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Berry Polyphenols Inhibit Digestive Enzymes: a Source of Potential Health Benefits?

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Abstract This paper provides an overview of work that suggests that polyphenol components from berries can inhibit crucial enzymes involved in starch and lipid digestion and potentially influence blood glucose levels and fat digestion. Polyphenols from certain berries have been found to inhibit lipase activity in vitro at low levels. By screening berries with differing polyphenol composition for their inhibitory effectiveness, certain polyphenol classes were implicated as active components. The inhibition was caused at low levels, certainly achievable in the gut after intake of a small amount of berries and approached inhibition achieved by the pharmaceutical lipase inhibitor, orlistat. Polyphenols from berries also have the potential to modulate starch digestion as they inhibit both α-amylase activity and α-glucosidase activity in vitro at low levels. Different berries showed very different levels of effectiveness against the two enzymes and a comparison of their polyphenol composition indicated which components were the most effective inhibitors. For α-amylase, tannins (ellagitannins and proanthocyanidins) were found to be the most effective components but their effectiveness may be modulated by other polyphenols. The active components in the inhibition of α-glucosidase were less obvious and a range of different polyphenol classes may be effective. In both cases, the berry components could act additively with the pharmaceutically used inhibitor, acarbose, and there is potential for berry components to substitute for acarbose or reduce the dose required for effective glycaemic control.

Keywords Polyphenols · Digestion · Inhibition · Glycaemic control · Diabetes · Obesity · Health

Introduction

The health benefits associated with a diet rich in fruits and vegetables [1] may reflect overall reductions in sugar and fat intake but may also be due to increased intake of non-nutritive phytochemical components which directly and beneficially influence health. Polyphenols are a major phytochemical component of fruits and are particularly enriched in berries [2]. Over the last 20 years, a theory has developed that the intake of natural antioxidants, such as polyphenols [3], could protect against the damage caused by free radicals to DNA, membranes and cellular components, which are involved in disease progression. However, this theory has been challenged by the growing evidence that dietary polyphenols have substantially different bioavailabilities [4]. For example, abundant berry polyhenol components such as anthocyanins and tannins [ellagitannins and proanthocyanidins] [2], have low serum bioavailability and it has been argued that systemic bioactivities ascribed to these polyphenols are largely mediated by their metabolites [5]. Therefore, a large proportion of potentially protective berry polyphenols are unable to enter the circulation and influence cellular interactions. In fact, most polyphenols from berries remain

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in the gastrointestinal tract (GIT) and pass through to the large intestine where they are subject to biotransformation by colonic microbiota [6]. Therefore, one could propose that the health benefits derived from a diet rich in polyphenol antioxidants may be partly delivered through effects carried out within the GIT. In particular, we have become interested in the possibility that polyphenols may modulate nutrient availability through the inhibition of digestive enzymes involved in lipid and starch breakdown, which could influence obesity and blood glucose control (see reviews [7] and [8]). In this overview, we summarise recent work that confirms our initial findings that polyphenol-rich extracts from berries can inhibit the two main enzymes involved in starch digestion, α-amylase and α-glucosidase, in vitro [7]. We also summarise our recent work on the inhibition of pancreatic lipase by berry polyphenols [8]. We discuss the specificity of enzyme inhibition and suggest possible candidate polyphenol classes as the most effective inhibitors [9]. We also describe comparisons and interactions between polyphenols and currently used drugs, i.e. acarbose and orlistat. Acarbose can inhibit both α-amylase and α-glucosidase and is used to control post-meal blood glucose levels in patients with poor glycaemic control. Orlistat can inhibit pancreatic lipase, reducing fat utilization with benefits for CVD and obesity.

Our long-term aims are to identify components important for enzyme inhibition and use this information to breed new varieties of berries with elevated levels of these components and/or to design novel products with high nutraceutical value.

