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Different Polyphenolic Components of Soft Fruits Inhibit α-Amylase and α-Glucosidase

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Polyphenol-rich extracts from soft fruits were tested for their ability to inhibit α-amylase and α-glucosidase. All extracts tested caused some inhibition of α-amylase, but there was a 10-fold difference between the least and most effective extracts. Strawberry and raspberry extracts were more effective α-amylase inhibitors than blueberry, blackcurrant, or red cabbage. Conversely, α-glucosidase was more readily inhibited by blueberry and blackcurrant extracts. The extent of inhibition of α-glucosidase was related to their anthocyanin content. For example, blueberry and blackcurrant extracts, which have the highest anthocyanin content, were the most effective inhibitors of α-glucosidase. The extracts most effective in inhibiting α-amylase (strawberry and raspberry) contain appreciable amounts of soluble tannins. Other tannin-rich extracts (red grape, red wine, and green tea) were also effective inhibitors of α-amylase. Indeed, removing tannins from strawberry extracts with gelatin also removed inhibition. Fractionation of raspberry extracts on Sephadex LH-20 produced an unbound fraction enriched in anthocyanins and a bound fraction enriched in tannin-like polyphenols. The unbound anthocyanin-enriched fraction was more effective against α-glucosidase than the original extract, whereas the α-amylase inhibitors were concentrated in the bound fraction. The LH-20 bound sample was separated by preparative HPLC, and fractions were assayed for inhibition of α-amylase. The inhibitory components were identified as ellagitannins using LC-MS-MS. This study suggests that different polyphenolic components of fruits may influence different steps in starch digestion in a synergistic manner.

KEYWORDS: α-Amylase; α-glucosidase; anthocyanins; ellagitannins; inhibition; starch digestion

INTRODUCTION

Berry fruits such as raspberries, strawberries, blueberries, and blackcurrants have high levels of polyphenolic compounds and are therefore rich sources of dietary antioxidants (*1*, *2*). There is substantial epidemiological evidence that insufficient intake of dietary antioxidants may predispose one to a number of chronic health disorders such as cancers and coronary heart disease (*3*−*7*). Recent Finnish health intervention programs have had particular success resulting in 60% reduction in heart disease and strokes (*8*). These health improvements can be largely explained by major lifestyle changes including reduced intake of salt and saturated fats and reduced rates of smoking in men, but there was also a 2−3-fold increase in the consumption of fruits and vegetables. In particular, there was a notable increase in consumption of antioxidant-rich wild and cultivated berries including cowberries, raspberries, strawberries, blackcurrants, and cloudberries (*9*).

The mechanism by which dietary antioxidants exert their effects is not fully understood, but a consensus has developed that antioxidants protect against damage to membranes, proteins, and DNA by scavenging free radicals generated through oxidative metabolism (see *10* for overview). Polyphenols probably have multiple beneficial effects and may also induce protective effects by chelating metal ions (*11*) or activating the expression of antioxidant enzymes (*12*).

There is increasing evidence that individual polyphenols or classes of polyphenols may cause other beneficial effects, independent of their antioxidant capacities, by directly influencing the activities of key enzymes. There have been reports that polyphenolic fractions from plants can cause insulin-like effects in glucose utilization (see *13* for review). Polyphenolic extracts from a number of plants were found to be effective inhibitors of intestinal α-glucosidase activity (*14*) with K_i values in the same range as synthetic inhibitors (acarbose and voglibose) already being used therapeutically to control non-insulin-dependent diabetes mellitus (NIDDM) (*15*). These extracts also inhibited α-amylase activity, which could prove to be synergistic to their potential therapeutic effect on post-meal blood glucose levels. The most effective inhibitory agents against α-glucosidase activity proved to be diacylated anthocyanins (*16*), which alone were capable of inducing an anti-hyperglycemic effect

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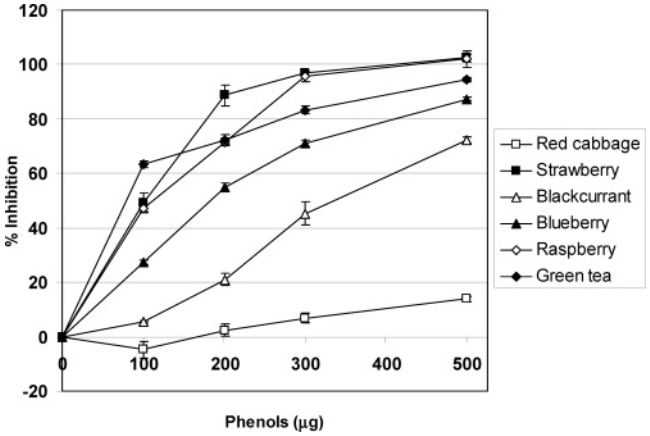


Figure 1. Effect of polyphenol extracts on pancreatic α-amylase activity. The % inhibition of assay is plotted against concentration of phenols/assay. Each value is the mean of triplicates ± standard error. Each graph is representative of a number of repeat assays.

