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Antioxidant properties of extracts from fermented and cooked seeds of Polish cultivars of *Lathyrus sativus*

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Abstract

Antiradical and total antioxidant activities of extracts from raw, prepared for inoculation, fermented (tempeh) and cooked seeds of grass pea (*Lathyrus sativus* Krab and Derek cultivars) were measured. Tempeh fermentation with *Rhizopus oligosporus* resulted in higher scavenging activity towards DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ radicals which correlated well with the content of total phenols. In Derek cultivar, fermentation caused a significant inhibition of linoleic acid oxidation by methanol extracts. In buffer extracts the highest TAA values were observed in raw seeds. Cooking of seeds lowered RSA values as compared to fermentation, especially for the DPPH$^{\cdot}$ assay. Methanol and buffer extracts from cooked seeds showed prooxidant activity towards linoleic acid.
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Keywords: Antiradical activity; *Lathyrus sativus*; *Rhizopus oligosporus*; Tempeh; Total antioxidant activity

1. Introduction

Grass pea is a legume plant widely consumed in developing countries of Asia and Africa. In Poland, its forms have only locally been grown in the Podlasie region. Great taste and nutritional value of the seeds together with little cultivation requirements have gained the interest of Polish scientists (Milczak & Masłowski, 1993) who in 1998 selected the first Polish grass pea cultivars – Derek and Krab with small and large seeds, respectively.

The factor that still reduces the interest of consumers is a toxic amino acid, β-Odap, present in grass pea seeds. Our preliminary research showed that the effective process of partial seed detoxification is tempeh fermentation with *Rhizopus* strains (data unpublished). Solid-state fermentation of legumes seeds is an ancient method that nowadays is still used for enhancing the nutritional and organoleptic qualities of legumes. Tempeh fermentation is a well known alternative to traditional methods of food processing like cooking, which cause partial loss of nutritionally valuable ingredients, e.g. vitamins.

An interesting application of solid-state fermentation is the production of foods enriched in non-enzymatic antioxidants. It has been proven that tempeh fermentation of legume seeds, especially soybeans, may increase the concentration of antioxidant phenols capable of scavenging free radicals and chelating metal ions (Sheih, Wu, Lai, & Lin, 2000). There are epidemiological studies showing a relationship between the consumption of products rich in phenols and a low incidence of diseases like certain forms of cancer, coronary heart disease or atherosclerosis (Randhir, Wattem, & Shetty, 2004). Apart from compounds with very strong antiradical properties, other ingredients of antioxidant activity for example aromatic amino acids and peptides (e.g. glutathione), are also present in fermented seeds. For consumers, only non-enzymatic antioxidants present in fermented foods are significant, as it has been shown that antioxidant enzymes do not add to the antioxidant value of tempeh (Fernandez-Orozco, Zieliński, & Piskuła, 2003). The most important is the overall antioxidant potential of the fermented product which includes combined and possible synergic activity of all compounds.

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The purpose of the study was to investigate if the fermentation with *Rhizopus oligosporus* DSM 1964 may enhance the antioxidant properties of Krab and Derek seeds. The objective of this research was also to compare the antioxidant value of tempeh with cooked seeds of grass pea.

To our knowledge, this study is the first that concerns tempeh made from Polish cultivars of *Lathyrus sativus* Derek and Krab and, thus, the antiradical and antioxidant activities of extracts obtained from this product are the main issue of investigations presented here.

2. Materials and methods

2.1. Materials

The seeds of *L. sativus* (Polish cultivars Derek and Krab) were obtained from the company 'Spójnia Hodowla i Nasiennictwo Ogrodnicze' in Nochowo, Poland. The starter for the tempeh fermentation (type B), containing *R. oligosporus* DSM 1964 culture, was purchased from 'Top Cultures', Zoersel, Belgium.

2.1.1. Tempeh production

L. sativus seeds (250 g) were cleaned with tap water, cooked for 30 min and then soaked for 18 h. Next, they were dehulled by hand, parted into halves and cooked for 30 min (with addition of 13 cm^3 of 6% vinegar). After drying, the seeds were cooled to 35 °C, supplied with 3.5 cm^3 of vinegar and mixed thoroughly with 0.975 g of *Rhizopus* starter culture. The inoculated material was put in perforated plastic bags, 3 cm in height, and fermented at 32 °C for 31 h (until a tight 'cake' was formed).

