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Effect of black bean tannins on in vitro carbohydrate digestion and absorption

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Legume seeds are an important source of protein and carbohydrate for both human and animal consumption. Nevertheless the bioavailability of these nutrients is affected by several antinutritional factors present in edible pulses. Most of them, except tannins, are destroyed, at least partially, during cooking. Tannins impair macronutrient utilization by forming tannin-protein complexes with various digestive enzymes precluding the formation of products absorbable by the small intestine. There is ample experimental evidence on the effect of these compounds on protein utilization, but little attention has been paid to the impairment of starch and disaccharide assimilation. In this study, it is shown that α-amylase, maltase, sucrase, and lactase were strongly inhibited in vitro by condensed tannins isolated from black beans. These pigments also affected in vitro glucose uptake by rat-everted intestinal sleeves, although the transport system was less susceptible to tannin inhibition than the hydrolases mentioned above. The inhibition of α-amylase and sucrase by bean tannins appeared to be of the mixed type with changes in both Vmax and Km. The effect of these compounds on enzyme activity was decreased by treating the tannin solution with polyvinyl-polypyrrolidone but not by heating. Tannin interference with the digestive machinery may explain the decrease in carbohydrate bioavailability observed in animals fed diets of high tannin content. (J. Nutr. Biochem. 7:445–450, 1996.)

Key words: tannins; Phaseolus vulgaris; black beans; disaccharidases; glucose absorption

Introduction

In many regions of the world beans are an important source of protein, carbohydrates, vitamins, and minerals for both human and animal consumption. Nevertheless, they contain antinutritional and toxic factors that impair, to various degrees, the biological utilization of their nutrients. Most of these deleterious agents are proteins, i.e., protease and amylase inhibitors, lectins, toxins, etc. These proteins are usually destroyed by effect of various treatments, such as ordinary cooking, which increase the nutritive value of the seeds.[1–3] However, most colored seeds contain heat-stable pigments which may be responsible for the relatively low digestibility of these grains.[1,4–6]

These pigments belong to the group of plant polyphenols. Among them, tannins, an ill-defined and chemically diverse class, have received considerable attention because of their ability to complex and precipitate proteins.[7] Based on its chemical character, two major tannin categories have been identified: hydrolyzable and condensed tannins. The second group, abundant in vegetable foods,[8] is formed by polymers of flavan-3ols (catechins) or flavan-3,4-diols (leucoanthocyanidins).[9,10]

Tannic acid, a hydrolyzable tannin commercially available, has been used as a model compound in various in vivo and in vitro studies depicted to evaluate the antinutritional effects of the whole tannin group. It was found to inhibit intestinal enzymes and transport systems concerned with carbohydrate assimilation such as amyloglucosidase/maltase,[11] sucrase,[12] and the intestinal sodium-dependent glucose uptake system.[13] Nonetheless, because tannic acid is not a usual dietary component,[8] the results of such studies should be interpreted with caution. For instance, diets

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based on high tannin sorghum do not cause depression of food intake as it has been reported for those containing tannic acid.[14–16] In addition, condensed tannins are more effective than tannic acid regarding the reduction of body weight in chicks.[17]

Bean tannins have been shown to strongly inhibit pancreatic trypsin, chymotrypsin, and α-amylase activities.[18–20] Most of these studies were performed using tannin-rich bean extracts that may contain other antinutritional factors. In this study, purified bean tannins were tested for their in vitro effects on carbohydrates digestive enzymes and the glucose uptake system. Results suggest that bean tannins may impair carbohydrate assimilation, contributing to the relatively low digestibility of cooked beans.

