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ANTIOXIDANT ACTIVITY OF EXTRACT OF PEA AND ITS FRACTIONS OF LOW MOLECULAR PHENOLICS AND TANNINS

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Phenolic compounds were extracted from pea (*Pisum sativum*) seeds using 80% aqueous acetone. Crude extract was applied onto a Sephadex LH-20 column. Fraction I of low molecular phenolic compounds was eluted from the column by ethanol. Fraction II of tannins was obtained using water-acetone (1:1; v/v) as mobile phases. Phenolic compounds present in extract and its fractions showed antioxidant and antiradical properties investigated using a β-carotene-linoleate model system, Total Antioxidant Activity (TAA) method, DPPH radical scavenging activity and reducing power. The results of assays performed were the highest when tannins (fraction II) were tested. For example, TAA of tannin fraction was 2.48 μmol Trolox/mg, whereas the extract and fraction I showed only 0.30 and 0.22 μmol Trolox/mg, respectively. The content of total phenolics in fraction II was found the highest (113 mg/g). The content of tannins in this fraction determined using the vanillin method and expressed as absorbance units at 500 nm per 1 g was 368. The HPLC analysis of pea crude extract showed the presence of such phenolic compounds as vanillic, caffeic, *p*-coumaric, ferulic and sinapic acids (after basic hydrolysis), quercetin and kaempherol, procyanidin B_2 and B_3.

INTRODUCTION

Legumes have bean investigated as a source of phenolic compounds showing antioxidant activity. A strong activity was found in the hydrophilic phenolics. The antioxidant activity of hydrophilic phenolics present in the extract of pea was found by Tsuda *et al.* [1993]. Navy bean hull extracts investigated in a bulk oil system showed better antioxidant activity than that of a mixture of BHA and BHT when used at similar concentration [Onyeneho & Hettiarachchy, 1991]. Antioxidant properties of extracts of several legumes were investigated using a β-carotene-linoleate model system. The same method gave positive results for the phenolic fractions separated using Sephadex LH-20 column chromatography from extracts of everlasting pea, faba bean, broad bean [Amarowicz *et al.*, 1996b], pea [Amarowicz *et al.*, 2001], lentil [Amarowicz *et al.,* 2003], and beach pea [Chavan *et al.*, 1999].

Antioxidative activity of extract of phenolics from green bean was observed by Raab *et al.* [1996]. An EPR spin traping method was used for monitoring hydrophilic oxygen radical scavengers in leguminous seeds [Yoshiki *et al.*, 1996]. The antioxidative efficacy of leguminous seed extracts, evaluated with chemiluminescence methods, did not dependent upon their content of phenolic compounds [Amarowicz & Raab, 1997]. The fluorometric method to study the antioxidative properties of legume seeds was used by Drużyńska [2000].

The aim of the present study was to evaluate the antioxidant activity of an extract of pea and its fractions of low molecular phenolics and tannins using several assays.

MATERIAL AND METHODS

Material. Pea (*Pisum sativum*) seeds were obtained from the Plant Breading Station in Olsztyn, Poland.

Preparation of crude extract. Phenolic compounds were extracted from ground seeds according to Amarowicz *et al.* [1995] with 80% (v/v) aqueous acetone at 80°C for 15 min at a solid to solvent ratio of 1:10 (w/v). Extraction was carried out in dark-coloured flasks using a shaking water bath. The extraction was repeated twice more, supernatants combined and acetone evaporated under vacuum at 40°C in a rotary evaporator. The remaining water solution was lyophilized.

Column chromatography. Phenolic compounds present in crude extract were separated into fractions I (low molecular phenolics) and II (tannins) according to the method described by Strumeyer & Malin [1975]. A 2 g portion of the crude extract was suspended in 20 mL of 95% (v/v) ethanol and applied onto a chromatographic column (5 x 40 cm) packed with Sephadex LH-20 and equilibrated with 95% (v/v) ethanol. Low molecular phenolic compounds (fraction I) were eluted from the column using 1 L of 95% (v/v) ethanol. To obtain tannins (fraction II), the column was washed with 500 mL of 50% (v/v) acetone. Organic solvents were evaporated and water solution of tannin fraction was lyophilized.

Total phenolics. The content of total phenolic compounds in each fraction was estimated using the Folin & Ciocalteau's reagent [Naczk & Shahidi, 1989].