Materials and Methods

Plant Material and Extraction

Ripe fruit of strawberries, black currants, arctic brambles, cloudberries, blueberries and rowanberries was obtained from growers, frozen then transported frozen to the James Hutton Institute. Blackberries, pomegranates and red wine (Echo Falls, a Merlot variety wine from Mission Bell Winery, Madera, California, USA) were purchased from a local supermarket. Red wine was concentrated by rotary evaporation to remove alcohol.

Fruits were extracted and polyphenol-rich extracts, which have been shown to be devoid of minerals, sugars, lipids, vitamin E and vitamin C, were obtained by solid-phase extraction as outlined previously [9]. Phenol content was measured using a modified Folin-Ciocalteau method [9] against a standard curve of gallic acid and phenol content expressed as gallic acid equivalents (GAE). Samples were dried in aliquots to set phenol content using a SpeedVac (Thermo Scientific, Waltham, MA).

Fractionation on Sephadex LH-20 Sorption to Sephadex LH-20 in aqueous ethanol and step-wise elution with solutions of aqueous ethanol and acetone is an established method [7, 9] for fractionation of polyphenol components into tannin-rich and tannin-depleted fractions.

α-Glucosidase Assay The α-glucosidase assay was as described previously [10] and was used to measure inhibition of α-glucosidase by berry extracts, pure compounds and pharmaceuticals. The buffer for this assay was 100 mM HEPES (pH 6.8) and the substrate was 2 mM *p*-nitrophenyl α-D-glucopyranoside. The enzyme source was rat intestinal acetone powder (Sigma-Aldrich) dissolved in ultra pure water (UPW) at 10 mg/μL then centrifuged at 13,000×*g* for 5 min, and the supernatant used. Inhibitors were added in a fixed total volume to obtain the concentration ranges required for individual experiments. Controls lacking inhibitors were run and defined the control activity in each experiment. Each treatment was accompanied by a treatment blank containing all components apart from the enzyme to account for the possible absorbance of the berry extracts/inhibitors. This assay was time- and substrate-sensitive therefore all components were added in a specific order; buffer, enzyme, inhibitor then substrate. The substrate was added last to start the reaction. The assay was linear over for 2 h at 37 °C. Then samples were centrifuged at 13,000×*g* for 2 min and the absorbance at 410 nm read in a UV spectrophotometer. All samples were carried out in triplicate and values were presented as % control activity ± standard errors. Statistically significant differences were assessed using Student's *t* test.

Amylase Assay This assay was conducted as described previously [9]. Briefly, stock starch solution was prepared by suspending 1 % (*w*/*v*) soluble potato starch (Sigma Chem. Co. Ltd., product S-2360) in synthetic saliva buffer and gelatinizing the mixture for 15 min at 90 °C. Porcine pancreatic α-amylase (Sigma Chem. Co. Ltd., product A-3176) was dissolved in synthetic saliva buffer at 380 mg/L. The control assay contained 800 μL of synthetic saliva buffer and 100 μL of α-amylase and 100 μL of UPW or extract, and the reaction was started by addition of 500 μL of starch solution. The plus extract control assays contained various amounts of extracts in the 100 μL volume. To estimate IC_{50} values (the amount of phenols that gave 50 % inhibition of amylase), assays were carried out with a range of phenol contents. Acarbose (Sigma Chem. Co. Ltd, product number A8980) was dissolved at 1 mg/mL in UPW. Bovine serum albumin (BSA, Sigma Chem. Co. Ltd, product number A4503) was dissolved at 5 mg/mL in UPW.

Assay for Reducing Termini Using PAHBAH A 5 % (*w*/*v*) stock solution of *p*-hydroxybenzoic acid hydrazide

(PAHBAH) in 0.5 M HCl was diluted 1:4 with 0.5 M NaOH to give the working PAHBAH reagent. Triplicate samples (50 μL) of assays were taken at fixed times and added to 1 mL of PAHBAH reagent in a 1.5-mL tube. After heating for 10 min at 100 °C, the absorbance at 410 nm was measured. Controls lacking enzyme were used as blanks. An assay time of 5 min was taken as the standard as the rate of production of reducing termini was linear up to this point. Percent of control amylase activity was calculated as the difference between the control and the + extract reactions divided by the control reaction.