Methods and materials

Materials

Black beans (Phaseolus vulgaris, var. Cubagua), were obtained from Centro de Investigaciones Agropecuarias (CENIAP; Maracay, Venezuela). Lactose, maltose, sucrose, phloridzin, hog pancreatic α-amylase, and polyvinyl-polypryrrolidone were from Sigma Chemical Co. (St. Louis, MO, USA). Tannic acid, bovine pancreatin, tri-sodium-citrate dihydrate, tri-(hydroxymethyl)-aminomethane and 3.5-dinitro salicilic acid were from Merck (Darmstadt, Germany). Anhydrous D-glucose was from BDH Chemicals LTD (Poole-Dorset, U.K.) and soluble potato starch was from Fisher Scientific (Pittsburgh, PA, USA). The glucose oxidase-peroxidase reagent (Glox Novum) was from Kabi Diagnostica (Stockholm, Sweden). U-[^{14}-C]-glucose was purchased from New England Nuclear (Boston, MA, USA), and Sephadex LH-20 from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade.

Methods

Polyphenol extraction. Beans were placed above a steam current for 1 h, dried at 55°C for 25 hr and peeled off manually. Coats were grounded to a mean particle size of 820 μm, which allows the highest polyphenol recovery.[21] This raw extract was evaporated to dryness and the residue redissolved in 8 volumes of 95% ethanol (based on the original seed coat weight) and filtrated to remove insoluble materials. Aliquots of the ethanolic solution were used to prepare purified tannins by adsorption chromatography.

Fractionation of polyphenols. Tannins were purified by adsorption chromatography on Sephadex LH-20, using a modification of the procedure of Strumayer and Malin.[23] Briefly, 10 mL of the ethanolic polyphenol-extract were diluted with 30 mL of 95% ethanol and mixed with 10 g of Sephadex LH-20 swelled with the same solvent. The slurry was filtered under vacuum and washed with 30 ml portions of 95% ethanol until the absorbance at 280 nM of the eluate was close to zero. The elution of condensed tannins was completed after washing the gel 10 times with 30 mL portions of 50% acetone (fractions 17 to 26). The tannin-fractions were pooled, concentrated under vacuum, freeze dried and stored, and protected from light, at 4°C.[24] Just before use for the enzyme assays, the purified tannins were dissolved in water or in 0.1 M citrate buffer and cleared by low-speed centrifugation to remove insoluble materials. Actual tannin content (around 1 mg/mL) was estimated as described.

Estimation of polyphenols. Polyphenol content of raw HCl-methanol extracts, Sephadex LH-20 eluate fractions and purified tannin-solutions were estimated by both the Prussian blue and the quantitative anthocyanidin formation procedures.[10,22,25] Polyphenol content, expressed either as tannic acid equivalents (TAE) or black bean tannin equivalents (BTE), was obtained by comparison with the appropriate standard curve.

Preparation of the brush-border fraction. Female Sprague-Dawley rats, weighing 200 ± 20 g, were obtained from the colony kept at the School of Biology, Universidad Central de Venezuela. The rats were fed ad libitum a commercial diet (Ralston Purina, Valencia, Venezuela). Lights were on from 7:00 a.m. to 7:00 p.m. The rats were killed by decapitation and the small intestine were immediately excised and flushed with cold 0.15 M NaCl. The mucosa was scraped off with a glass slide and homogenized with 4 volumes (v/w) of the same saline solution[26] in a Potter-Elvehjem homogenizer flask with 10 strokes at 1,725 rpm. The homogenate was centrifuged for 30 min at 11,000 × g and the supernatant (brush-border fraction) stored frozen at −70°C until used. The protein contents of the brush-border fractions were determined using the procedure of Lowry et al.[27]

α-amylase assay. A modification of the procedure of Bergmayer was used.[28] The incubation mix (1 mL final volume) contained phosphate buffer (22 mM, pH 7.0), NaCl (3 mM), starch (5 mg), and 50 μg of either bovine pancreatin or purified hog pancreatic α-amylase. Samples were incubated at 25°C for 5 min. Incubations were ended adding 1 mL of the color reagent (1% 3,5–dinitrosalicylic acid and 30% Na^+-K^+-tartrate in 0.4 N sodium hydroxide). Color was developed by boiling the tubes in a water bath for 5 min. After cooling, mixtures were brought to 10 mL with water and read at 546 nM against a proper blank. The amount of maltose released was determined by comparison with a standard curve.