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(+)-Catechin was used as a standard in this work. Analysis were triplicated.

UV spectra. UV spectra of individual fractions were recorded using a Beckman DU 7500 diode array spectrophotometer.

Content of tannins. The content of tannins in the crude extract and its fractions was determined using the modified vanillin method assay [Price *et al.*, 1978] and expressed as absorbance units per 1 g of extract (A_{500}/g). Briefly, 1 mL of solution containing 1 mg of the extract of its fraction was mixed with 5 mL of vanillin/HCl regent. After 20 min, the absorbance was read at 500 nm.

Separation of phenolic acids from extract. Separation of phenolic acids was carried out according to Amarowicz and Weidner [2001]. An aqueous suspension of the extract (100 mg in 10 mL) was dissolved in 10 mL of 2 mol/L NaOH and hydrolysed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 using 6 mol/L HCl, free phenolic acids and constituents liberated from esters were extracted 5 times into 15 mL of diethyl ether using a separatory funnel. Then, ether was evaporated to dryness. The dry residues of free phenolic acids and phenolic acids liberated from esters were dissolved in 2 mL methanol and filtered trough a 0.45 μm filter. The samples obtained this way were injected onto an HPLC column.

HPLC analysis of phenolic acids. Phenolic acids were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a LC-10AD pump, SCTL 10A system controller and SPD-M 10A photo-diode array detector. Phenolic acids separation was performed by a prepacked LiChrospher 100 RP-18 column (4 x 250 mm, 5 m; Merck, Darmstad, Germany). The mobile phase water--acetonitrile-acetic acid (88:10:2; v/v/v) [Amarowicz & Weidner, 2001] was delivered at a rate of 1 mL/min. The detection was monitored at 320 nm.

Acidic hydrolysis of flavonoids from extract. Acidic hydrolysis of flavonoids from the crude extract was carried out according to Crozier *et al.* [1997]. Briefly, 50 mg of the extract was dissolved in 5 mL of a solution of 1.2 mol/L HCl in 50% (v/v) aqueous methanol containing 0.2% (m/v) of TBHQ. The solution was heated at 90°C for 2 h. After hydrolysis, the sample was adjusted to 25 mL with distilled water.

Analysis of flavonoids. The same Shimadzu HPLC system was used for the analysis of flavonoids liberated after acidic hydrolysis. A sample was filtered through a 0.45 μm filter and injected on the prepacked LiChrospher 100 RP-18 column (4 x 250 mm, 5 m). The mobile phase was composed of water-methanol-acetic acid (50:47:2.5; v/v/v) [McMurrough & Byrne, 1992]; a flow rate was 0.8 mL/min; and the detector was set at 280 nm.

HPLC analysis of procyanidins. The content of procyanidins in the crude extract was determined using the method described by Oszmiański & Bourzeix [1995]; C_{18} LUNA column (Phenomenex) was used for this perpouse.

Total Antioxidant Activity. The determination of the Total Antioxidant Activity (TAA) was carried out using the Randox kit (Randox Laboratories Ltd., Crumlin, UK) according to the procedure attached to it by the supplier; the concentration of 2 mg extracts/mL methanol was used in the assay.

Antioxidant activity of extract of pea and its fractions in β-carotene-linoleate model system. Antioxidant activities of extract and its fractions were evaluated using a β-carotene--linoleate model system [Miller, 1971]. Methanolic solutions (0.2 mL) containing 2 mg of the extract, fraction I and II or 0.3 mg of butylated hydroxyanisole (BHA) were added to a series of tubes with 5 mL of prepared emulsion of linoleic acid and β-carotene. Samples were incubated in a water bath at 50°C for 120 min measuring absorbance. The measurements were made at 470 nm every 15 min.

Reducing power. Reducing power of phenolic compounds was determined as described by Oyaizu [1986]. The suspension of the crude extract and fractions I and II (0.2 – 1.0 mg) in 1 mL of distilled water was mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Following this, 2.5 mL of trichloroacetic acid (TCA) was added and the mixture was then centrifuged at 1 750 g for 10 min. A 2.5 mL aliquot of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ and the absorbance of the mixture was read at 700 nm.