Lipase Assay This assay was as described previously [8]. Lipase from porcine pancreas type II (Sigma product L3126) was dissolved in UPW at 10 mg/mL then the supernatant was used after centrifugation at 13,000×*g* for 5 min. The assay buffer was 100 mM Tris buffer (pH 8.2) and *p*-nitrophenyl laurate (pNP laurate) was used as the substrate. The substrate stock was 0.08 % *w/v* pNP laurate dissolved in 5 mM sodium acetate (pH 5.0) containing 1 % Triton X-100 and was heated in boiling water for 1 min to aid dissolution, mixed well then cooled to room temperature.

The control assay contained 400 μL assay buffer, 450 μL substrate solution and 150 μL lipase. Berry extracts were dissolved in UPW and added in 50 μL total volume. The buffer, enzyme and berry extracts were added and then substrate was added to start the reaction. The samples were incubated at 37 °C for 2 h. Then samples were centrifuged at 13,000×*g* for 2.5 min and read at 400 nm in a UV spectrophotometer. All samples were assayed in triplicates and an inhibitor blank was prepared for each sample.

Orlistat was dissolved in methanol and diluted 50-fold to achieve the working solutions in the assays. This level of methanol did not affect lipase activity.

Liquid Chromatography–Mass Spectrometry The polyphenol composition of extracts were assessed by liquid chromatography–mass spectrometry (LC-MS) as described previously [8]. Polyphenol components were putatively identified using their UV characteristics, MS and MS^2 properties using data gathered in-house and from literature.

Results and Discussion

Lipase Inhibition

Polyphenol-rich extracts of berries, red wine and other fruits were tested for their ability to inhibit pancreatic lipase in vitro (Fig. 1). At 50 μg/ml GAE phenols, the extracts had different effects; blackcurrant and rowan had little effect, the blueberry extract caused slight but significant inhibition whilst lingonberry, arctic bramble, cloudberry, strawberry and raspberry were more effective. The most effective berry types were three members of the *Rubus* family (raspberry, arctic bramble and cloudberry) and strawberry. Published polyphenolic compositions of these berry types [2, 11] suggested that ellagitannins could be important for lipase inhibition. As raspberry and cloudberry differ mainly in their anthocyanin content [11], this suggests that anthocyanins, which are present in only small amounts in cloudberry [10], are not necessary for lipase inhibition. Nevertheless, it is possible that synergistic interactions between polyphenols occur, even in terms of protecting the stability of the active components.

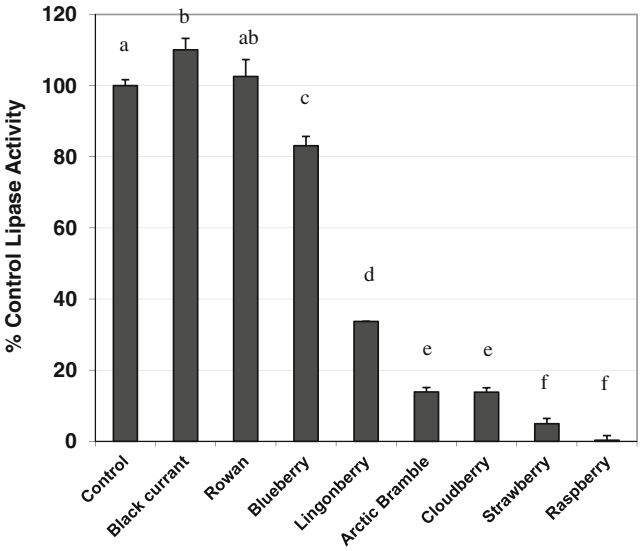

Fig. 1 Screen for inhibition of lipase inhibition by berry extracts. All extracts were tested at 50 μg/mL phenol content (GAE). A representative experiment is shown and values are means of triplicate assays ± standard error. Different *letters* (*a–f*) denote significantly different samples at $p<0.05$ (Student's *t* test)

The inhibition by the cloudberry extract had an IC_{50} (concentration for half maximal activity) of ~5 μgGAE/mL phenols (Fig. 2). This is in the same range as the IC_{50} value noted for orlistat in the same assay at 1.0 μg/mL.