Disaccharidase assay. Lactase was measured as described by Asp and Dahlqvist[29] in 0.1 M citrate buffer (pH 6.0). Maltase and sucrase were assayed in the same buffer adjusted at pH 6.3.[30] Stock solutions of lactose, maltose, and sucrose (50 mg/mL) were prepared in citrate buffer at the appropriate pH. The assay mixture contained 0.5 mL of diluted brush-border fraction (used as enzyme source), 0.36 mL of substrate solution, and 0.14 mL of citrate buffer at the indicated pH. When the effects of tannins were tested, a given amount of buffer was replaced by the tannin solution. Reactions, started by adding the substrate, were carried out at 37°C for 60 min and stopped by placing the samples in a boiling water bath for 5 min. The amount of free glucose produced was measured using a glucose-oxidase peroxidase reagent.[11]

For comparative enzyme studies, we selected an enzyme concentration (determined by the dilution factor of the brush-border fraction) and an incubation length that resulted in a linear glucose release. Corrections were made for the small amount of free glucose present in the brush-border fraction and in the substrate and tannin solutions. The background color contributed by the tannin solutions was also subtracted.

Glucose uptake by everted intestinal sleeves. Intestinal sleeves (1 cm long) were prepared from everted rat jejunum and attached to the end of stainless-steel rods (300 × 4 mm).[31] The sleeves were pre-incubated 4 min at 37°C in 8 mL of Krebs-Ringer buffer (pH 7.4). They were continuously gassed with a 95:5 O_2-CO_2 mixture. At the end of this period, the rods were transferred to similar vials containing ^{14}C-glucose (10 mM; 0.005 μCi/μmol) in the same buffer and incubated for 4 additional min. When tannins or phloridzin were tested, 0.8 mL of Krebs Ringer buffer was substituted for 0.8 mL of either the condensed tannin or tannic acid solution

(20 mg/mL) or 10 mM phloridzin prepared in the same buffer. The sleeves were rinsed for 20 sec in cold 10 mM glucose in Krebs-Ringer buffer, blotted dry, released from the rods, and dried at 70°C in pre-weighed scintillation vials. Upon determining the dry weight, the intestinal pieces were digested in 0.1 mL of water and 1 mL of tissue solubilizer (Solvable, Dupont, Boston, MA, USA) for at least 4 hr at 70°C. Samples were mixed with 10 mL of scintillation fluid (Scint AXF, Packard Instrument Co., Downers Grove, IL, USA) and counted in a spectrophotometer (Packard, Model 2000 CA, USA). Glucose uptake was expressed as nmols/mg dry weight/4 min.

Results and Discussion

Fractionation of black bean polyphenols

Black bean polyphenols had been successfully fractionated by adsorption chromatography on Sephadex LH-20 columns.[20,22] Through this simple and reliable procedure, seed tannins can be separated from other polyphenols while obtaining a good yield and extensive purification. However, it is time consuming and requires between 8 to 10 hr to complete the chromatography. To overcome this problem we attempted the separation of tannins from other polyphenols using a batch-wise fractionation procedure *(Figure 1)*, which takes approximately 2 hr.

Removal of non-tannin polyphenols and other compounds extracted with 1% HCl in methanol was accomplished washing the gel 15 times with 30 mL portions of 95% ethanol until the A280 nM of the eluate was near zero *(Figure 1)*. The elution of condensed tannins was accomplished after 10 washings (30 mL each) with 50% acetone (fractions 17 to 26). Usually, the first fraction eluted with ethanol was contaminated with loosely bound tannins, which are drifted away along with other polyphenols at the beginning of the alcohol elution. Overall recovery was close to 86%, making this approach quite appropriate for preparative purposes.