DPPH radical scavenging activity. The capacity of the prepared extract and its fractions to scavenge the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano *et al.* [1988]. A 0.1 mL methanolic solution containing 0.5–2.5 mg of extract and fraction I and 0.1–0.5 mg of fraction II was mixed with 2 mL of distilled water and then added to a methanolic solution of DPPH (1 mmol/L; 0.25 mL). The mixture was vortexed for 1 min, then left to stand at room temperature for 20 min and the absorbance of this solution was then read at 517 nm.

RESULTS AND DISCUSSION

The content of total phenolics in fraction I was 10 times lower than in fraction II (Table 1). This phenomenon is typical of Sephadex LH-20 column chromatography with the solvent systems used. Ethanol is able to elute from the column sugars which are dominating chemical compounds present in crude extract of legume seeds [Amarowicz *et al.*, 1995]. A high content of total phenolics in tannin fractions separated from crude extract using this kind of chromatography was previously described for several legumes [Amarowicz *et al.*, 2000]. The content of total phenolics in fraction I was similar to the results observed in the case of the pea phenolic fractions separated using a Sephadex LH-20 column chromatography with methanol as a mobile phase. In this material, total phenolics ranged from 2.6 mg/g to 9.2 mg/g [Amarowicz *et al.*, 2001]. Troszyńska *et al.* [1997] reported the content of total phenolics in Sephadex LH-20 fractions to range from 7.2 to 110 mg/g.

TABLE 1. Characteristics of pea crude extract and its fractions: content of total phenolics and tannins, Total Antioxidant Activity, and UV spectral data.

Analyzed material	Total phenolics [mg/g]	Tannins [A_{500}/g]	Total Antioxidant Activity [μmol Trolox/mg]	λmax [nm]
Extract	22.6	71	0.30	265
Fraction I	11.2	52	0.22	265
Fraction II	113	368	2.48	266, 272

A high content of tannins in fraction II (A_{500}/g = 368) expressed as the absorbance value at 500 nm per g confirmed a good separation of these phenolic compounds from the crude extract using a Sephadex LH-20 column chromatography with ethanol and 50% acetone as mobile phases. In the case of low molecular phenolic fraction, a vanillin positive reaction could be given by catechin and other flavan-3-ols wich are able to be eluted from a Sephadex LH-20 column with ethanol [Amarowicz & Shahidi, 1995].

The maximum of UV spectra of phenolic compounds from extract and fraction I (Table 1) were 265 nm. Two maxima, at 266 and 272 nm, were noted for fraction II. Similar spectra of pea extract and its fractions and fractions of pea coats were recorded by Amarowicz *et al.* [2001] and Troszyńska *et al.* [1997].

The highest value of the Total Antioxidant Activity was noted for tannin fraction: 2.48 μmol Trolox/mg (Table 1). Much less active were the extract (0.30 μmol Trolox/mg) and fraction I (0.20 μmol Trolox/mg). When assaying methanolic extracts of wheat, barley, rye and oat, Zieliński & Kozłowska [2000] reported lower values of TAA (0.054 – 0.222 μmol Trolox/mg) than those obtained in this study. The extract of seed coats from pea was characterised by TAA of 3.6 μmol Trolox/mg [Troszyńska & Kubicka, 2001]. Vetch extract and its low molecular and tannin fractions exhibited TAA values of 0.79, 0.40 and 6.40 μmol Trolox/mg, respectively [Amarowicz & Troszyńska, 2003]. The high value of TAA reported by the mentioned authors was caused probably by the tannins which were dominating phenolic compounds in the investigated material.

TABLE 2. The content of some phenolic compounds in pea crude extract.

Phenolic compounds	Content [mg/g]
Vanillic acid[1]	0.07
Caffeic acid[1]	0.02
p-Coumaric acid[1]	0.06
Ferulic acid[1]	0.32
Sinapic	0.07
Quercetin[2]	0.14
Kaempherol[2]	0.51
Procyanidin B_2	3.85
Procyanidin B_3	3.22

[1]After basic hydrolysis; [2]after acidic hydrolysis.

Using a HPLC method, such phenolic compounds as vanillic, caffeic, *p*-coumaric, ferulic and sinapic acids, quercetin and kaempherol, procyanidin B_2 and procyanidin B_3 were determined in pea extract (Table 2, Figures 1–4). Dominating compounds were procyanidins B_2 and B_3. The results of the chromatographic analysis are in accordance with UV spectra with bands typical of flavonoids and tannins and without bands at approximately 310–330 nm typical of phenolic acids [Amarowicz *et al.*, 2001; Naczk *et al.*, 1992]. Orsák *et al.* [2000] reported caffeic, sinapic and vanillic acids with the highest representation in pea seeds. Phenolic acids and procyanidins B_1, B_2 and B_3 were determined in lentil seeds [Bartolome *et al.*, 1994]. Raab *et al.* [1996] noted the presence of quercetin and kaempherol in green bean. White and Xing [1996] listed phenolic acids and flavonoids as the main natural antioxidants of legumes.