After fractionation of cloudberry on Sephadex LH-20, the lipase inhibitory activity was recovered in a fraction that was enriched in ellagitannins and ellagic acid derivatives [8]. Lipase activity was also effectively inhibited by a similarly produced ellagitannin-rich extract from raspberry. Inhibition by a strawberry tannin fraction may have been influenced by the presence of ellagitannins and proanthocyanidins (PACs). PACs in grape seed extracts have been reported as effective lipase inhibitors [12] and PACs from apple have been shown to be the main components responsible for inhibition of pancreatic lipase in vitro and for the prevention of triglyceride absorption in humans and in mice models [13]. It also seems likely that PACs present in lingonberry extracts [14] are responsible for their lipase inhibition.

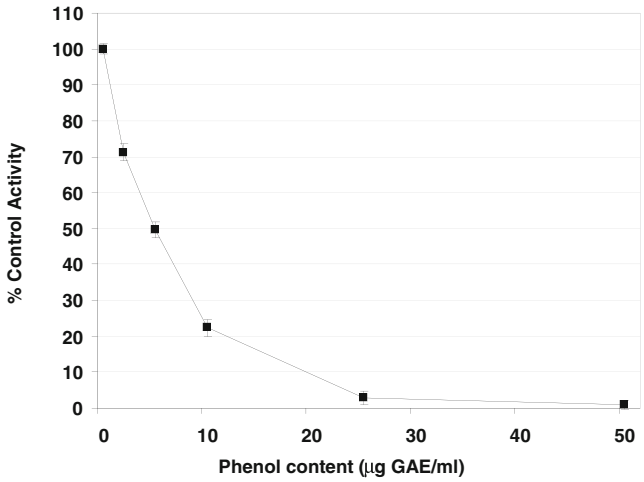

Fig. 2 Inhibition of lipase activity by cloudberry extract. Values are means of triplicate assays ± standard error. A representative experiment is shown

Amylase Inhibition

A range of polyphenol-rich extracts from berries inhibited α-amylase activity in vitro (Fig. 3) with various levels of effectiveness. The extract from rowanberries was particularly potent with an IC_{50} value of 4.5 µg GAE/mL. The original rowanberry extract had a similar composition to previous work [15] containing mainly chlorogenic acids, flavonols, anthocyanins and PACs. After fractionation by step elution chromatography on Sephadex LH-20, eight main fractions were obtained [9]. LC-MS showed that Fraction 1 was mainly composed of chlorogenic acids, fractions 2 and 3 contained chlorogenic acids and anthocyanins in varying amounts, fraction 4 contained mainly quercetin hexoses, fraction 5 contained as yet unidentified flavonol components, fraction 6 contained low levels of unidentified phenolics, fraction 7 mainly contained quercetin coumaroyl hexoses and fraction 8 was mainly composed of PACs. Only the PAC-rich fraction 8 caused substantial inhibition of amylase (results not shown) and yielded an IC_{50} value of ~5 µgGAE/mL compared to 4.5 µg GAE/mL for the unfractionated rowanberry extract. The PACs were a characteristic mix of A- and B-type proanthocyanidins composed of epicatechin units known to be present in rowanberry [15]. Although the rowanberry PACs were as potent inhibitors as the whole rowanberry extract, it is clear that this fraction was, at least, tenfold enriched in PACs compared to the original rowanberry extract. This suggests that the presence of the other non-PAC components may have influenced amylase inhibition. Synergism between polyphenol components may also partly explain the great difference in effectiveness between the rowanberry extracts and the lingonberry extracts which also contain PACs but differ considerably in their polyphenolic composition [14].

