3] Baxter, N. J., Lilley, T. H., Haslam, E., Williamson, M. P., *Biochemistry* 1997, *36*, 5566 – 5577.

[4] Arts, M. J., Haenen, G. R., Voss, H. P., Bast, A., *Food Chem. Toxicol.* 2001, *39*, 787 – 791.

[5] de Freitas, V., Mateus, N., *J. Agric. Food Chem.* 2001, *49*, 940 – 945.

[6] Warner, T. F., Azen, E. A., *Med. Hypotheses* 1988, *26*, 99 – 102.

[7] Lu, Y., Bennick, A., *Arch. Oral Biol.* 1998, *43*, 717 – 728.

[8] Prinz, J. F., Lucas, P. W., *J. Oral Rehabil.* 2000, *27*, 991 – 994.

[9] Naurato, N., Wong, P., Lu, Y., Wroblewski, K., Bennick, A., *J. Agric. Food Chem.* 1999, *47*, 2229 – 2234.

[10] Boulton, D. W., Walle, U. K., Walle, T., *J. Pharm. Pharmacol.* 1998, *50*, 243 – 249.

[11] Charlton, A. J., Baxter, N. J., Khan, M. L., Moir, A. J., *et al.*, *J. Agric. Food Chem.* 2002, *50*, 1593 – 1601.

[12] Guharay, J., Sengupta, B., Sengupta, P. K., *Proteins* 2001, *43*, 75 – 81.

[13] Prigent, S. V., Gruppen, H., Visser, A. J., Van Koningsveld, G. A., *et al.*, *J. Agric. Food Chem.* 2003, *51*, 5088 – 5095.

[14] Rawel, H. M., Meidtner, K., Kroll, J., *J. Agric. Food Chem.* 2005, *53*, 4228 – 4235.

[15] de Freitas, V., Mateus, N., *J. Sci. Food Agric.* 2002, *82*, 113 – 119.

[16] Sengupta, B., Sengupta, P. K., *Biochem. Biophys. Res. Comm.* 2002, *299*, 400 – 403.

[17] Zhang, J., Kashket, S., *Caries Res.* 1998, *32*, 233 – 238.

[18] Rawel, H. M., Czajka, D., Rohn, S., Kroll, J., *Int. J. Biol. Macromol.* 2002, *30*, 137 – 150.

[19] Epps, D. E., Raub, T. J., Caiolfa, V., Chiari, A., Zamai, M., *J. Pharm. Pharmacol.* 1999, *51*, 41 – 48.

[20] Holm-Hansen, C., Tong, G., Davis, C., Abrams, W. R., Malamud, D., *Clin. Diagn. Lab. Immunol.* 2004, *11*, 909 – 912.

[21] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., *et al.*, *Anal. Biochem.* 1985, *150*, 76 – 85.

[22] Laemmli, U. K., *Nature* 1970, *227*, 680 – 685.

[23] Kahkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., *et al.*, *J. Agric. Food Chem.* 1999, *47*, 3954 – 3962.

[24] Bernfeld, P., *Method Enzymol.* 1955, *1*, 149 – 158.

[25] Peters, T., *All about Albumin Biochemistry, Genetics and Medical Applications*, Academic Press Inc., San Diego 1996.

[26] Papadopoulou, A., Green, R. J., Frazier, R. A., *J. Agric. Food Chem.* 2005, *53*, 158 – 163.

[27] Mahesha, H. G., Singh, S. A., Srinivasan, N., Rao, A. G., *FEBS J.* 2006, *273*, 451 – 467.

[28] Bian, Q., Liu, J., Tian, J., Hu, Z., *Int. J. Biol. Macromol.* 2004, *34*, 333 – 337.

[29] Bennick, A., *Mol. Cell Biochem.* 1982, *45*, 83 – 99.

[30] Liang, H., Wang, Y., Wang, Q., Ruan, M. S., *J. Chromatogr. B Biomed. Sci. Appl.* 1999, *724*, 381 – 388.

[31] Kandra, L., Gyemant, G., Zajacz, A., Batta, G., *Biochem. Biophys. Res. Comm.* 2004, *319*, 1265 – 1271.

[32] Ramasubbu, N., Ragunath, C., Mishra, P. J., Thomas, L. M., *et al.*, *Eur. J. Biochem.* 2004, *271*, 2517 – 2529.

[33] Soares, R. V., Lin, T., Siqueira, C. C., Bruno, L. S., *et al.*, *Arch. Oral Biol.* 2004, *49*, 337 – 343.