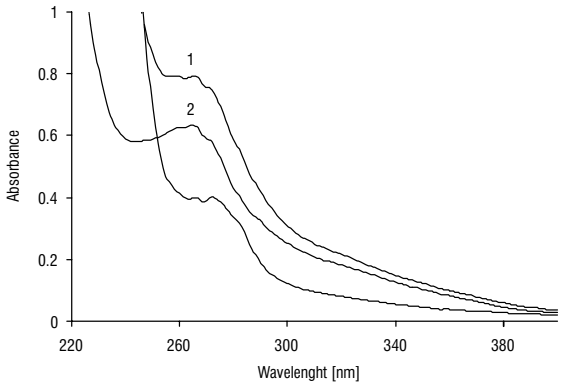


FIGURE 1. UV spectra of pea crude extracts and its fractions (1 - extract; 2 - fraction I; 3 - fraction II).

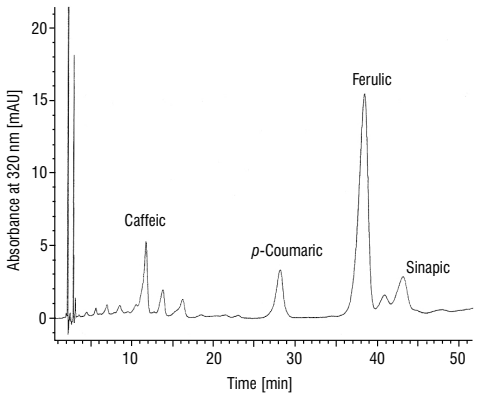


FIGURE 2. HPLC chromatogram of phenolic acids in pea crude extract after basic hydrolysis.

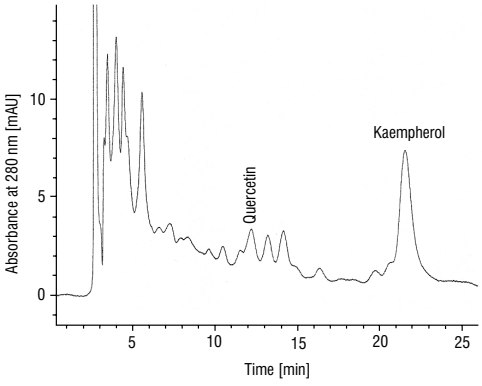


FIGURE 3. HPLC chromatogram of flavonoids in pea crude extract after acidic hydrolysis.

The crude extract of pea and fractions I and II exhibited antioxidant activity in a β-carotene-linoleate model system (Figure 5). The effect of the extract on the couple oxidation of linoleic acid and β-carotene was the highest (Figure 5). Using the same method, similar or lower antioxidant

activities were found for extracts of faba bean, broad bean, lentil, and everlasting bean [Amarowicz *et al.*, 1996a, 1996b, 2003; Chavan *et al.*, 1999]. Tannin fraction was probably too polar for the interaction with drops of linoleic acids present in the emulsion used. On the other hand, this fractions could be able to be rapidly oxidised and therefore to be not effective as an antioxidant in emulsion model.

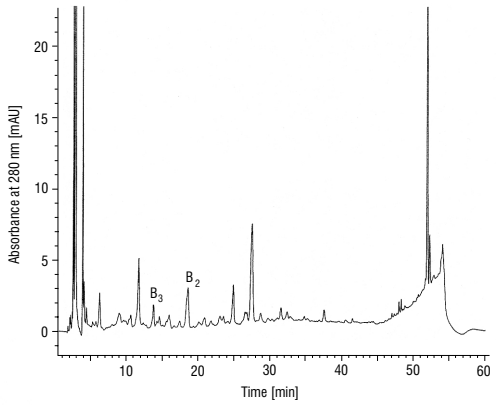


FIGURE 4. HPLC chromatogram of procyanidins in pea extract.

