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Effect of salivary proteins on the transport of tannin and quercetin across intestinal epithelial cells in culture

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Abbreviations:
PRP, proline-rich protein
Hst, histatin
DMEM, Dulbecco's modified Eagle's medium
5GG, pentagalloyl glucose
DMSO, dimethyl sulfoxide
TEER, transepithelial electrical resistance
HBSS, Hank's balanced salt solution
HEPES, (N-2)hydroxyethylpiperazine-N′-2-ethanesulfonic acid
dC/dt, rate of transport
P_{app}, apparent permeability coefficient
SGLT1, sodium-dependent glucose transporter
MRP2, multidrug resistance-associated protein 2

ABSTRACT

Polyphenols including tannins and flavonoids are common in plant foods. While tannins may be deleterious to animals, flavonoids can have beneficial effects on the cardio-vascular system. Since salivary proteins can form complexes with polyphenols and thereby interfere with their intestinal absorption, the effect of salivary proteins on transport of tannins and flavonoids across intestinal epithelial cells (Caco-2 cells) was investigated. In presence of the salivary protein Histatin5 (Hst5) the rate of transport (dC/dt) of pentagalloyl glucose (5GG), a tannic acid, across Caco-2 cells in culture was reduced maximally 3.6-fold to 5×10^{-7} μM/s in apical to basolateral direction. Replacing the basolateral salt solution with serum caused a 13-fold increase in dC/dt of 5GG in the absence of Hst5, but addition of Hst5 decreased dC/dt l4-fold. Transport of 5GG in basolateral to apical direction was decreased in half in the presence of Hst5. Decreases in dC/dt were closely paralleled by formation of insoluble 5GG–Hst5 complexes. In contrast, Hst5 and 1B4, a salivary proline-rich protein, had little if any effect on the transepithelial transport of the flavonoid quercetin in apical to basolateral as well as basolateral to apical direction. Taken together with previous studies [Cai K, Hagerman AE, Minto RE, Bennick A. Decreased polyphenol transport across cultured intestinal cells by a salivary proline-rich protein. Biochem Pharmacol 2006;71:1570–80] it appears that although Hst5 and 1B4 are synthesized in salivary glands they have an important biological function in the intestines as scavenger molecules preventing uptake of tannin but without notably affecting absorption of flavonoids.

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1. Introduction

The occurrence of polyphenolic compounds in plants is widespread. They include tannins which are generally believed to act as deterrent to foraging animals due to their bitter astringent taste. Moreover, tannins can inhibit animal growth and metabolism and various toxic effects on animals including humans have been described [1–5]. Flavonoids constitute another large, diverse group of plant polyphenols and they are widely present in coloured flowers and fruits [6]. Because they are common plant components both tannins and flavonoids are found in many plant derived foods. The interest in polyphenols has increased considerably in recent years because of evidence that flavonoids can lower the incidence of heart disease and may also have an anti-carcinogenic effect [7,8]. In contrast it seems undesirable for food tannins to gain access to the organism because of potentially harmful effects.

Human saliva contains two families of proteins, Basic Proline-rich Proteins* (basic PRPs) and Histatins (Hsts) that readily precipitate tannins [9,10]. Feeding tannin to rats and hamsters causes growth retardation, but normal growth resumes within days in rats concomitant with the onset of synthesis of salivary PRPs [11]. In contrast, growth in hamsters remains retarded as long as they are fed tannin and there is no induction of synthesis of PRPs [12,13]. The protection against tannins provided by PRPs may well be related to the ability of PRPs to form complexes with tannins most of which remain insoluble under conditions similar to those prevailing in the stomach and intestines [9]. In fact, formation of insoluble tannin–PRP complexes correlates with a marked decrease in transport of tannin across intestinal epithelial cells (Caco-2 cells) in culture [14]. Thus, the function of PRPs may be to prevent intestinal uptake of tannins. In view of this effect it is of interest to evaluate if Hsts also decrease the transport of tannin across Caco-2 cells. Because of the beneficial effects that flavonoids may have it would be desirable if their intestinal uptake were unimpeded. However, flavonoids share structural characteristics with condensed tannins, so it is possible that salivary proteins may modulate their intestinal uptake. Little is known about the interaction of salivary proteins with flavonoids. The transport of flavonoids across intestinal cells has been investigated [15] but it is not known if this transport is affected by salivary proteins.