The even-numbered fractions were analyzed for total polyphenols (Prussian blue test) or condensed tannins (quantitative anthocyanidin formation). A very close correlation (r = 0.96) was observed between both assays. The condensed tannins reacted strongly with the Prussian blue reagent and generated large amounts of anthocyanidins *(Figure 1)*. In addition, they induced the precipitation of proteins and were adsorbed by the complexing agent polyvinyl-polypyrrolidone (results not shown).

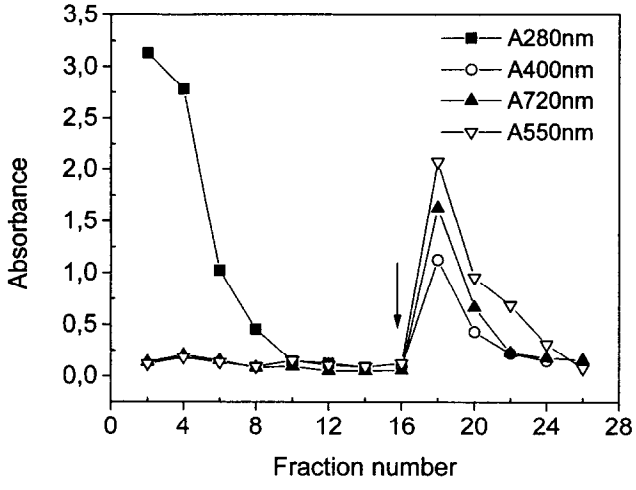

Figure 1 Batch-wise fractionation of black bean polyphenols. Aliquots of the 95% ethanol polyphenol solution containing 7.5 mg TAE of black bean polyphenols in 30 mL of 95% ethanol were mixed with 10 g of Sephadex LH-20 swelled in the same solvent. Tannins were eluted with 50% acetone (30 mL each time), as described in the text. Arrow indicates the shift from 95% ethanol to 50% acetone. Aliquots (200 µL) of the even numbered fractions were assayed for its polyphenol content with the Prussian Blue test, recording the absorbance at 720 nM. For the quantitative anthocyanidin formation test, 1 mL portions of the same fractions were analyzed as indicated in the text. Absorbance of samples was determined at 550 nM and corrected subtracting that of unheated blanks.

Effect of bean tannins on α-amylase activity

α-amylase plays, along with the brush-border glucoamylase, an important role in the degradation of dietary starches. *Figure 2* shows that the α-amylase activity of bovine pancreatin was inhibited to the same extent by either commercial tannic acid or black bean tannins. Treatment of condensed tannins in autoclave (120°C, 15 psi) for 15 min did not affect their inhibitory potency. Under the conditions of the in vitro assay, the amount of tannins required to inhibit starch degradation by 50% was close to 20 µg/mL. This confirms previous observations regarding the high sensitivity of α-amylase to tannin inhibition.[18,20,32,33]

Effect of bean tannins on brush-border disaccharidase activities

Disaccharides, whether present in foods or produced during the digestion of polysaccharides, must be hydrolyzed to their component monosaccharides. This task is performed by the intestinal disaccharidases that are located in the brush-border of enterocytes. Maltase, sucrase, and lactase are arranged as three bi-glycosidase complexes[34]: sucrase-

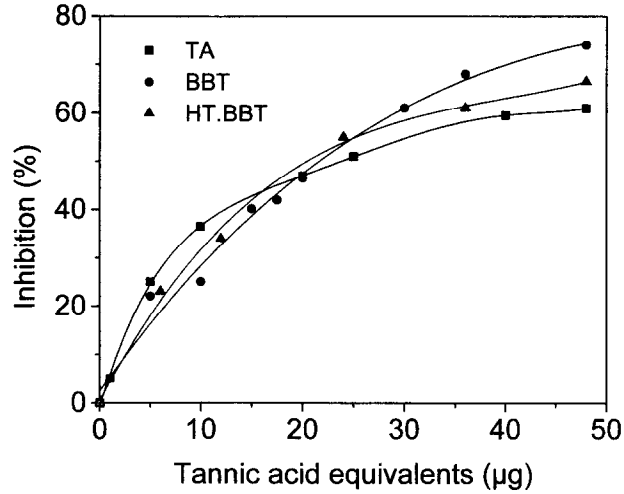

Figure 2 Effect of tannins on α-amylase activity. The α-amylase activity of bovine pancreatin (50 µg/mL) was determined as described in the text. TA: Tannic Acid; BBT, Black Bean Tannins; HT-BBT: Heat-treated Black Bean Tannins.