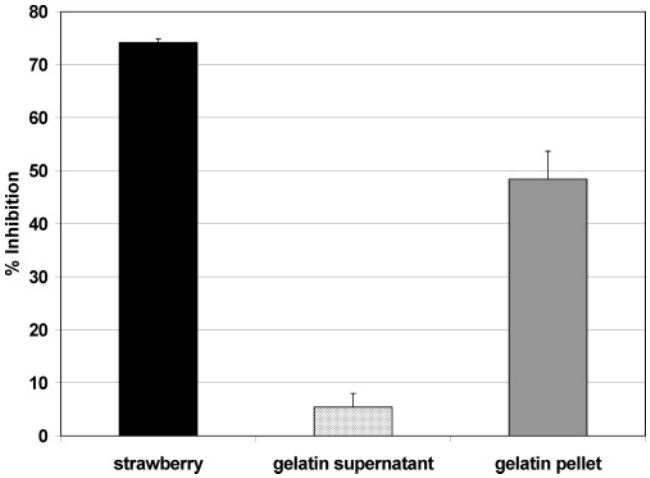


Figure 2. Effect of gelatin on inhibition of salivary α-amylase by strawberry polyphenols. Each assay carried out with 50 μg of phenols/assay. Each value is the mean of triplicates ± standard error.

in rats (*17*), but no further information on their inhibition of α-amylase was presented. This paper tests polyphenol-rich extracts from a range of fruits for α-glucosidase and α-amylase inhibition to identify compounds that may influence the digestion of starch in vivo.

MATERIALS AND METHODS

Plant Material and Extraction. Strawberries (*Fragaria ananasia*, SCRI breeding variety 932034), blueberries (*Vaccinium corymbosum* L. variety Berkley), and blackcurrants (*Ribes nigrum* L. variety Ben Lomond) were grown at SCRI, picked when ripe, and handled fresh or after freezing. Larger amounts of strawberries (variety Elsanta) were obtained from local growers (Abbey Fruit, Arbroath, U.K.). Raspberries (*Rubus idaeus* L. variety Glen Ample) were purchased from local farmers. Red cabbage, red grape juice, red wine, and green tea were purchased from supermarkets. Phenolic-rich fractions were obtained using a previously described method (*18*). Briefly, 100 g of fresh or frozen plant material was homogenized in a Waring Blender using 100 mL of 0.5% (v/v) glacial acetic acid in water. The extract was filtered through a glass sinter. Alcohol was removed from red wine by evaporation, and red wine and red grape juice were adjusted to 0.5% acetic acid before chromatography. Initially the extracts were passed through a 15-mL bed volume column of Polyamide S (Riedel de Haen Ltd, Seelze, Germany) preconditioned in 0.5% (v/v) acetic acid in methanol then equilibrated in 0.5% (v/v) acetic acid in water. The column was then washed with acetic acid/water to remove unbound material. The bound material, obviously enriched in anthocyanins, was then eluted with 2 × 15 mL of acetic acid/methanol. The bound material was evaporated to dryness and resuspended in acetic acid/water. To ensure that no free sugars were present which could interfere with amylase or glucosidase assays, these samples were applied to 10-mL bed volume columns of C18 packing material (Sigma Chem. Co. Ltd.), and the procedure was repeated as above. The C18-bound extracts were evaporated to dryness with repeated additions of methanol to drive off acetic acid and resuspended in small volumes of water to give the polyphenol-rich extracts. Green tea extracts were prepared by placing one tea bag (50 g) of green tea in 100 mL of freshly boiled water and brewing for 5 min. The bag was removed and squeezed dry, and the resulting solution was stored at 4 °C before use.

