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TANNINS FROM DIFFERENT FOODSTUFFS AS TRYPSIN INHIBITORS

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The trypsin inhibitory effect of phenolic extracts from different plant foodstuffs: two bean varieties, black and green tea and quince fruits as well as of standard phenolic compounds: (+)catechin, (-)epicatechin, gallic acid and tannic acid, was investigated. Monomeric tannin precursors exhibited 1000-times lower activity in the trypsin inhibition than high-molecular tannic acid. All of the plant phenolic extracts used in the experiments showed high level of the trypsin inhibition except for white bean one. Black tea polyphenols exhibited the highest inhibitory potency. The salting out of high molecular polyphenols reduced trypsin inhibiting effect of the examined extracts by 14–68%.

INTRODUCTION

The main chemical property of condensed as well as hydrolysable tannins is their ability to form complexes with proteins. The astringent sensation is assumed to be due to a cross-linking between tannins and proteins or glycoproteins in the mouth.

Condensed tannins – proanthocyanidins – have been defined by Bate-Smith and Swain as water soluble phenolic compounds – oligomers and polymers of flavan-3-ols which have molecular weights between 500 and 3000 and possess at least 10 hydroxyl groups.

Proanthocyanidins, as polyphenolic compounds, show positive biological properties, such as antioxidant and radical scavenging activities. Many experimental studies have suggested their protective role against cancers and cardiovascular diseases [Santos-Buelga & Scalbert, 2000].

Condensed tannins may also have adverse nutritional effects. A decrease in the digestibility of certain food ingredients, especially proteins, is attributed to direct interactions with the dietary nutrients or the inhibition of digestive enzymes, proteolytic ones in particular.

The products of plant origin rich in protein influence the quality of the human diet and of the feed, to a great degree, in developing countries in particular. Nevertheless, raw materials being rich sources of protein, contain often considerable quantities of condensed tannins which are potent trypsin inhibitors and decrease the digestibility of proteins by preventing their complete hydrolysis in the gut. Nguz *et al.* [1998] showed that trypsin inhibition is correlated with the concentration of condensed tannins according to a quadratic relation.

The investigations *in vivo* carried out with the use of tannin-rich feeds in animal feeding, have confirmed considerable decrease in protein and dry matter digestibility due to inhibition effect of tannins on the activity of trypsin and other digestive enzymes in the intestines of rats and poultry [Griffiths & Moseley, 1980; Longstaff & McNab, 1991; Nyamambi *et al.*, 2000]. Much has been written also on the adverse role of tannins in ruminant feeding and on various methods of removal of condensed tannins from feed [Kumar & Singh, 1984].

The inhibition of trypsin by polyphenols, especially condensed tannins of carob pods [Tamir & Alumot, 1969], lucerne [Milić *et al.*, 1972] and other fodder plants [Horigome *et al.*, 1988], sorghum [Daiber, 1975; Nguz *et al.*, 1998; Nyamambi *et al.*, 2000], seed legumes, mainly faba bean [Griffiths, 1979; Helsper *et al.*, 1993; Carbonaro *et al.*, 1996; Alonso *et al.*, 2000], has been also reported *in vitro*.

Although humans ingest lower amounts of tannins than animals, *in vitro* studies on the inhibitory potency of these compounds against trypsin are very important.

Trypsin (EC 3.4.21.4.) is the main proteolytic enzyme in pancreatic fluid. It is capable of hydrolysing only these peptide linkages which have been formed by carboxyl group belonging to arginine or lysine.

Several authors have reported on the difficulties involved in studying trypsin inhibition on account of the possible interaction of phenolic inhibitors with protein

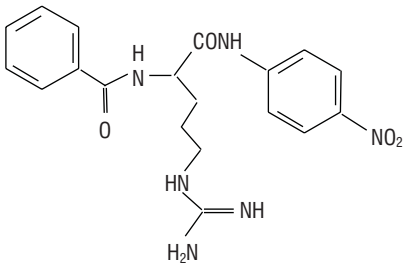

FIGURE 1. Structure of BAPNA.