The purpose of this study was to evaluate the effect of Hst on the transport of a representative tannin, pentagalloyl glucose (5GG) across intestinal epithelial cells (Caco-2 cells) in culture and to determine if salivary proteins impede the transport of quercetin, a common food flavonoid across Caco-2 cells.

2. Experimental procedures

2.1. Materials

The following supplies for tissue culture were obtained from Life Technologies, Burlington, ON, Canada: Dulbecco's modified Eagle's Medium (DMEM), antibiotic-antimycotic solution 100 × concentrated, 10 mM MEM non-essential amino acids solution and 100 mM Sodium Pyruvate. Human serum was prepared from outdated Human plasma obtained from the Hospital for Sick Children, Toronto as previously described [14]. Fetal bovine serum was purchased from Sigma Canada Ltd., Oakville, ON, Canada and 12-well Costar Transwell tissue culture plates with polyester membranes from Corning Inc., Corning, NY, USA. Caco-2 cells were obtained from American Type Culture Collection, Manassas, VA, USA.

Other supplies included 1,2,3,4,6-penta-0-galloyl-[U-^{14}C]-D-glucopyranose (5GG) with a specific activity of 8.28×10^7 Bq/m mol or 4.51×10^8 Bq/m mol synthesized as described [16]. ^{14}C quercetin (specific activity 196×10^7 Bq/m mol) was obtained from Midwest Research Institute, Kansas City, Missouri. It was dissolved in DMSO and stored under Argon at −20 °C. The Human salivary Basic Proline-rich Protein IB4 (56 residues) with the sequence SPPGKPQGPPQQEGNNPQGPPPAGGNPQQPQAP PAGQPQGPPRPPQGGRPSRPPQ and Human salivary Histatin5 (Hst5), with the sequence DSHAKRHHGYKRKFHEKHHSHRGY (24 residues) were synthesized and purified by the Biotechnology Service Centre, University of Toronto as described [14,17].

2.2. Cell culture

Caco-2 cells obtained from American Type Culture Collection, Manassas, VA, USA were cultured in DMEM supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Passage number 50–70 were used in the cell culture experiments. The cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. They were subcultured at 80% confluence.

2.3. Transport studies

Caco-2 cells were seeded in 12 mm i.d. (filter area 1.1 cm^2) polyester filter cell culture inserts with pore size 0.4 μm at a density of 10^5 cells/cm^2. The basolateral (serosal) and apical (mucosal) compartments contained 1.5 ml and 0.5 ml culture medium, respectively. Culture medium was replaced every second day for 14 days and daily thereafter.

For transepithelial permeability experiments cells were used 21–28 days after seeding. Transepithelial electrical resistance (TEER) of the cell layer was measured with a Millicell-ERS voltohmmeter (Millipore Ltd., Missisauga, ON, Canada). Inserts with TEER larger than 350 Ω/cm^2 were used for transport studies. Mannitol and propanolol transport studies confirmed the integrity of the Caco-2 cell monolayer [14]. The inserts were washed with Hank's balanced salt solution (HBSS) containing 25 mM HEPES for 5 min followed by another wash for 30 min.