isomaltase, glucoamylase, and β-glycosidase. The sucrase isomaltase complex is responsible for all the sucrase activity and 80% of the maltase activity, whereas the remaining 20% is accounted for by the glucoamylase complex. All the neutral lactase activity is contained in the β-glycosidase complex.

The specific activity of brush-border disaccharidases in rats fed the commercial diet was 34.4 ± 5.3 U/mg Prot. for maltase, 4.8 ± 0.5 for sucrase, and 2.2 ± 0.2 for lactase. A similar relationship among the specific activities of these enzymes was reported by Thomsen and Tasman-Jones.[35]

The effect of condensed tannins on intestinal dissacharidases is depicted in *Figure 3*. Sucrase, maltase, and lactase were strongly inhibited by black bean tannins. Under the conditions of the in vitro assay, the amount required to inhibit each enzyme by 50% was 53, 75, and 97 μg/mL TAE, respectively. In the literature there are only a few reports concerning the effect of tannic acid on these enzymes. Welsch et al.[12] found that the amount of tannic acid required to inhibit sucrase in vitro by 50% was close to 50 μg/mL. Similarly, Björck and Nyman[11] observed a 66% inhibition of human maltase at 65 μg/mL. Considering that the amount of brush-border-extract-protein used in the assays changed for each enzyme, it is not possible to clearly establish the degree of sensitivity of any particular disaccharidase towards bean tannins. Nonetheless, it seems that maltase is less susceptible than sucrase to tannin inhibition. The former was assayed using a more diluted brush-border extract than sucrase but required a larger tannin concentration to reduce its activity by 50%.

Determination of the type of tannin inhibition of α-amylase and sucrase

Figure 4 depicts the effect of black bean tannins on α-amylase and sucrase activities presented in Lineweaver-Burk type plots. Without tannins, the Vmax and Km for sucrase (Panel A) were respectively 0.019 ± 0.0009 (μmol/min) and 0.059 ± 0.007 M. In the presence of bean tannins (60 μg/mL TAE), Vmax decreased by 37%, whereas Km increased by 56%. These results show that the inhibition of sucrase by bean tannins was of the mixed type. A similar situation was observed for purified hog pancreas α-amylase when incubated in the presence of bean tannins or tannic acid (Panel B).

The formation of non-specific tannin-protein complexes has been considered as the direct cause of enzyme inhibition.[6] The early findings of Tamir and Alumot seemed to confirm this hypothesis.[33] They showed that the inhibition of α-amylase and trypsin by carob tannins were noncompetitive, affecting only Vmax. In contrast, the interaction of black bean tannins with α-amylase and sucrase changed both Vmax and Km *(Figure 4)*. These results agree with those of Björck and Nyman who found that the inhibition of human maltase by tannic acid was of the mixed type.[11] Mixed type inhibition is observed when the enzyme and tannins are pre-incubated in the absence of the substrate. Apparently, binding of the substrate to the active site protects it from interacting with tannins leading to noncompetitive inhibition when the three components are mixed simultaneously.[9]

Effect of tannins on glucose uptake

Monosaccharide absorption is the final step of carbohydrate assimilation. Most of the glucose absorbed in the small intestine is taken up by the enterocytes through the activity of the Na^+-glucose co-transporter.[36] Bean tannins and tan-