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substrates. For that reason, methods based on hydrolysis of non-protein substrates, among others synthetic benzoyl--DL-arginine-p-nitroanilide (BAPNA) [Kakade *et al.*, 1974], have been developed. Structure of BAPNA (Figure 1) bears similarity to the peptide bond formed by arginine.

The purpose of this work was to compare the trypsin inhibitory effect of low- and high-molecular phenolic compounds from certain foodstuffs in terms of their antinutritional importance.

MATERIALS AND METHODS

Materials. Porcine pancreas trypsin and non-protein substrate BAPNA were obtained from Sigma. Standard phenolic compounds were purchased from: Sigma – catechin and epicatechin, Koch Light Laboratories – gallic acid, and Fluka – tannic acid.

The following plant foodstuffs were used: bean (*Phaseolus vulgaris* L.) – white variety Kontra and coloured variety Tip-top, quince fruit (*Chaenomeles japonica* L.), green and black tea (*Camellia sinensis*).

Analytical methods. Trypsin activity was determined according to the modified method of Kakade [Kakade *et al.*, 1974]. The reaction mixture was prepared by mixing 2 mL of trypsin solution with 2 mL of water (or 2 mL of an inhibitor solution) in a water bath at 37°C. A 5-mL portion of BAPNA solution previously warmed to 37°C, was added. After 10 min, the reaction was terminated by adding 1 mL of 30% acetic acid solution. The absorbance was measured at 410 nm against the reagent blank which was prepared by adding 30% acetic acid prior to BAPNA. It represented p-nitroaniline released as a result of enzymatic hydrolysis of BAPNA.

The percentage of inhibition was expressed as: (D-E)/D x 100, where D is the absorbance without an inhibitor and E – the absorbance in the presence of an inhibitor.

Total polyphenols were determined using Folin--Ciocalteu reagent [Sejder & Datunasvili, 1978]. The results were expressed as catechin equivalents.

Sample preparation. Polyphenolic extracts were isolated from foodstuffs according to the scheme presented in Figure 2 [Masquelier *et al.*, 1980].

RESULTS AND DISCUSSION

At the beginning of the studies the effects of standard polyphenols on the trypsin activity included: (+)catechin and (-)epicatechin as condensed tannin precursors as well as high-molecular tannic acid and gallic acid which is one of the basic units of hydrolysable tannins. The inhibition curves are shown in Figure 3A and 3B. As it was assumed, the monomeric phenols (catechins and gallic acid) exhibited very low levels of the trypsin inhibition. In the presence of 10 mg of epicatechin, catechin and gallic acid in the reaction mixture, the degrees of the trypsin inhibition achieved 38, 50 and 60%, respectively. On the contrary, 0.1 mg of tannic acid caused almost complete inhibition of the enzyme and therefore 50-fold lower concentrations of tannic acid (from 0.01 to 0.2 mg/mL) were used.

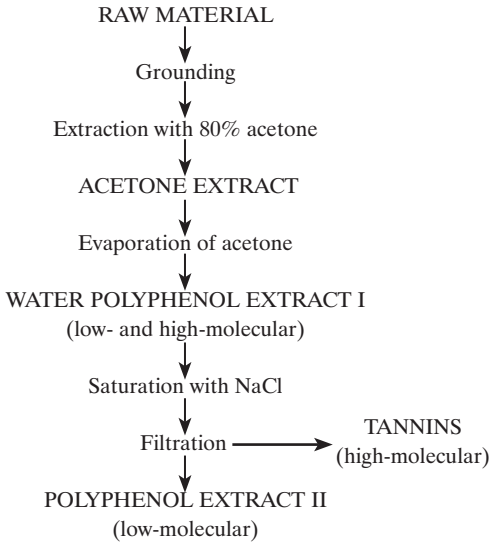

FIGURE 2. Isolation of polyphenolic extracts from foodstuffs.