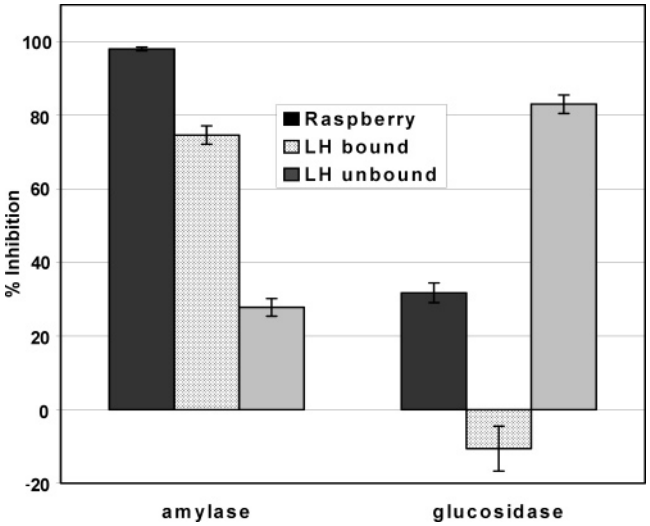


Figure 3. Effect of partition of raspberry polyphenols on Sephadex LH-20 on inhibition of α-amylase and α-glucosidase. Each assay carried out with 50 μg of phenols/assay. Each value is the mean of triplicates ± standard error.

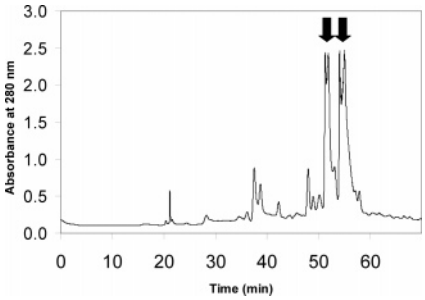


Figure 4. Separation of raspberry polyphenols on preparative reverse phase HPLC. Absorbance at 280 nm is shown. The black arrows mark the position of the compounds inhibitory to α-amylase.

For removal of tannins, an equal volume of 0.5% (w/v) suspension of porcine skin gelatin (Sigma Chem. Co. Ltd., product G-1890) was added to the sample and shaken for 5 min at room temperature (adapted from *19*). The gelatin was precipitated by centrifugation at 12 000 rpm in a microfuge, and the supernatant was decanted into a fresh tube. The gelatin pellet was extracted with an excess of 0.5% (v/v) acetic acid in ethanol. The supernatant was recovered by centrifugation as above and dried by rotary evaporation.

Sorption to Sephadex LH-20 in aqueous ethanol and selective debinding with aqueous acetone is an established method for separating tannins from non-tannin phenolics (*20*). The method was adapted from the Tannins handbook (kindly made available from the Hagermann laboratory at www.users.muohio.edu/hagermae/tannin.pdf. Briefly, a column of Sephadex LH-20 was washed in 80% (v/v) ethanol/water and then 50% (v/v) acetone/water before being equilibrated with three volumes of 80% ethanol. The extract in 80% ethanol was applied to the column, and the run-through material plus three volumes of 80%