To measure the transepithelial transport of 5GG or quercetin, following washing of the wells as described [^{14}C]-5GG or [^{14}C]-quercetin in HBSS was added to either the apical or basolateral compartment. The final concentration of DMSO in the quercetin solution was 0.2% which has been shown not to affect transport of quercetin [18]. HBSS or human serum was added to the opposite side and the plates incubated for 1 h (quercetin) or 3 h (5GG) at 37 °C. Within these time periods transport has been shown to be linear [14,19]. At the end of incubation the TEER was measured and the amount of radionuclide transported determined by removing samples from the receiving side and counting them on a liquid

scintillation counter (Beckman-Coulter, LS 6500 Multi-Purpose Scintillation Counter). In other experiments the influence of 1B4 and Hst5 on the transport of [^{14}C]-5GG or [^{14}C]-quercetin was evaluated by adding the protein mixed with the radioactive polyphenol to either the apical or basolateral compartment and HBSS or serum to the receiving side. For each condition results from three experiment were combined and mean and S.D. calculated.

Since the salivary proteins form complexes with polyphenols, transport solutions containing both of these components were triturated several times to suspend evenly any insoluble complexes that might be present before counting on the liquid scintillation counter. In addition, aliquots of such transport solutions containing 5GG were centrifuged at 15,000 × *g* for 15 min and the resultant supernatant removed. In the case of quercetin the transport solutions were centrifuged in a Beckman Optima ultracentrifuge at 300,000 × *g* for 60 min. The pellet from either centrifugation was washed two times with HBSS, centrifuged and the washes combined with the supernatant and counted on the liquid scintillation counter. The pellet was dissolved in 10 mM triethanolamine containing 0.1% SDS and subjected to liquid scintillation counting as well. This allowed determination of the distribution of polyphenol in solution and in insoluble complexes. To evaluate the rate of transport of quercetin the apparent permeability coefficient (P_{app}) was calculated using the following equation [20]:

$$P_{app} = \frac{V}{AC_0}\frac{dC}{dt} = \text{cm} \times \text{sec}^{-1}$$

where V is the volume of the solution in the receiving compartment, A the membrane surface area, C_0 the initial concentration in the donor compartment and dC/dt the change in concentration of radioactive compounds in the receiving solution over time (1 h). Under these conditions quercetin remains stable [19]. The use of P_{app} allows comparison with other studies on quercetin transport where this parameter is commonly used. In experiments on 5GG transport dC/dt rather than P_{app} was evaluated since salivary proteins form soluble and insoluble complexes with 5GG. Consequently the concentration of free 5GG (C_o) is not known. It has been found that 5GG is metabolized during transepithelial transport giving rise to various compounds including tri- and tetragalloyl glucose as well as glucose [14] both in the donor and receiving wells, so dC/dt reflects the sum of transport of 5GG and its metabolites rather than a single component. Both dC/dt and P_{app} allow evaluation of factors that affect transepithelial transport of the polyphenols.

For both P_{app} and dC/dt all values obtained at a given concentration were used to calculate mean ± S.D.

3. Results

3.1. *Effect of Hst5 on apical to basolateral transport of 5GG*

3.1.1. *Transport of 5GG when the basolateral receiving solution is HBSS*

Fig. 1A illustrates the effect on dC/dt of adding Hst5 to a 90 μM solution of 5GG. This is the same concentration as used in a previous study on the salivary proline-rich protein 1B4 [14]. In the absence of Hst5 dC/dt was 18 × 10^{-7} μM/s, but it drops gradually to a value of 5 × 10^{-7} μM/s in the presence of 50 μM Hst5. Increasing the concentration of Hst5 beyond 50 μM had little if any effect on dC/dt. The distribution of soluble 5GG and 5GG bound in insoluble complexes to Hst5 is shown in Fig. 1B. Addition of Hst5 caused precipitation of 5GG which reached 81% of recovered 5GG at 50 μM Hst5. Further addition of Hst5 caused precipitation of 96% of 5GG at a concentration of 330 μM Hst5. It is apparent that the decrease in dC/dt parallels the formation of insoluble 5GG–Hst5 complexes.