This study confirms previous work [7] that polyphenol-rich extracts from berries can inhibit α-amylase in vitro at low concentrations. Our initial work strongly suggested that ellagitannins (ETs) in raspberry were the main active components for amylase inhibition [7] and purified ETs from strawberry have also been shown to have amylase inhibitory activity [16].

The effect of the PACs and acarbose were tested at various combinations of their IC_{50} values [IC_{50} for acarbose was 0.8 µg/mL, similar to previous reports [17]]. Combining PACs and acarbose at 100 % of their IC_{50} values expectedly gave considerable inhibition to around 15 % control activity (Fig. 4). Combining them at 50 % of their individual IC_{50} values, gave inhibition at lower than 50 % control activity (~30 % control), which suggests some

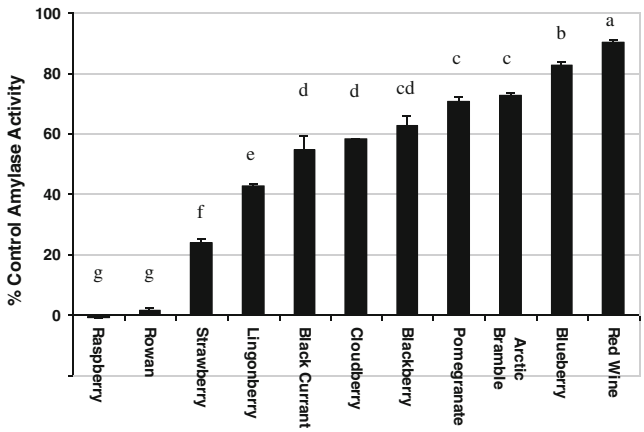

Fig. 3 Inhibition of α-amylase activity in vitro by berry, red wine and fruit extracts. Extracts were assayed at 50 µg GAE/ml. The values shown are averages of triplicate assays ± standard error. Different *letters* (*a*–*g*) denote significantly different samples at $p<0.05$ (Student's *t* test)

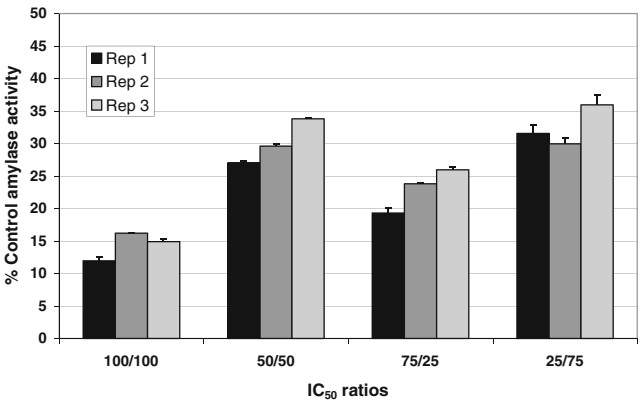

Fig. 4 Effect of co-incubation with acarbose and rowanberry PACs on amylase activity. The values are averages of triplicate assays ± standard error. Acarbose and rowanberry PACs were added at various defined ratios of their IC_{50} values: e.g., 100/100=each at 100 % of their IC50 values, 75/25=75 % IC_{50} value of rowanberry and 25 % IC_{50} value of acarbose. Three replicate assays run on different days are shown

additive or synergetic effects between the two treatments. Adding them at 75 % of the IC_{50} value for the PACs and 25 % of the IC_{50} value for acarbose gave greater inhibition than the 50:50 addition or indeed the inhibition caused by addition of 75 % acarbose:25 % PACs. Taken together, this indicates that combination with PACs could reduce the effective dose of acarbose required for inhibition.