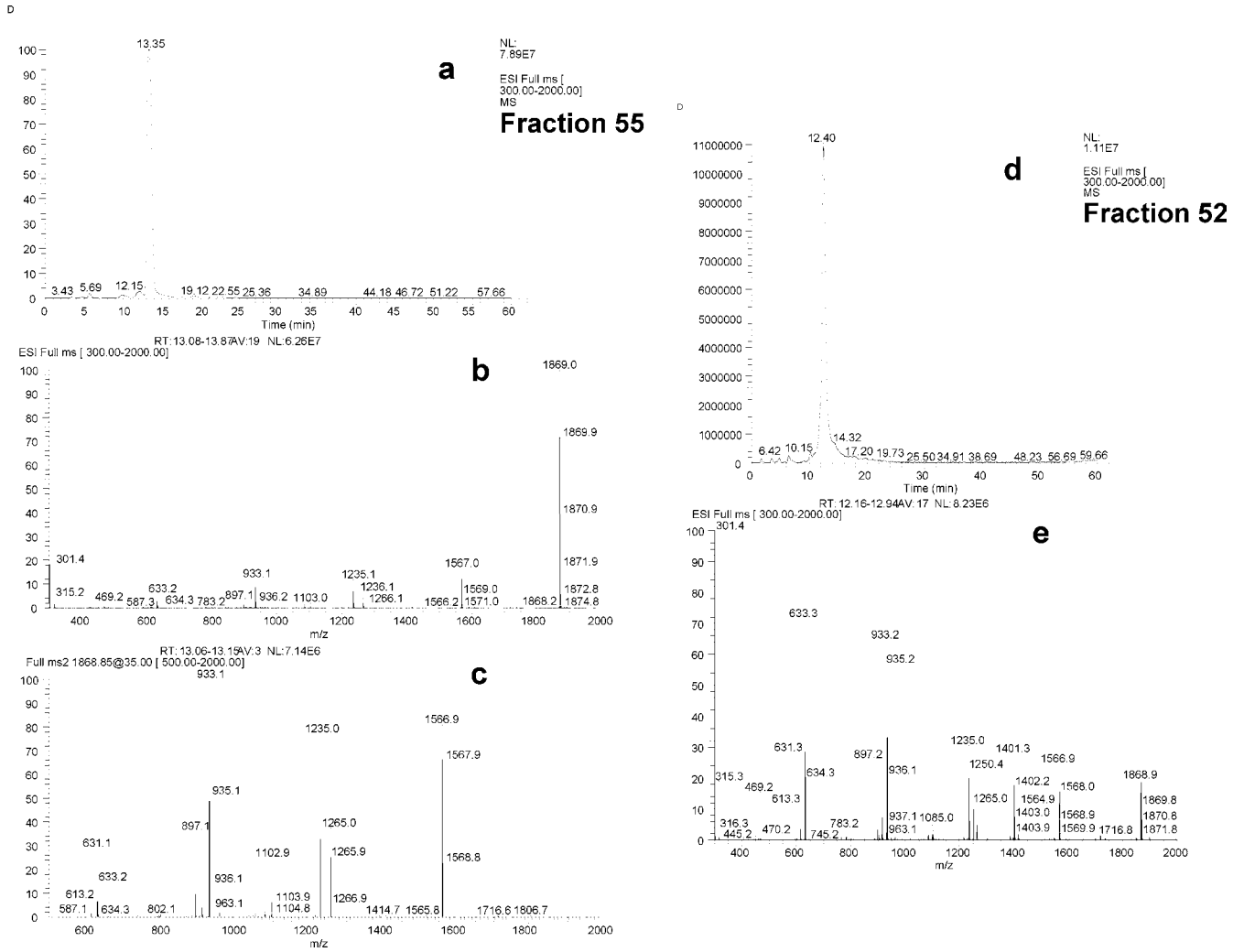


Figure 5. Analysis of α-amylase inhibitory fractions 55 and 52 by LC-MS. (a) Total ion count for fraction 55, (b) full MS spectrum across the main peak, and (c) MS^2 spectrum of the *m/z* ion at 1869.0. (d) Total ion count for fraction 52, and (e) full MS spectrum across the main peak.

ethanol were collected as the unbound fraction. The bound fraction was eluted with three volumes of 50% acetone. The unbound and bound fractions were evaporated to near dryness and then stored frozen.

Anthocyanin and Phenol Assays. The total anthocyanin concentration was estimated by a pH differential method (*1*). The absorbance value was related to anthocyanin content using the molar extinction coefficient calculated in-house for cyanidin-3-glucoside (CyG). Phenol content was measured using a modified Folin−Ciocalteau method (*1*). Phenol contents were estimated from a standard curve of gallic acid.

Amylase Assay. Stock starch solution was prepared by suspending 1% (w/v) soluble potato starch (Sigma Chem. Co. Ltd., product S-2360) in synthetic saliva buffer (*21*) and gelatinizing the mixture for 15 min at 100 °C. Artificial saliva was made by dissolving porcine pancreatic α-amylase (Sigma Chem. Co. Ltd., product A-3176) in synthetic saliva buffer at 380 mg/L. Human saliva was diluted 1:20 with synthetic saliva buffer prior to assay. The control assay contained 900 μL of synthetic saliva buffer and 100 μL of α-amylase, and the reaction was started by addition of 500 μL of starch solution. The + extract assays contained various amounts of extracts in the same volume. To estimate K_i values (the amount of phenols that gave 50% inhibition of amylase) assays were carried out with phenol contents ranging from 10 to 1500 μg.

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 inhibition of amylase activity was calculated as the difference between the control and the + extract reactions divided by the control reaction.

Assay for α-Glucosidase. The assay method was taken from *14*. Acetone powder from rat intestine (Sigma Chem Co. Ltd., product I1360) was extracted as a source of α-glucosidase. The rate of release of *p*-nitrophenol from *p*-nitrophenyl α-D-glucopyranoside (Sigma Chem. Co Ltd., product N1377) was measured at 30 °C. To estimate K_i values (the amount of phenols that gave 50% inhibition of amylase), assays were carried out with phenol contents ranging from 5 to 150 μg.