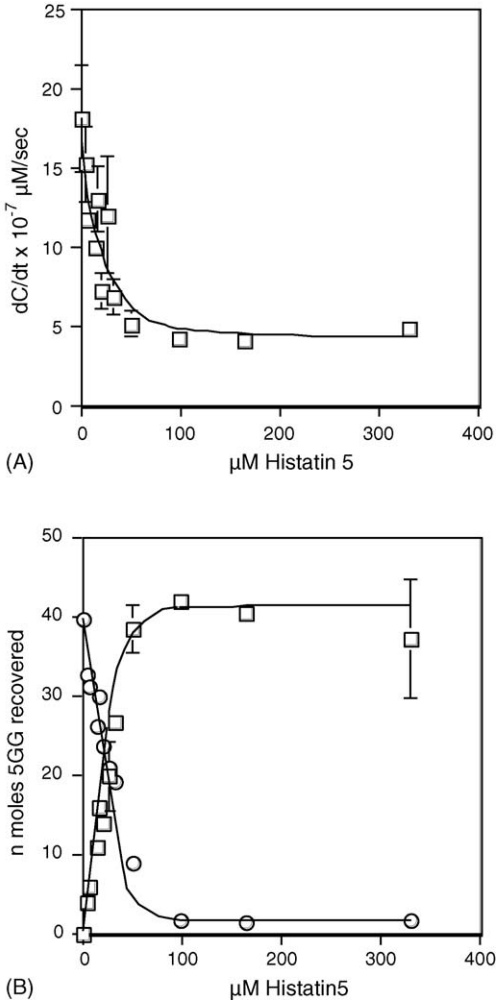

Fig. 1 – (A) Effect of the salivary protein Hst5 on apical to basolateral transport of 5GG across Caco-2 cells. The receiving basolateral well contained HBSS. The apical compartment was loaded with 90 μM 5GG containing varying concentrations of Hst5. Mean dC/dt ± S.D. (*n* = 2, 4 or 6) has been plotted as a function of Hst5 concentration. (B) Distribution of 5GG in insoluble (pellet, □) and soluble (supernatant, ○) fractions in the apical loading solution. The amount of 5GG in the two fractions is plotted as a function of the Hst5 concentration in the loading solutions. The average values of two experiments are shown. Average recovery of 5GG was 100% of total amount initially present in the medium.

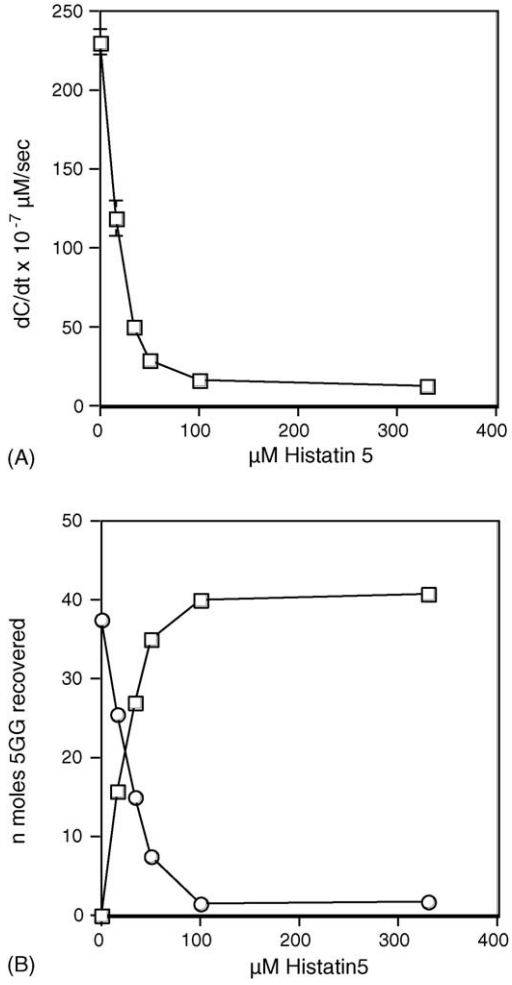

Fig. 2 – (A) Effect of the salivary protein Hst5 on apical to basolateral transport of 5GG across Caco-2 cells. The receiving basolateral well contained human serum. The apical compartment was loaded with 90 µM 5GG containing varying concentrations of Hst5. Mean dC/dt ± S.D. (*n* = 4) has been plotted as a function of Hst5 concentration. (B) Distribution of 5GG in insoluble (pellet, □) and soluble (supernatant, ○) fractions in the apical loading solution. The amount of 5GG in the two fractions is plotted as a function of the Hst5 concentration in the loading solutions. The average values of two experiments are shown. Average recovery of 5GG was 94% of total amount initially present in the medium.