Addition of bovine serum albumin was protective against inhibition by PACs (Fig. S1), which suggests that the PACs may operate by binding to amylase and preventing interaction with the starch substrate as suggested previously [7]. However, the protective effect was maximal at 100 μg/mL when the assay contained 28.5 μg/mL amylase (and 4.5 μg GAE/mL PACs) and increasing BSA to 250 μg/mL did not increase protection. A slight but significant increase in the control amylase activity with BSA addition suggested that part of the protection against inhibition by PACs may have been due to improved amylase activity.

Glucosidase Inhibition

Polyphenol-rich extracts from black currants were effective inhibitors of α-glucosidase with an IC_{50} value of 20 μg GAE/mL (Fig. 5). Acarbose inhibited α-glucosidase in a dose-dependent manner giving an IC_{50} value of 40 μg/mL, which is similar to previous reports [18]. Therefore, black currant extracts were, at least, as effective as acarbose in the same assay system. On the other hand, raspberry and cloudberry extracts were poor inhibitors with IC_{50} values estimated >200 μgGAE/mL, which is supported by previous work [7, 10]. The rowanberry PAC fraction (IC_{50}> 200 μgGAE/mL) was considerably less effective than the whole rowanberry extract (IC_{50}=30 μgGAE/mL). This is different to the situation with amylase inhibition [9] and suggests that these tannin components are not influential in inhibition of α-glucosidase. Given their high contents of ellagitannins [19], the lack of inhibition by raspberry and cloudberry extracts strongly suggests that these tannins are poor inhibitors of α-glucosidase, which is supported by previous work [7].

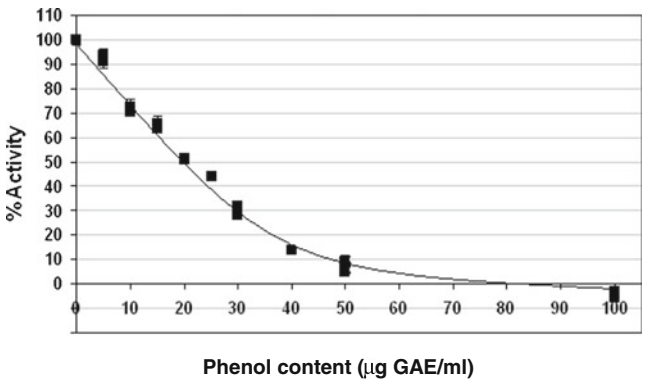

Fig. 5 Effect of black currant extracts on α-glucosidase activity. The scatter plot contains data from three separate experiments. Values are averages of assays ± standard error. The *trend lines* were fitted by Excel software

The black currant extracts were rich in anthocyanins [especially the characteristic glucosides and rutinosides of cyanidin and delphinidin; [20]] which made up approximately 70 % polyphenol content with smaller amounts of flavonols and hydroxycinnamate derivatives. Pure anthocyanins, such as cyanidin *3-O*-glucoside, were relatively ineffective (IC_{50} value of 205 μg/mL) and an anthocyanin-rich preparation from black currants was no more effective than cyanidin-3-*O*-glucoside alone. Taken together, these results suggest that anthocyanins alone are not responsible for the α-glucosidase inhibition of black currants. The more potent inhibition by the berry extracts suggests that other components present in the berry extracts enhance inhibition. Indeed, flavonol glycosides have been shown to inhibit α-glucosidase in vitro [21]. Black currants also contain a range of flavonol derivatives, albeit in lesser amounts than anthocyanins, which could act in concert with the more abundant anthocyanins. Future work in this area could extend the study of inhibition of α-glucosidase with combinations of anthocyanins and purified flavonols.