Preparative Reverse-Phase HPLC. Samples of raspberry extracts were diluted 1:1 in 5% (v/v) acetonitrile in 1% formic acid and 5 mL aliquots separated using a Gilson 305 liquid chromatography system with a 250 mm × 21.2 mm octadecyl silica (C-18) column (Phenomenex Ltd., Macclesfield, U.K.). A linear gradient of 5−25% (v/v) acetonitrile in 1% formic acid was applied over 60 min at a flow rate of 5 mL/min followed by a wash at 80% acetonitrile. The elution was monitored at 280 and 510 nm using a Gilson 170 diode array detector, and fractions (5 mL) were collected every minute. Each fraction was assayed for phenol content.

Liquid Chromatography−Mass Spectroscopy (LC-MS). Samples were analyzed on a LCQ-Deca system comprised of a Surveyor autosampler, pump, and photodiode array detector (PDAD) and a ThermoFinnigan mass spectrometer ion trap. The PDAD scanned two discrete channels at 280 and 365 nm. The column (Synergi Hydro C18 with polar end capping, 2 mm × 150 mm, Phenomonex Ltd.) was heated to 40 °C, and the autosampler tray was cooled to 4 °C. Samples were eluted over a gradient from 5% (0.5% formic acid) to 40% acetonitrile (0.5% formic acid) over 60 min at a rate of 200 μL/min.

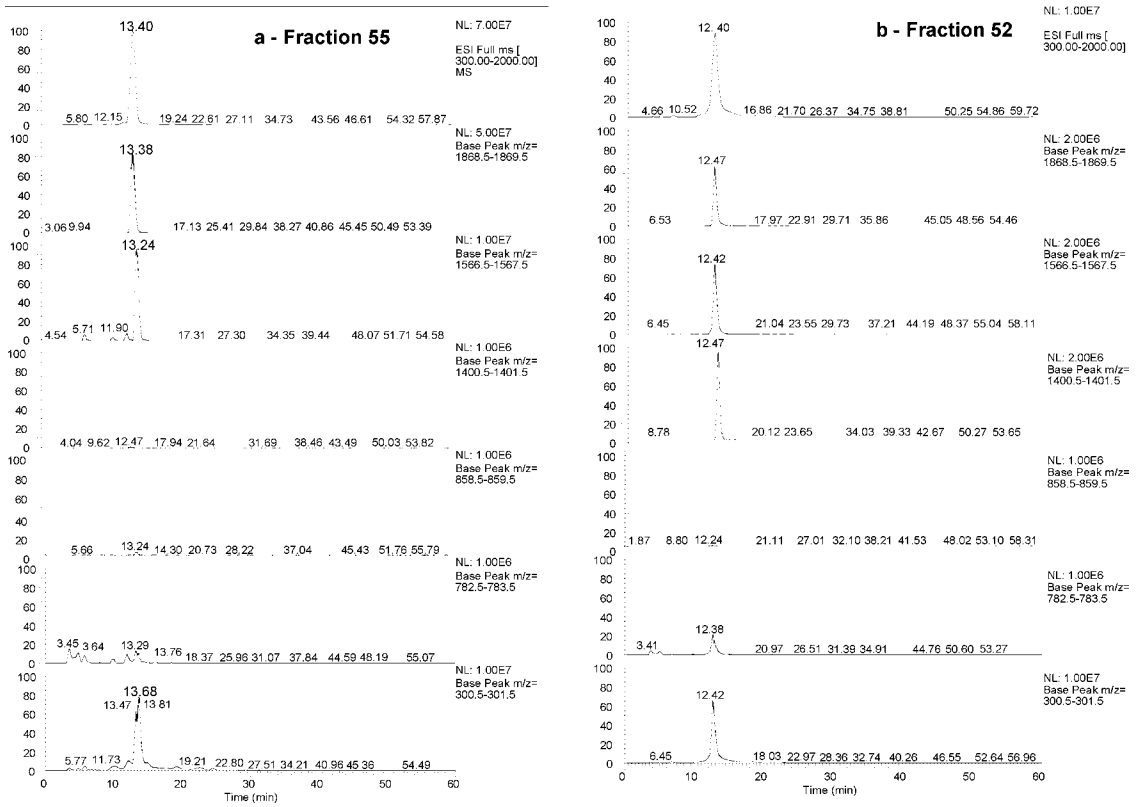


Figure 6. Analysis of fractions 55 and 52 at specific *m*/*z* values. The chromatographs show the abundance of specific *m*/*z* values. The putative structures are given in Chart 1.

The LCQ-Deca LC-MS was fitted with an ESI (electrospray ionization) interface and analyzed the samples in negative-ion mode. There were three scan events; full scan analysis, followed by MS/MS of the most intense ions, and then MS^3 of selected ions. The data-dependent MS/MS used collision energies (source voltage) of 35% and MS^3 at 50%. The capillary temperature was set at 250 °C with sheath gas at 60 psi and auxiliary gas at 10 psi.