2.1.2. Cooking

Whole seeds were cleaned, soaked for 18 h and then cooked in tap water until soft (50 min for Krab and 40 min for Derek).

2.1.3. Sample preparation

The raw seeds (S) of both cultivars were ground in a seed mill (1 mm in mesh diameter). The cooked seeds (C), the seeds prepared for inoculation with *Rhizopus* starter (P) and the fermented product (tempeh, T) of both cultivars were dried at 60 °C for 24 h and ground in a seed mill. The flours from S, C, P and T were stored at 2–4 °C in closed vessels until analyzed.

2.2. Analytical methods

2.2.1. Reagents

1,1-Diphenyl-2-picryl-hydrazyl (DPPH); 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 97%; 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) diammonium salt, 98%; linoleic acid, 95%; polyoxyethylenesorbitan monolaurate (Tween 20) and Folin–Ciocalteu reagent were obtained from Sigma. Tannic acid and other reagents were of analytical grade from Chempur or Przedsiębiorstwo Odczynniki Chemiczne (POCh), Poland.

2.2.2. Radical scavenging activity (RSA)

The reduction of synthetic stable free radicals $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ by compounds present in flour extracts was monitored by a spectroscopic assay of discoloration of initial radicals' solutions. The radical scavenging activity of extracts was compared with the activity of Trolox solutions and expressed as μmol Trolox g^{-1} dm.

Scavenging of $DPPH^{\cdot}$ radical: 1.25 g of flour was shaken with 45 cm^3 of extraction mixture (96% ethanol, glycerin and distilled water 1:1:1 v/v) for 2 h at 60 °C. After centrifugation (15 min, 4000 rpm), the supernatant was made up to final volume of 50 cm^3 with distilled water. The $DPPH^{\cdot}$ radical scavenging activity was measured according to Pekkarinen, Stockmann, Schwarz, Heinonen, and Hopia (1999). A 0.05 cm^3 portion of the extract was mixed with 2.950 cm^3 of $DPPH^{\cdot}$ radical (0.1 mmol dm^{-3} in 80% methanol) and the absorption was measured at 516 nm after 5 min against an 80% methanol blank.

Scavenging of $ABTS^{\cdot+}$ radical: 0.125 g of flour was homogenized (supersonic homogenizer Labsonic P) with 5 cm^3 of phosphate buffer (0.1 mol dm^{-3}, pH 7.4) and centrifuged. The $ABTS^{\cdot+}$ radical scavenging ability was determined as described by Cano, Acosta, and Arnao (2003) with some modifications. To prepare the $ABTS^{\cdot+}$ solution, 10 mg of $ABTS^{\cdot+}$ was dissolved in 1.3 cm^3 of 0.0049 mol $K_2S_2O_8$ and 1.3 cm^3 of distilled water. After mixing, the tube was closed and left for 16 h at room temperature and then, stored at 2–4 °C. Just before analysis, $ABTS^{\cdot+}$ solution was diluted with phosphate buffer (0.1 mol dm^{-3}, pH 7.4) so that the absorbance of 0.7 ± 0.02 at 734 nm was achieved. The antiradical activity assay of extracts was performed in tubes tightly covered with aluminum foil. $ABTS^{\cdot+}$ (2 cm^3) solution and 200 μl of extract was mixed and incubated for 6 min at room temperature. The absorption of mixture was measured at 734 nm, with phosphate buffer as a reference.

2.2.3. Total antioxidant activity (TAA)

Total antioxidant activity was estimated according to Toivonen and Sweeney (1998). The method is based on the spectrophotometric assay of primary products of linoleic acid peroxidation (conjugated dienes) which may be inhibited or stimulated by compounds present in extracts.