3.1.2. *Transport of 5GG when the basolateral solution is serum*

Fig. 2A shows the results obtained with a 90 µM solution of 5GG. In the absence of Hst5 dC/dt was 230 × 10^{-7} µM/s which is 13 times higher than when the receiving solution is HBSS. Adding increasing amounts of Hst5 to the 5GG containing apical solution caused a gradual decrease in dC/dt until a value of 16 × 10^{-7} µM/s was obtained at 100 µM Hst5. Further increase in Hst5 concentration had no additional effect on P_{app}. Thus, even in the presence of a saturating amount of Hst5 dC/dt is three times larger when the receiving solution is serum rather than HBSS. As shown in Fig. 2B the decrease in dC/dt closely parallels the increase in insoluble 5GG–Hst5 complexes. In the presence of 100 µM Hst5 99% of recoverable 5GG is present as insoluble complexes.

3.2. *Effect of Hst5 on basolateral to apical transport of 5GG*

The transport of 5GG in a basolateral to apical direction is illustrated in Fig. 3A. A 10 µM solution of 5GG was used since it has been found that if 90 µM 5GG is added to the basolateral solution the Caco-2 cell layer is not intact as evaluated by TEER measurements [14]. In the absence of Hst5 dC/dt is 56 × 10^{-7} µM/s and it drops to 23 × 10^{-7} µM/s in the presence

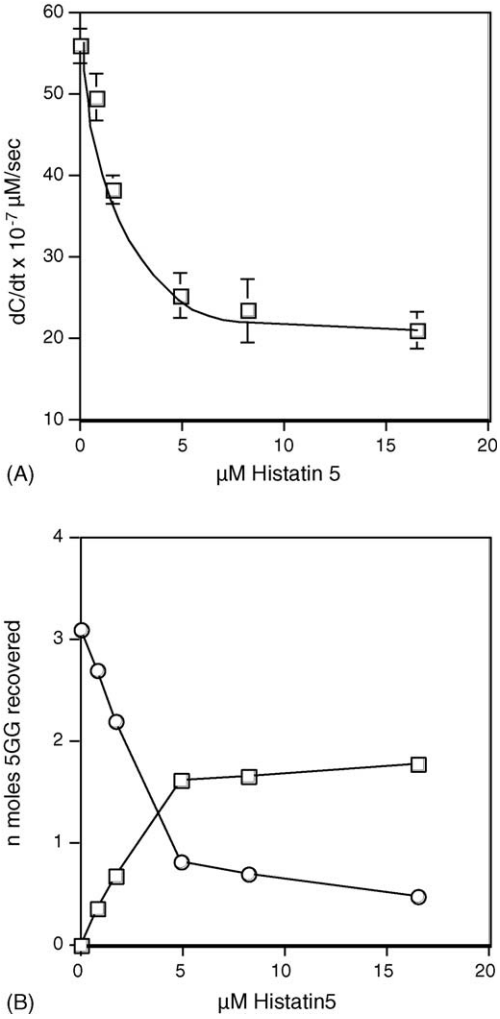

Fig. 3 – (A) Effect of the salivary protein Hst5 on basolateral to apical transport of 5GG across Caco-2 cells. The receiving apical well contained HBSS. The basolateral compartment was loaded with 10 µM 5GG containing varying concentrations of Hst5. Mean dC/dt ± S.D. (*n* = 4) has been plotted as a function of Hst5 concentration. (B) Distribution of 5GG in insoluble (pellet, □) and soluble (supernatant, ○) fractions in the basolateral loading solution. The amount of 5GG in the two fractions is plotted as a function of the Hst5 concentration in the loading solutions. The average values of two experiments are shown. Average recovery of 5GG was 91% of total amount initially present in the medium.