RESULTS

Strawberry and raspberry extracts were the most effective inhibitors of α-amylase followed by blueberry, blackcurrant, and then red cabbage with estimated K_i values of 120, 150, 300, 400, and 1100 μg of phenols/assay, respectively (**Figure 1**). The inhibition by strawberry extracts ($K_i \approx 120$ μg/assay) approached that of green tea ($K_i = 60$ μg/assay), a known inhibitor of salivary amylase (*19*).

The order of inhibition of salivary α-amylase by extracts was the same as that of artificial saliva. However, the extracts were more effective on salivary amylase, with K_i values of 50 μg/assay for the strawberry extract, probably due to dilution of the amylase. Phenolic-rich extracts from red grape juice and red wine were also effective inhibitors of salivary α-amylase with K_i values of ∼20 μg/assay (results not shown). Neither gallic acid nor ellagic acid inhibited α-amylase at concentrations ranging from 50 to 500 μg/assay (results not shown).

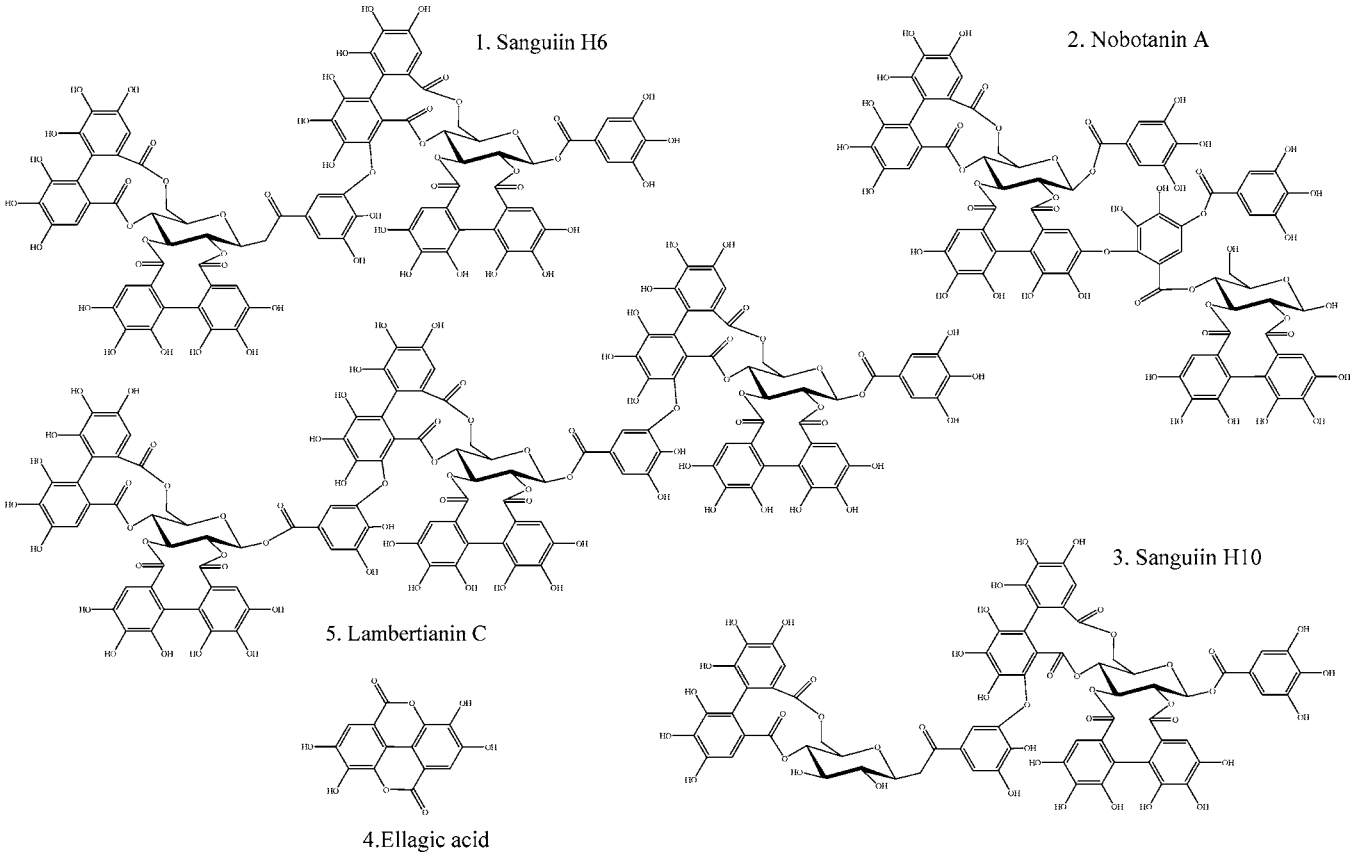
The extracts also inhibited rat intestinal α-glucosidase activity, but the order of effectiveness was different than with α-amylase. Blueberry and blackcurrant were the most effective followed by strawberry, raspberry, and red cabbage with apparent K_i values of 18, 22.5, 42, 87, and 145 μg of phenols/assay, respectively. The K_i values were influenced by the assay conditions (notably by variation in the protein content of the α-glucosidase preparation), but the order of effectiveness was always the same.