The assay was conducted in extracts of two kinds. The methanol extracts were obtained by homogenizing of 1.25 g of flour with 7 cm^3 of 80% methanol for 5 min. After centrifugation the extracts were filled up to final volume of 4.5 cm^3 with 80% methanol. The buffer extracts were obtained in the same manner, except that 8 cm^3 of phosphate buffer pH 7.0 (0.02 mol dm^{-3}) was used for homogenization and the centrifuged extracts were made up to total volume of 5.5 cm^3 with buffer solution. The solution of linoleic acid was prepared daily by diluting 0.56 g of

linoleic acid and 1.5 g of Tween 20 in 8 cm^3 of 96% ethanol. Each extract of flour (100 μl) was mixed with 200 μl of linoleic acid solution and 3 cm^3 of 0.02 mol dm^{-3} phosphate buffer pH 5.6 for methanol extracts, and pH 7.0 for buffer ones. Controls contained 100 μl of methanol or phosphate buffer pH 7.0 instead of the flour extract, respectively. All samples were homogenized for 3 min in a supersonic homogenizer Labsonic P. Stable emulsions were placed in a water bath at 37 °C. Then, 1.5 cm^3 of 50 μmol dm^{-3} $FeCl_2$ solution (0.0994 g $FeCl_2$ and 0.168 g EDTA diluted to 1000 cm^3 with distilled water) was added to induce the oxidation of linoleic acid. After 1 h of incubation, 2 cm^3 of 0.1 mol dm^{-3} NaOH in 10% ethanol was added to 0.5 cm^3 of the mixture to stop the oxidation process. After mixing, 10 cm^3 of 10% ethanol was added and the absorbance measured at 232 nm against 10% ethanol blank.

The percent of antioxidant activity was calculated according to equation: $100 - (B \cdot 100 - A)$, where A is the difference between the absorbance of control sample after 24 h and 1 h of incubation, B is the difference between the absorbance of extract sample after 24 h and 1 h of incubation.

2.2.4. Total soluble phenolic content assay

The content of soluble phenols in the flour extracts was estimated according to the method given by Swain and Hillis (1959) based on the reduction of Folin–Ciocalteu reagent by compounds present in samples. As a standard, 0.05 g kg^{-1} tannic acid was used.

The flour extracts were prepared as described for DPPH$^{\cdot}$ and $ABTS^{\cdot+}$ assays, respectively. For soluble phenols assay, 5 cm^3 of properly diluted extracts was mixed with 0.25 cm^3 of Folin–Ciocalteu reagent and 0.5 cm^3 of saturated Na_2CO_3 solution. After 15 min of incubation, absorption of the samples was measured at 700 nm against the reagents blank. The result was expressed in g kg^{-1} dm.

2.3. Statistical analysis

For each determination, four replications were made. Data were analyzed using Statgraphics Plus for Windows. The results were statistically evaluated using Student's t-test. To determined significant differences, the LSD test was used at $p < 0.05$.

3. Results

3.1. Radical scavenging activity (DPPH$^{\cdot}$ and $ABTS^{\cdot+}$ assays) and phenols content

All samples (from the raw, pretreated for inoculation, fermented and cooked seeds) showed radical scavenging activity in investigated models (Tables 1 and 2). The approximate activity of extracts from both grass pea cultivars against $ABTS^{\cdot+}$ radical was about 4-fold higher than the activity measured in the DPPH$^{\cdot}$ assay (22 and 5 μM Trolox g^{-1} of dry matter, respectively).

Extracts from the raw seeds of Krab cultivar were more effective in scavenging the DPPH$^{\cdot}$ radical (Table 1) and less active against the $ABTS^{\cdot+}$ radical (Table 2), than extracts from the Derek cultivar. Treatments before inoculation with *R. oligosporus* starter culture resulted in a significant decrease of antiradical activities, as compared to raw seeds (Tables 1 and 2). The inoculation of seeds with *R. oligosporus* and subsequent fermentation caused a pronounced increase in the RSA values. Fermented product of both cultivars had significantly higher antiradical activities than raw grass pea seeds. The ability of tempeh extracts to scavenge the DPPH$^{\cdot}$ radical was over 2-fold higher in the Krab cultivar than in the Derek cultivar (Table 1). As for the $ABTS^{\cdot+}$ assay, extracts from fermented seeds of both cultivars had similar activities (Table 2).