Black currant extracts potentiated the inhibition of α-glucosidase caused by acarbose (Fig. 6). This was demonstrated by the increased inhibition caused by combining acarbose with black currant extracts at their IC_{50} values. Moreover, combination of berry extracts at 75 % of their IC_{50} values could replace the inhibition "lost" by reducing the acarbose levels to 25 % IC_{50} value i.e. addition of black currant extracts returned inhibition to around 50 % of control activity. Overall, these results suggest that acarbose and

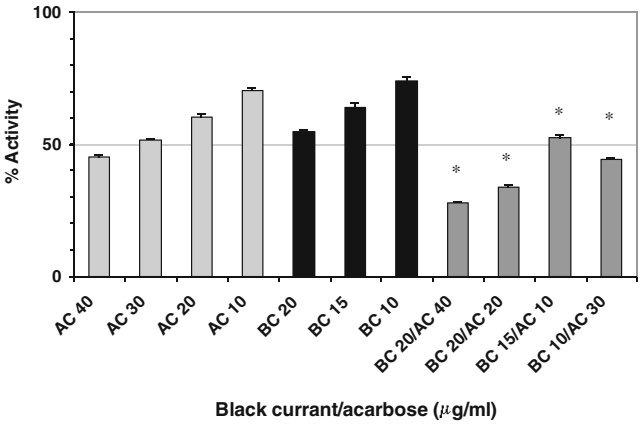

Fig. 6 Effect of co-incubation of black currant extracts and acarbose. A single representative experiment is shown but the experiment was repeated three times with the same results. Values are means of triplicate assays ± standard error. *BC* black currant, *AC* acarbose. The values of the combined samples denoted by *asterisks* were statistically different ($p<0.05$; Student's *t* test) from the values obtained from the single components; e.g., the value of the combination of BC 20/AC 40 was significantly different than the value for BC 20 or AC 40 alone

black currant extracts can act in a synergistic manner to inhibit α-glucosidase in vitro.

Conclusions and Perspectives

Polyphenol-rich extracts from berries inhibit lipase, α-amylase and α-glucosidase activities in vitro at low levels certainly achievable in the GIT after the intake of berries [22, 23]. Our studies have indicated certain candidate components for inhibition; e.g. tannin-like components (ellagitannins and proanthocyanidins) were effective against amylase and lipase but ineffective against α-glucosidase. These tannin components may operate by non-specific protein-binding and preventing the enzymes from interacting with their substrates [24]. The protection of amylase inhibition by the presence of proteins [bovine serum albumin [9] or gelatin [7]] or the reduction in potency of inhibition of lipase activity when pancreatin was used [8] raises questions about the possible effectiveness of these polyphenol components in vivo where "normal" mixtures of foods were ingested which could be protein-rich. However, the protection of amylase activity by BSA was only noted when the protein to polyphenol ratio reached a 20-fold excess. In addition, although many polyphenolic components survive gastrointestinal conditions [23], others, such as anthocyanins, are less stable. Nevertheless, these results are only indicative and confirmatory studies in animals or humans are required.

It is well accepted that polyphenol-rich foods or extracts can influence digestive processes [25, 26]. Polyphenols from red wine delay the absorption of dietary fat in humans [27] and reduce obesity in rats [28]. Similar effects on obesity have been identified for tea polyphenols [e.g. 29], which inhibit pancreatic lipase in vitro [30]. However, inhibition of lipase is only one of many mechanisms whereby polyphenols could influence obesity [31] and it is possible that inhibition of lipase would be overcome by compensatory increases in lipase secretion as seen in studies on PACs in rats [32] and some studies have suggested that high polyphenol intake does not increase faecal lipid excretion which may be expected if lipase was inhibited [33].

Animal model studies have shown that polyphenol-rich berry extracts can ameliorate hyperglycaemia [e.g. 34, 35] and there are indications that berry components may be effective in humans [36]. Our in vitro results confirm that polyphenols found in berries inhibit both α-amylase and α-glucosidase in vitro and they can act additively with acarbose. This indicates obvious potential for controlling starch digestion in the GIT. In addition, identifying berry extracts with high α-glucosidase but lower α-amylase inhibitory potential, such as the black currant extracts, could prevent certain side-effects of acarbose, which are largely due to undigested starch reaching the colon [37] and undergoing fermentation. Further work is required to confirm that berry components can inhibit these enzymes in vivo and cause beneficial effects on fat intake or glycaemic responses.

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