of 8 μM Hst5 and there is no further change in dC/dt when the concentration of 5GG is increased. The decrease in dC/dt is mirrored in an increase in insoluble 5GG which reaches 71% in the presence of 8 μM Hst5 and further increases to 79% of recoverable 5GG when the Hst5 concentration is increased to 17 μM (Fig. 3B).

3.3. *Effect of salivary proteins on quercetin transport*

3.3.1. *Effect on apical to basolateral transport of quercetin*

Using a 10 μM concentration of quercetin 1B4 has no significant effect on P_{app}, even at a concentration of 160 μM (data not shown). Analysis of the medium showed that with a recovery from 97% to 100% only 0.9% to 1.6% quercetin was sedimented by centrifugation, so only a minute amount of quercetin was precipitated by 1B4 (data not shown).

Addition of Hst5 to a 10 μM solution of quercetin (Fig. 4) also had no effect on P_{app}. The presence of Hst5 caused little precipitation of quercetin. With a recovery varying from 96% to 100% only 1.4% to 2.4% quercetin was recovered as insoluble complexes.

3.3.2. *Effect on basolateral to apical transport of quercetin*

There was no effect of 1B4 on basolateral to apical transport of quercetin as measured by P_{app} even if 1B4 in a concentration of 160 μM was added to 10 μM quercetin (data not shown). Compared with apical to basolateral transport P_{app} was about 2-fold larger. Centrifugation of the medium resulted in the sedimentation of a negligible amount of quercetin varying from 0.5% to 3% (97–100% recovery of quercetin). Similar results were obtained when 1B4 was replaced by Hst5 (Fig. 5). Even at the highest concentration of Hst5 (160 μM) there was no effect on basolateral to apical transport of quercetin. Only small amounts of quercetin was present as insoluble complexes in the media, varying from 2% to 8% except for 12% sedimented in the presence of 160 μM Hst5 (91–97% recovery of quercetin).

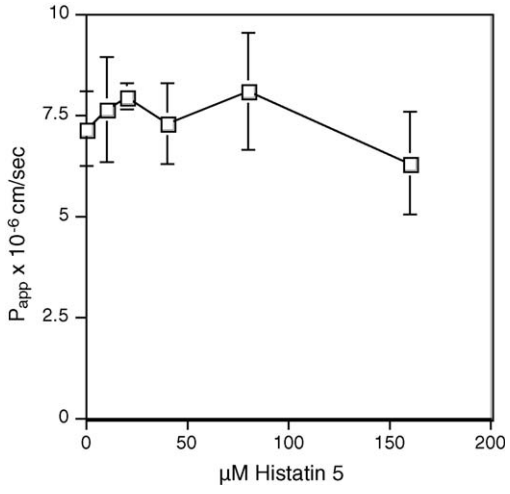

Fig. 4 – Effect of the salivary protein Hst5 on apical to basolateral transport of quercetin across Caco-2 cells. The receiving basolateral well contained HBSS. The apical compartment was loaded with 10 μM quercetin containing varying concentrations of Hst5. Mean P_{app} ± S.D. (n = 6) has been plotted as a function of 1B4 concentration.