Cooking reduced the ability of compounds present in extracts to scavenge both the DPPH$^{\cdot}$ radical and the $ABTS^{\cdot+}$ radical as compared to fermentation. The observed differences were more pronounced in case of the DPPH$^{\cdot}$ assay. The extracts from cooked seeds were about one third (the Derek cultivar) and one sixth (the Krab cultivar) of the activity of extracts from tempeh while in the

Table 1
RSA (DPPH$^{\cdot}$ assay) and total phenols in ethanol:glycerin:water extracts

Seeds		DPPH$^{\cdot}$ (μmol) Trolox (g^{-1} dm)		Phenols (g kg^{-1} dm)	
		–	SEM	–	SEM
Krab cultivar	Raw	2.87 c[A,B]	0.12	3.05 c[A,B]	0.06
	Pretreated	1.87 a	0.12	0.70 a	0.06
	Fermented	15.64 d	0.12	3.19 c	0.06
	Cooked	2.39 b	0.12	1.37 b	0.06
Derek cultivar	Raw	5.59 c	0.12	2.65 c	0.03
	Pretreated	1.36 a	0.12	1.04 a	0.03
	Fermented	8.87 d	0.12	2.65 c	0.03
	Cooked	2.45 b	0.12	1.50 b	0.03

[A] Statistical analysis within cultivars.
[B] Statistical analysis within columns, values with different letters differ significantly ($p < 0.05$).

Table 2
RSA ($ABTS^{\cdot+}$ assay) and total phenols in buffer extracts

Seeds		$ABTS^{\cdot+}$ (μmol) Trolox (g^{-1} dm)		Phenols (g kg^{-1} dm)	
		–	SEM	–	SEM
Krab cultivar	Raw	32.0 c[A,B]	0.47	5.10 c[A,B]	0.03
	Pretreated	5.90 a	0.47	1.18 a	0.03
	Fermented	34.94 d	0.47	4.65 c	0.03
	Cooked	15.54 b	0.47	1.89 b	0.03
Derek cultivar	Raw	26.46 c	0.94	5.13 d	0.03
	Pretreated	12.62 a	0.94	2.12 b	0.03
	Fermented	32.85 d	0.94	3.34 c	0.03
	Cooked	17.07 b	0.94	1.96 a	0.03

[A] Statistical analysis within cultivars.
[B] Statistical analysis within columns, values with different letters differ significantly ($p < 0.05$).

$ABTS^{\cdot+}$ assay – it was reduced by one half for both cultivars.

The extracts used for $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays were examined for their content of soluble phenols. The results are shown in Tables 1 and 2, respectively. Ethanol:glycerin:water extracts had lower levels of compounds reacting with the Folin–Ciocalteu reagent than buffer extracts. Processes prior to fermentation caused a loss of a major part of phenols in extracts of both kinds. Subsequent fermentation with *R. oligosporus* resulted in the rise of total phenols content to the levels similar (Table 1) or lower (Table 2) than those obtained in raw seeds. Cooking of grass pea seeds reduced the level of phenols in both kinds of extracts as compared to fermented seeds.

A positive nonlinear correlation was found between the activity against $DPPH^{\cdot}$ or $ABTS^{\cdot+}$ radicals and the level of phenols in corresponding extracts from raw, pretreated to inoculation, fermented and cooked seeds of both grass pea cultivars (Tables 1 and 2). For the $DPPH^{\cdot}$ assay, a model $y = \exp(a + bx)$ was used/fitted and the correlation coefficient was 0.81 (Fig. 1). For $ABTS^{\cdot+}$ assay, a model $y = 1/(a + b/x)$ was fitted and the correlation coefficient was 0.91 (Fig. 2).

3.2. Total antioxidant activity (TAA) assay

3.2.1. Methanol extracts

Extracts from the raw, pretreated to inoculation and fermented grass pea seeds inhibited linoleic acid oxidation in the investigated model system, with one exception (pretreated seeds of the Derek cultivar) (Fig. 3). Total antioxidant activity of methanol extracts from the fermented seeds of the Krab cultivar was over 2-fold higher than that of raw seeds, but the observed differences were not statistically significant. In the Derek cultivar, pretreating of seeds prior to inoculation resulted in reduction of the antioxidant activity of extracts. The subsequent fermentation resulted in significant increase of TAA.