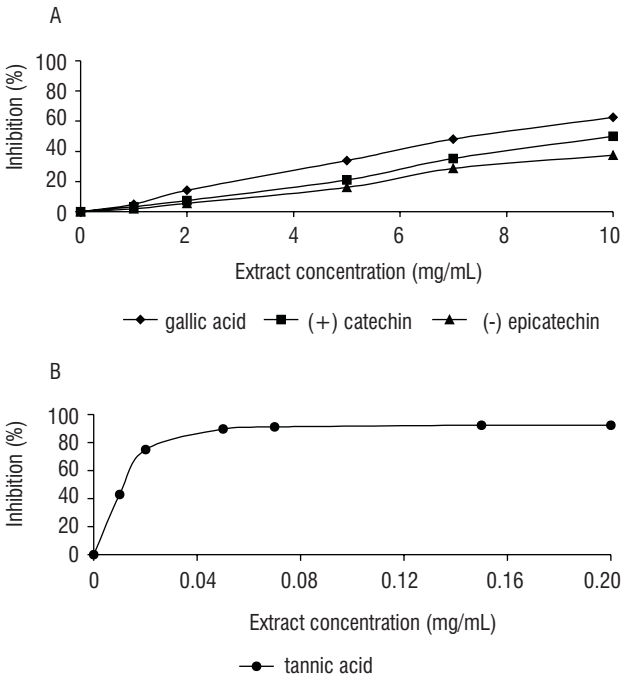

FIGURE 3. Trypsin inhibition curves for the standard phenolic compounds.

On the basis of the inhibition curves, the inhibitory potency I_{50} of each phenolic compound was calculated. The I_{50} value represents such concentration of the compound which is required to achieve 50% of the enzyme inhibition (Table 1).

TABLE 1. Values of I_{50} of standard phenolic compounds.

Compound	I_{50} (mg/mL)
(-) Epicatechin	>10
(+) Catechin	10
Gallic acid	7.4
Tannic acid	0.01

The I_{50} value for tannic acid was 0.01 mg/mL, whereas these values for catechins and gallic acid were about

10 mg/mL. Thus monomeric phenolics exhibited 1000-times lower levels of trypsin inhibition in comparison with high--polymeric tannic acid. This is in agreement with the results of Quesada *et al.* [1996] who found that simple phenolic compounds did not affect significantly trypsin and α-amylase activity levels except at extremely high concentrations. Inhibitory effects of other simple phenols (caffeic, chlorogenic and ferulic acids) on the trypsin activity have been also reported [Rohn *et al.*, 2002].

For the comparison of the trypsin inhibitory potency of low- and high-molecular polyphenols from different foodstuffs, the seeds of white and coloured bean varieties, green and black tea and quince fruits have been selected. Investigations were carried out using polyphenol extracts before (I) and after (II) salting out of the polymeric compounds. High-molecular tannin contents were calculated approximately as a difference in polyphenols I and II. The results are presented in Table 2.

To estimate the degrees of trypsin inhibition, phenolic extracts were added to reaction mixtures at the range of polyphenol concentrations from 0.01 to 0.2 mg/mL. The inhibition curves for polyphenol extracts I are shown in Figure 4 and the I_{50} values are presented in Table 2.

All of the polyphenol extracts used in the experiments, except for white bean one, exhibited a high level of the trypsin inhibition. The comparison of two examined bean varieties showed that polyphenols extracted from the coloured bean were considerably more active in inhibiting of trypsin (I_{50}=0.05 mg/mL).

Griffiths [1979] has suggested that the inhibitory activity demonstrated by extracts of the coloured field bean variety is due to the presence of tannins which are known to occur in considerably higher quantities than in white varieties. This is confirmed by the data presented in Table 2. The white bean "Kontra" has been shown to be poor in phenolic compounds.

Both green and black teas are characterised by high phenolic compounds content, polymeric in particular. The polyphenols of tea have been shown to be strong trypsin inhibitors; the I_{50} values were 0.01 and 0.02 mg/mL for black and green tea, respectively.