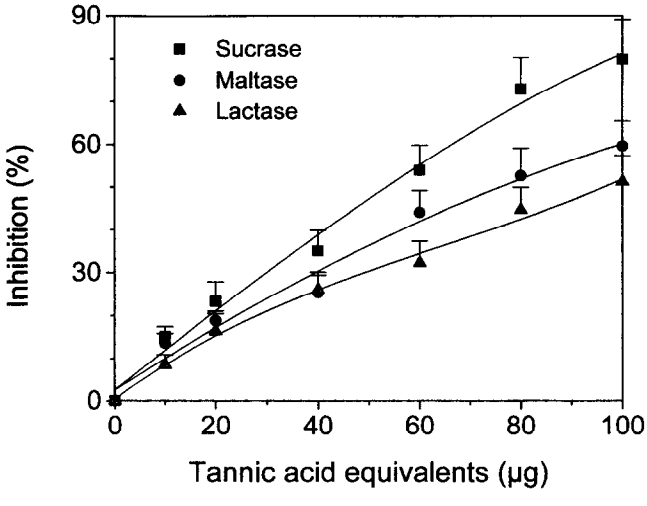

Figure 3 Effect of tannins on disaccharidase activity. Black bean tannins (10 to 200 μg TAE) were incubated, as described in the text, with an aliquot of the proper dilution of brush border fraction such that the assay mixture contained 15 to 20 mU of any given disaccharidase. Values are means ± S.D. for four experiments performed in duplicate in which different brush-border fractions were used.

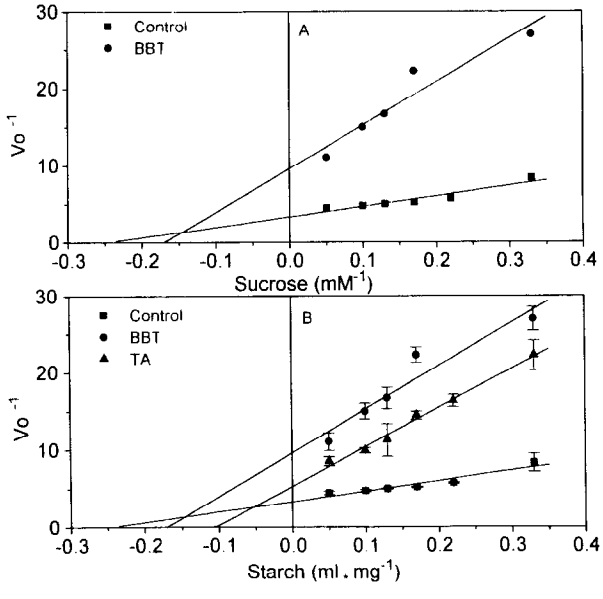

Figure 4 Lineweaver-Burk plot for sucrase and α-amylase. Sucrase activity was measured in the presence or absence of 60 μg TAE of bean tannins (A). Sucrose concentrations (30–80 mM). Hog pancreas α-amylase activity (B) was measured on soluble potato starch (1–10 mg/mL) in the absence of tannins or in the presence of either tannic acid (TA) or black bean tannins (BBT) (60 μg/mL). For sucrase values are means of two experiments performed in duplicate. For α-amylase means ± S.E.M. (n = 3) are given.

nic acid effects on glucose uptake by rat everted intestinal sleeves are shown in *Figure 5*. Both types of polyphenols (2 mg/mL) inhibited glucose incorporation by more than 50%, under conditions in which phloridzin (1 mM) decreased it by approximately 70%. Using brush-border vesicles from rat small intestine, Welsch et al.[37] found that the amount of tannic acid required to decrease glucose uptake by 50% was 0.5 mg/mL. The brush border vesicles used by these authors represent a simpler system as compared with the whole pieces of jejunum used in this study. In the former system the removal of some intestinal components (i.e., mucins) and the disruption of intestinal architecture may favor the interaction of tannins with the glucose uptake system. Therefore, the uptake inhibition could be observed at lower tannin concentrations.

Apparently, glucose absorption is less susceptible to inactivation by tannins than starch or disaccharide hydrolysis. The amount of tannin required to produce a 50% inhibition of the former is 10 to 100 times higher than those needed to block the hydrolases.