Treating strawberry extracts with gelatin prior to assay effectively removed the inhibitory effect against α-amylase (**Figure 2**) even when the extracts were assayed at the same level of phenols/assay. The inhibitory compounds could be recovered from the gelatin pellet by repeated washing with acidified ethanol. Gelatin "fining" has been traditionally used to remove tannins from plant extracts (*22*).

The raspberry extract was fractionated by sorption onto Sephadex LH-20. The LH-20 unbound material eluted from the column in 80% ethanol was bright red and contained the majority of anthocyanins. The LH bound material was eluted in 50% (v/v) acetone/water and very slightly pink. The LH unbound material had a much reduced ability to inhibit α-amylase compared to the original extract, and the inhibitory activity was recovered in the LH bound fraction (**Figure 3**). Sorption to Sephadex LH20 is a well-documented means of enriching plant tannins (*22*). Conversely, the inhibitory activity against α-glucosidase was recovered in the LH-20 unbound fraction with no apparent inhibitory activity in the LH-20 bound fraction (**Figure 3**). This strongly suggests that inhibition of α-amylase and α-glucosidase is caused by different polyphenolic components.

The LH-20 bound fraction was separated into distinct peaks by reverse-phase HPLC on a preparative C18 column (**Figure 4**). Only the late eluting peaks (**Figure 4**, see arrows) caused significant inhibition of α-amylase at 25 or 50 μg of phenols/assay (results not shown). To identify the inhibitory compounds, fractions 52 and 55 were analyzed by LC-MS (**Figure 6**). Fraction 55 gave one main peak (retention time 13.35) which gave one dominant ion at *m*/*z* 1869.0 (**Figure 5a** and **b**). The *m*/*z* 1869 ion gave MS^2 ions at 1869, 1567, 1266, 933, and 631 (*m*/*z*) (**Figure 5c**). The mass ion and the fragment ions were essentially identical to the pattern obtained (*23*) for Sanguiin H6 (**Chart 1**; structure 1). The other mass ions present in the

Chart 1. Structures of Ellagitannin Derivatives



full mass spectrum of the peak (**Figure 5b**) at 1567, 1235, 933, and 633 *m*/*z* also appear in the MS^2 spectrum of *m*/*z* 1869 (**Figure 5c**), suggesting that they are breakdown products of Sanguiin H6. Indeed, the *m*/*z* ion at 1567 can be assigned to Sanguiin H6 lacking one HHDP structure (loss of 302), whereas 1235, 933, and 633 can be assigned to further breakdown products of Sanguiin H6. Scanning the spectrum at *m*/*z* values characteristic of ellagitannins previously identified in raspberry extracts (*37*) suggested the presence of other coeluting ellagitannins in minor amounts (**Figure 6**). The $m/z = 859$ has been assigned to Nobatanin/Malabathrin B-like ellagitannins (structure 2), $m/z = 783$ to Sanguiin H10 (structure 3), and $m/z = 301$ to ellagic acid (structure 4).

Fraction 52 gave one major peak on total ion scan (retention time 12.40 min) and gave full MS peaks at *m*/*z* values characteristic of all the compounds found in fraction 55 (**Figure 5d** and **e**). In addition, there was an appreciable peak at $m/z = 1401$ which has been assigned to Lambertianin C (structure 5) (*23*). A similar "mining" exercise at the relevant base peak *m*/*z* values confirmed the presence of a number of coeluting ellagitannins (**Figure 6b**). In summary, fractions 52 and 55 contain a mixture of ellagitannins and ellagic acid. However, from the relative abundances it is clear that fraction 55 is predominantly Sanguiin H6 and fraction 52 contains more equal amounts of Lambertianin C, Sanguiin H10, and Sanguiin H6 and its breakdown products.