The unexpected results were found in the case of the quince extracts which exhibited high levels of trypsin inhibition (I_{50}=0.03 mg/mL) despite sufficiently low proportion of tannins in the total polyphenols (39%). In addition, only in the case of quince the extract II, after salting out of high-molecular phenolics, had high inhibitory effect (I_{50}=0.06 mg/mL).

This is probably due to the presence of flavanol oligomers which occur in quince fruits in considerable amounts. It may suggest that not only high-molecular tannins but also oligomers have been shown to be trypsin inhibitors.

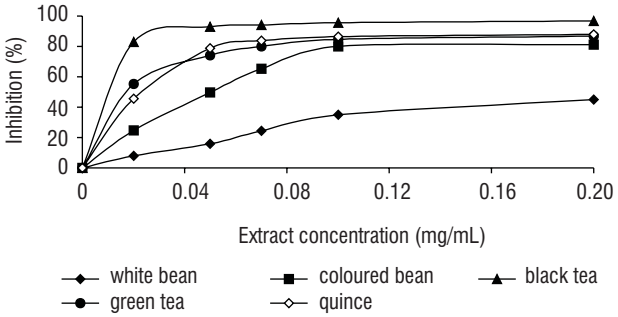

FIGURE 4. Trypsin inhibition curves for polyphenol extracts I.

Other extracts II after salting out (Figure 5) showed considerably lower inhibitory activity toward trypsin. The inhibitory potency I_{50} for these extracts was not possible to assign. The trypsin inhibition curve for white bean was not placed in Figure 5 as it is similar to that for black tea.

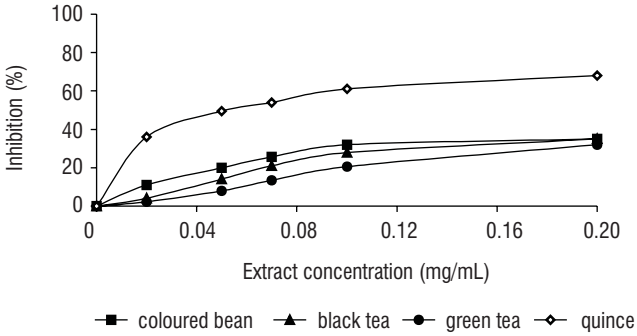

FIGURE 5. Trypsin inhibition curves for polyphenol extracts II.

The comparison of the percentage of trypsin inhibition of polyphenolic extracts I (before salting out) with that for extracts II (after salting out) at polyphenol concentration of 0.1 mg/mL is presented in Figure 6.

It can be observed the decrease in trypsin inhibiting effect of the polyphenol extracts after salting out by 14% (for white bean, which is poor in tannins) to 68% (for black tea being a rich source of high-molecular phenolic compounds). It confirms that low-molecular phenolics present in ingested food had no significant effect on trypsin activity.

The presented data confirm the results of studies by Griffiths [1979], Longstaff and McNab [1991], Quesada *et al.* [1996] and others who have reported that condensed tannins present in foodstuffs are first of all responsible for the inhibition of trypsin and other digestive enzymes.

TABLE 2. The characteristics of the polyphenol extracts tested.

Foodstuff	Total polyphenols (mg/mL)		Tannins (mg/mL)	Tannins in total polyphenols (%)	I_{50} (mg/mL) before salting out
	before salting out	after salting out			
White bean	0.20	0.15	0.05	25	>0.2
Coloured bean	0.55	0.25	0.30	55	0.05
Green tea	3.28	1.55	1.73	53	0.02
Black tea	2.17	0.50	1.67	77	0.01
Quince	2.37	1.45	0.92	39	0.03