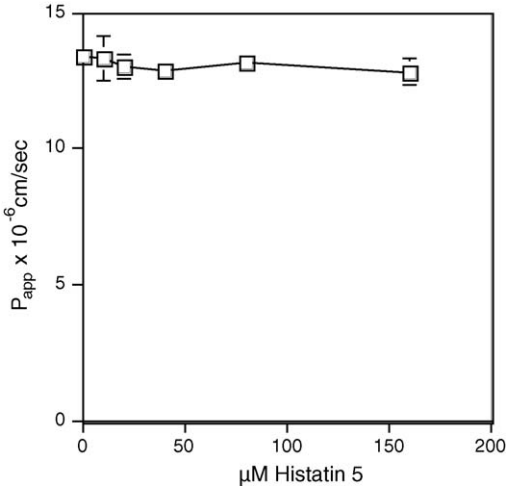

Fig. 5 – Effect of the salivary protein Hst5 on basolateral to apical transport of quercetin across Caco-2 cells. The receiving apical well contained HBSS. The basolateral compartment was loaded with 10 μM quercetin containing varying concentrations of Hst5. Mean P_{app} ± S.D. (n = 4) has been plotted as a function of Hst5 concentration.

4. Discussion

Hsts constitute 2.6% of total salivary proteins [21]. Two Hsts, Histatin 1 and Histatin 3 are synthesized in salivary glands. Before secretion from the glands the 32 residues Hst 3 is cleaved between residues 24 and 25 giving rise to the 24 residues Hst5 [22]. Following secretion from the glands Hsts undergo further cleavages giving rise to smaller Hst components [23]. For this study Hst5 was chosen because it is a major component of the Hst family [22]. Most of the studies on Hsts have concentrated on their antibacterial and antifungal effects [24,25], but it has also been shown that Hsts share with PRPs the ability to effectively precipitate tannins [10]. IB4 was selected as a representative basic PRP because it is a prominent member of this protein family and shares tannin binding characteristics with other basic PRPs [9]. Compared with previously published results [14] it is clear that Hst5 as well as 1B4 have the ability to inhibit transport of 5GG across Caco-2 cells. In the presence of Hst5, dC/dt for transport in an apical to basolateral direction into HBSS was decreased maximally 3.8-fold compared to a 4-fold reduction in the presence of 1B4 [14]. From Fig. 1A it can be calculated that a half maximal reduction in dC/dt was obtained at a molar ratio of Hst5/5GG of 0.08. In comparison it can be calculated from previously obtained results [14] that for half maximal reduction in dC/dt by 1B4 a 1B4/5GG molar ratio of 0.04 is necessary. Calculated on weight basis the ratio of salivary protein/5GG needed to achieve half maximal reduction in dC/dt is 0.25 for Hst5 and 0.26 for 1B4. Thus, the two salivary proteins showed similar ability to diminish the transport of 5GG. As previously found for 1B4 [14] the inhibition of 5GG transport is closely mirrored in the formation of 5GG–Hst5 insoluble complexes indicating that such complexes are not transported across the cells.

The much higher dC/dt measured for apical to basolateral transport of 5GG when the receiving well contains serum (Fig. 2A) may at least partly be due to binding of 5GG to the serum proteins albumin and apolipoprotein Al as shown in a previous study [14]. In the presence of Hst5 transport of 5GG was maximally reduced 20-fold when the receiving well contained serum (Fig. 2A). The value is comparable to that obtained previously when 1B4 was used instead of Hst5 [14]. From Fig. 2A it can be calculated that half maximal reduction in dC/dt was obtained at a Hst/5GG molar ratio of 0.19 whereas previously obtained results for 1B4 [14] gave a corresponding ratio 0.07. Calculated on a weight basis the ratios are 0.61 for Hst5 and 0.40 for 1B4 indicating that when serum is present in the receiving well the two salivary proteins have similar effects on the transport of 5GG across Caco-2 cells.

In agreement with previous results [14] it is apparent that Caco-2 cells have effective means of transporting 5GG in a basolateral to apical direction and that this transport is inhibited by Hst5 to the same extent as reported for 1B4 [14], most likely due to formation of insoluble protein–5GG complexes. The presence of 5GG binding proteins in the serosal compartment would therefore be expected to substantially increase the net uptake of 5GG by increasing apical to basolateral transport but diminish flow in the opposite direction. Interestingly it has been found that the efflux of quercetin in cultured Caco-2 cells is decreased in the presence of albumin, emphasizing the effect of polyphenol-binding proteins on the transport of 5GG and quercetin [18].