DISCUSSION

The inhibitory effectiveness of extracts against α-glucosidase was related to their anthocyanin content. The raspberry LH-20 bound fraction, which was effectively anthocyanin free, showed no detectable inhibition of α-glucosidase at 100 μg/assay. However, the raspberry LH-20 unbound sample, which was enriched in anthocyanins, gave a lower K_i for α-glucosidase than the original raspberry extract (47 versus 87 μg/assay). This agrees with previous findings (*14*) that polyphenolic extracts of extracts from sweet potato (*Ipomoea batatas* L.) roots and Morning glory (*Pharbitis nil* cv. Scarlett O'Hara) flowers enriched in anthocyanins were effective inhibitors of rat intestinal α-glucosidase *and* human α-amylase. Subsequently, they showed that diacylated anthocyanins from these sources were most effective against α-glucosidase (*16*) and capable of inducing an anti-hyperglycemic effect in rats (*17*). However, no further information on the ability of these compounds to inhibit α-amylase was given. Therefore, it is possible the initial report of inhibition of α-amylase (*14*) was caused by other components of the original extracts.

It has long been claimed that polyphenolic fractions from plants can alter glucose utilization in mammals, causing insulin-like effects (*13*). The active constituents in green and black teas have been identified as tannins (*19*). Inhibition of α-amylase by phenolic extracts of pears, cocoa, and lentils has been noted (*24*), and these authors also found that commercially available tannic acids and condensed tannins were also inhibitory. Indeed, the condensed tannin content of seeds is well known to be an antinutritional factor due to their inhibition of amylase and other digestive enzymes (*25*). The inhibition of salivary amylase by green tea extracts was attributed to tannin components (*19*). There have also been reports that tannins and ellagic acid derivatives from Banaba (*Lagerstroemia speciosa* L.) leaves are potent inhibitors of α-amylase (*26*). Therefore, it is perhaps not surprising that extracts that contain tannins (red grape, red wine, green tea, raspberry, and strawberry) were more effective α-amylase inhibitors than other extracts. The removal of inhibition by gelatin treatment and binding of the inhibitory components from extracts by sorption on Sephadex LH-20

strongly suggest that the amylase inhibitors are soluble, hydrolyzable tannins (*20*, *22*).

The α-amylase inhibitors in the LH-20 bound sample eluted late in the gradient (fractions 52/53 and 55/56) on RP-HPLC, and ellagitannins are known to elute late in similar HPLC conditions (*27*). The inhibitory fractions contained a mixture of ellagitannins and ellagic acid as shown by LC-MS; the second eluting peak (fraction 55) was predominantly composed of Sanguiin H6, which was previously found to be the major ellagitannin in raspberries (*27*, *28*). Fraction 52 contained a more equal mixture of Lambertianin C, Sanguiin H10, and Sanguiin H6 and its breakdown products.

Dietary ellagitannins are the main dietary source of ellagic acid, which has been reported to have antiviral (*29*) and anticarcinogenic (*30*, *31*) properties. Ellagitannins from raspberry have also been found to exert potent vasodilatory properties (*27*). Raspberries and strawberries are a good source of dietary ellagitannins, and there is interest in utilizing natural variation in ellagitannin content to select new soft fruit varieties (*32*, *33*).

Although the tannin-rich raspberry and strawberry extracts were effective against both salivary and pancreatic amylase, it is unclear whether they would be effective against pancreatic α-amylase in situ. Work on the bioavailability of dietary tannins has been limited (*34*), but gallo- or ellagitannins may not be stable in the neutral to mildly alkaline conditions of the small intestine (*35*). In addition, binding of tannins to proteins (*22*) (e.g., from food or digestive enzymes) in the small intestine would also reduce their effective concentration. Nevertheless, tannic acid and the tannin-rich nonalcoholic components of red wine have been shown to reduce serum glucose levels after starch-rich meals in a study of patients with non-insulin-dependent diabetes mellitus (*36*). As the mechanism involved in this anti-hyperglycemic effect is unknown, it is possible that the tannins are inhibiting α-amylase activity in situ. However, tannins have also been reported to be effective inhibitors of intestinal α-glucosidase activity (*37*), and it is becoming clear that a range of polyphenols also inhibit glucose uptake in intestinal cells (*38*).

The low K_i values against salivary α-amylase suggest that strawberry and raspberry extracts could influence the breakdown of starch in the oral cavity and perhaps influence the levels of fermentable sugars available to cariogenic bacteria. Indeed, tannins in green tea extracts have been shown to reduce the oral breakdown of starch (*19*), and tannins in strawberry and raspberry extracts may provide a similar effect.

ACKNOWLEDGMENT

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