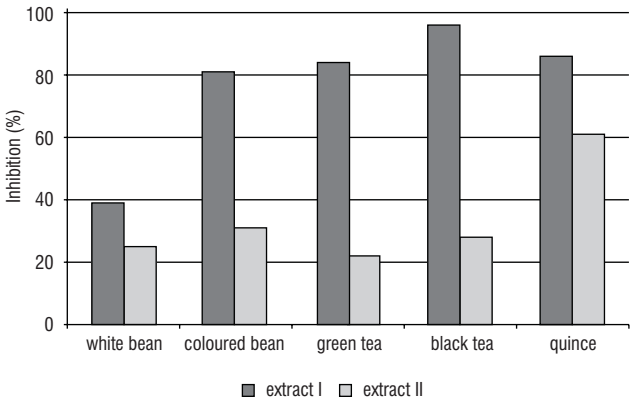

FIGURE 6. Comparison of the percentage of trypsin inhibition for extracts I and II (at polyphenol concentration of 0.1 mg/mL).

CONCLUSIONS

1. Monomers of tannins such as catechins and gallic acid have been shown to be 1000-times less active in the inhibition of trypsin (I_{50} about 10 mg/mL) compared to high-molecular weight tannic acid (I_{50}=0.1 mg/mL).

2. Of polyphenol extracts tested, only that from white been variety "Kontra" exhibited a low level of trypsin inhibition (I_{50}>0.2 mg/mL). It may result from low participation of high-polymerised tannins in the total phenols (25%).

3. The black tea extract showed the highest trypsin inhibitory effect comparable with that for tannic acid (I_{50}=0.01 mg/mL). In this extract, the proportion of tannins in the total phenols amounted to 77%.

4. The salting out of tannins reduced the trypsin inhibitory activity of the examined extracts by 14–68%. It confirmed that first of all the high-molecular weight phenolic compounds were responsible for the inhibition of trypsin.

5. The polyphenol extracts from the quince fruits rich in catechin oligomers had a considerable effect on the trypsin activity before as well as after salting out, indicating that not only high-molecular tannins but also oligomers have been involved in the trypsin inhibition.

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TANINY Z WYBRANYCH PRODUKTÓW JAKO INHIBITORY TRYPSYNY

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Badano wpływ ekstraktów polifenoli z wybranych surowców roślinnych (fasoli białej i kolorowej, herbaty czarnej i zielonej, pigwowca) oraz standardowych związków fenolowych (katechiny, epikatechiny, kwasu galusowego i taninowego) na aktywność trypsyny. Aktywność antytrypsynową wyrażano jako wskaźnik I_{50}, czyli stężenie inhibitora, które wywołuje inhibicję trypsyny w 50%. Badane monomeryczne związki fenolowe tylko w niewielkim stopniu hamowały aktywność trypsyny, natomiast wysokospolimeryzowany kwas taninowy odznaczał się wysoką efektywnością inhibicyjną; wskaźnik I_{50} (0,01 mg/mL) był około 1000-krotnie niższy w porównaniu z pozostałymi polifenolami (rys. 3, tab.1).

Użyte w doświadczeniach ekstrakty roślinne wykazywały wysoką zdolność inhibicji trypsyny (tab. 2, rys. 4); wskaźniki I_{50} wynosiły od 0,01 do 0,05 mg/mL. Wyjątek stanowił ekstrakt z białej fasoli z niskim wskaźnikiem I_{50} (>0,2 mg/mL).

Po wysoleniu tanin zanotowano zmniejszenie stopnia inhibicji trypsyny przez badane ekstrakty o 14–68% (rys. 5 i 6), co potwierdza fakt, że głównie wysokocząsteczkowe związki fenolowe, obecne w tych ekstraktach, są odpowiedzialne za efekt inhibicyjny w stosunku do trypsyny.

Ekstrakty z owoców pigwowca w dużym stopniu hamowały aktywność enzymatyczną, zarówno przed jak i po wysoleniu (odpowiednio 86 i 61%), co wskazuje na działanie inhibicyjne nie tylko tanin wysokocząsteczkowych, ale również oligomerów katechin, które występują w tych owocach w znacznych ilościach.