5GG and quercetin have markedly different structures. 5GG consists of a glucose molecule to which are attached 5 molecules of gallic acid in ester linkages. Quercetin on the other hand has the typical C6–C3–C6 ring structure of flavonoids. In spite of these differences they share characteristics of transport across Caco-2 cells such as inhibition by phlorizin in an apical to basolateral direction indicating a role of sodium-dependent glucose transporter SGLT1, and transport in the opposite direction is inhibited by MK571 suggesting involvement of the multidrug resistance-associated protein MRP [14,26]. A closer comparison is complicated because of the partial cleavage of 5GG during transcellular transport [14], whereas quercetin is transported intact [19]. In contrast to 5GG there is little or no effect of 1B4 and Hst5 on the transport of quercetin in either direction. This is most likely due to lack of formation of insoluble salivary protein–quercetin complexes. While centrifugation of mixtures of protein and 5GG at $15{,}000 \times g$ for 15 min readily sedimented insoluble protein–5GG complexes, little if any sedimentation of protein–quercetin complexes occurred even when centrifuging at $300{,}000 \times g$ for 60 min. This inability of quercetin to form insoluble complexes with salivary proteins is likely due to the smaller size of quercetin decreasing the number of protein binding sites. Moreover, the binding of quercetin to the salivary proteins may be weak. In a comparison of binding of 5GG and (−)-epicatecin that has a structure similar to quercetin it was found that the Kd for 5GG binding to a PRP fragment was 2 orders of magnitude smaller than the Kd obtained for interaction of (−)-epicatechin with the PRP fragment [27].

The structure of flavonoids such as quercetin is closely related to flavan-3-ols which can be found liked together by C–C bonds into polymers of varying sizes to form condensed tannins. In contrast to quercetin, condensed tannins precipitate salivary proteins as readily as the tannic acid 5GG [9,10]. Studies on condensed tannins have shown that catechin and proanthocyanidin dimers and trimers are transported across Caco-2 cells with P_{app} $0.9–2.0 \times 10^{-6}$ cm/s whereas the value for proanthocyanidin polymers is approximately 10 times lower [28]. It would be interesting to evaluate the effect of PRPs and Hsts on the transport of such molecules across Caco-2 cells to determine the effect of polyphenol size on the inhibition of this transport by salivary proteins.

Previously it was found that PRPs as well as Hsts form insoluble complexes with hydrolysable and condensed tannins under conditions prevailing in the mouth and that most of these complexes remain stable under conditions similar to those of the stomach and small intestine [9,29]. This taken together with the ability of 1B4 and Hst5 to inhibit the transport of 5GG across Caco-2 cells, but leaving the transport of quercetin unimpeded suggests that the proteins act as scavenging molecules preventing absorption of 5GG from the intestines without affecting absorption of quercetin. Other than their ability to precipitate tannins little is known about the functions of basic PRPs, although in some individuals salivary protein fractions enriched in a subset of basic PRPs inhibit HIV infectivity [30]. Basic PRPs account for 23% of parotid proteins [31]. Thus, while these major parotid proteins are synthesized in the salivary glands they appear to exert an important physiological role in the intestines. While in humans basic PRPs are only synthesized in the parotid gland [31], Hsts are synthesized in the parotid as well as the submandibular gland and they account for 2–3% of total salivary protein [21]. Unstimulated saliva secreted between meals is a mixture of parotid and submandibular saliva, whereas stimulated saliva secreted during meals has a significantly higher proportion of parotid saliva [32]. Given this distribution of basic PRPs and Hsts in the salivary glands, Hsts may be particularly important in neutralizing tannins that enters the mouth between meals, for example by inhalation of tannin-containing plant dust, whereas a much larger amount of basic PRP would be present in saliva secreted during meals allowing a more effective precipitation of food tannins.

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