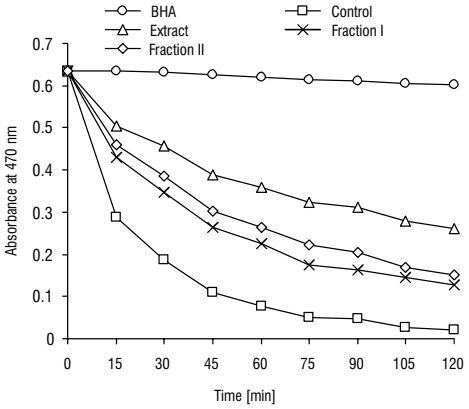
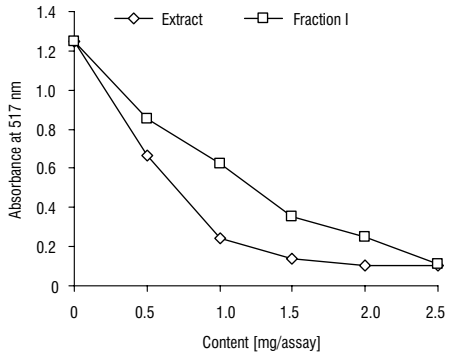


FIGURE 5. Antioxidant activity of pea crude extract and its fractions in β-carotene-linoleate model system.



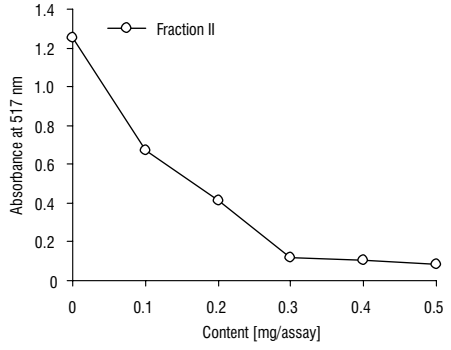


FIGURE 6. Scavenging effect of pea crude extract and its fractions on DPPH radical.

The strong scavenging effect of the pea crude extract and its fractions was confirmed in a DPPH assay (Figure 6). The fraction II exhibited several times higher antiradical activity than crude extract and its low molecular phenolics fraction I. Scavenging effect of beach pea, canola hulls, evening primrose and faba bean condensed tannins on DPPH radical was described by Amarowicz *et al.* [2000]. Radical scavenging activity of condensed tannin polymers as determined by the DPPH assay was reported by Muir [1996]. In the case of fraction I, probably phenolic acids were the active scavengers of DPPH radical. This suggestion is in accordance with the results of Brand-Williams *et al.* [1995] and Nenandis *et al.* [2003].

Figure7 displays the reduction power of the pea crude extracts and its fractions. The results indicate that fraction II exhibited a greater reduction power than the crude extract and fraction I. The reducing power of tannins (fraction II) was similar to that of canola reported by Amarowicz *et al.* [2000] for tannins of canola hulls. In the cited study, the reduction power of tannins from beach pea, evening primrose and faba bean was more than two times greater than of fraction II in the presented report. The same tendency between reducing power of extracts and its low molecular phenolics and tannin fractions was observed in the case of vetch [Amarowicz & Troszyńska, 2003].

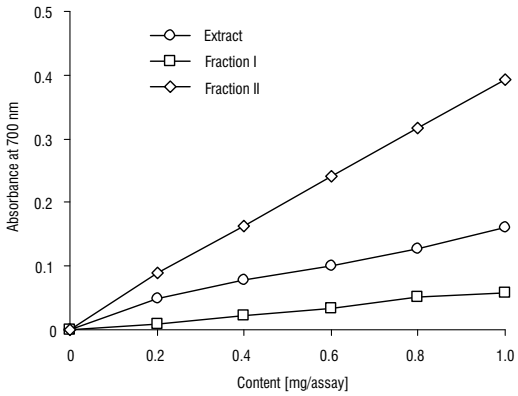


FIGURE 7. Reducing power of pea crude extract and its fractions.

CONCLUSIONS

The results presented confirmed the antioxidant and antiradical properties of phenolic compounds of pea seeds. The strong antiradical activity of tannins separated from the crude extract should be emphasised. Vanillic, caffeic, *p*-coumaric, ferulic and sinapic acids, quercetin and kaempherol, procyanidin B_2 and procyanidin B_3 were found as active phenolic compounds in the investigated material.

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