The results presented in this paper demonstrate that carbohydrate assimilation could be severely impaired by condensed tannins, explaining the low bioutilization of carbohydrates from bean diets.[16,38] Particularly in the case of starch, inhibition of α-amylase decreases the production of maltose whose hydrolysis may also be affected. Therefore, tannins may increase the proportion of resistant starch reaching the large bowel. It has not been established whether bean tannins affect bacterial fermentation. However, the increase in carbohydrate susceptible to be fermented by the colonic flora may be a contributing factor to the flatulence frequently associated with bean consumption.

Inhibition of disaccharidases may also be of practical significance. In some rural regions of Venezuela it is common to add black bean broth, rich in tannins, to infant bottles.[39,40] The inhibition of lactase and sucrase by bean tannins speaks against this practice because it would decrease the bioavailability of both the lactose from milk and the sucrose added for sweetness. Both sugars would reach the large bowel becoming available for bacterial fermentation as the undigested starch. Therefore, the energy value of the milk-broth mix could be reduced, whereas the risk of diarrhea could be increased in the case of undernourished infants affected by frequent infections. Besides the direct inhibition of maltase and sucrase by tannins, these polyphenols could also block the process of disaccharidase activation by intestinal proteases.[34] In contrast, the inhibition of glucose uptake may be less significant because a very high tannin concentration is required to reduce sugar uptake by 50% (*Figure 5*).

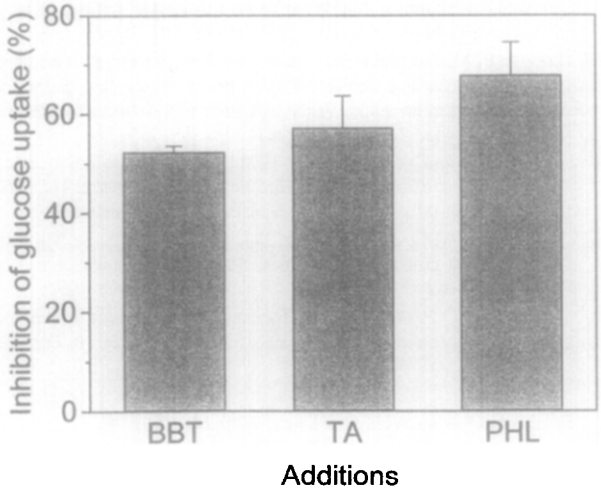

Figure 5 Effect of tannins on glucose uptake. [^{14}C]-Glucose uptake was measured using jejunal intestinal sleeves (1 cm in length) as described in the text in the presence of black bean tannins (BBT) or tannic acid (TA) (2 mg/ml) or phloridzin (PHL) (1 mM). Values are means ± S.E.M. for four experiments. Control uptake: 62.6 ± 5.3 nmols/mg dry weight.

As expected, heating of the tannin fraction did not affect its inhibitory properties (*Figure 2*), confirming the thermostable character of these compounds. Therefore, condensed tannins are likely to remain active even after cooking foods. Nonetheless, complexing of tannins with seed proteins during soaking and cooking of the seeds may render them unable to interact with the digestive machinery. This latter aspect is currently under investigation.

Only for purified hog pancreas α-amylase there was a clear difference between the effect of condensed tannins from black beans and that of tannic acid. The former produced a larger decrease in Vmax and a larger increase in Km than the latter (*Figure 4*). These results are in agreement with those of Carmona, who found that bean tannins were more effective promoting bovine serum albumin precipitation than tannic acid.[41] In addition, experiments performed with chicks and rats have revealed that bean tannins do not cause depression of food intake as tannic acid does while causing a larger inhibition of growth.[15–17]

It is not clear why the effects of black bean tannins and tannic acid on the α-amylase activity of bovine pancreatin and the rat glucose uptake system were essentially identical (*Figures 2* and *5*). It is likely that, in complex systems, the interaction of tannins with proteins of different affinities may lead to effects of similar intensity for both kinds of polymeric polyphenols.

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