In contrast to antioxidant properties of tempeh, the cooking resulted in prooxidant activity of methanol extracts from seeds of both grass pea cultivars.

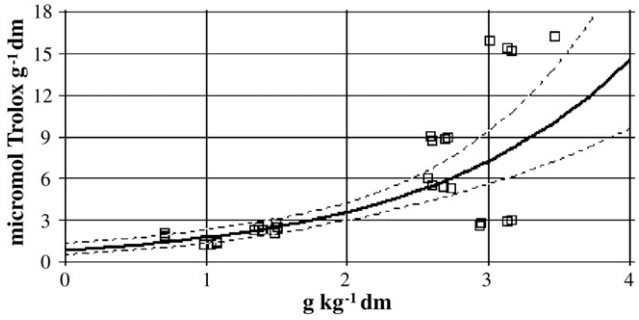

Fig. 1. Correlation between the activity against $DPPH^{\cdot}$ and the level of phenols. Model $y = \exp(a + bx)$, correlation coefficient = 0.81, standard error of prediction = 0.48.

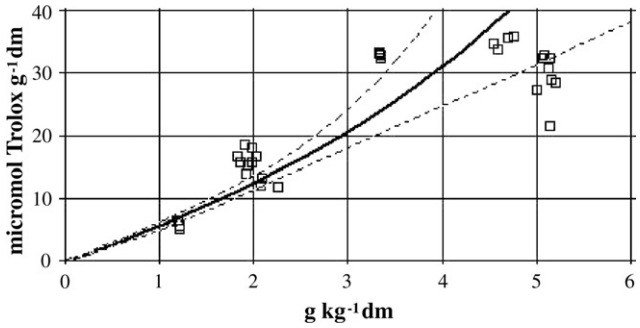

Fig. 2. Correlation between the activity against $ABTS^{\cdot+}$ and the level of phenols. Model $y = 1/(a + b/x)$, correlation coefficient = 0.91, standard error of prediction = 0.02.

3.2.2. Buffer extracts

Total antioxidant activity of buffer extracts from the raw seeds of both grass pea cultivars was about 10-fold higher than values obtained in methanol extracts (Figs. 4 and 3). Processes prior to inoculation with *R. oligosporus* resulted in significantly less effective inhibition of linoleic acid peroxidation (the Derek cultivar) or prooxidant activity (the Krab cultivar) of buffer extracts (Fig. 4). The subsequent fermentation did not significantly change the TAA values. The antioxidant activity of buffer extracts from tempeh samples were similar to data obtained in methanol ones. The product obtained from the Krab cultivar showed about 6% of linoleic acid inhibition and tempeh from the Derek cultivar–about 3% (methanol extracts) and about 4% (buffer extracts).

In the experiment, significant differences were found between buffer extracts from the fermented and cooked seeds of the Krab cultivar. Extracts from the cooked seeds of both grass pea cultivars showed prooxidant activity in the investigated model.

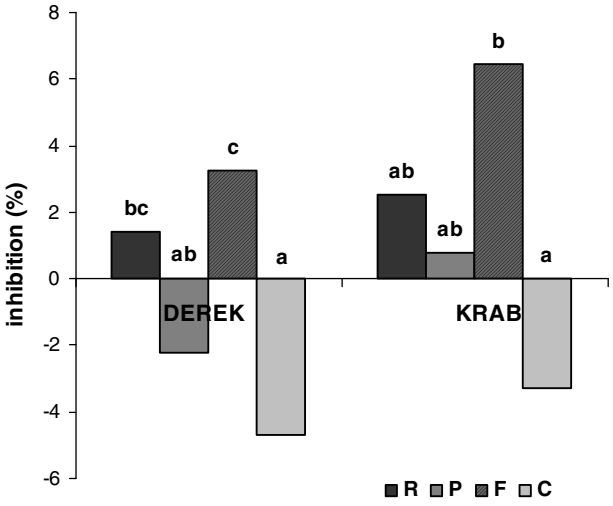

Fig. 3. TAA of methanol extracts. R–raw seeds, P–seeds prepared for inoculation, F–fermented seeds (tempeh), C–cooked seeds. Statistical analysis within varieties – values with different letters differ significantly ($p < 0.05$). SEM: Derek – R, P, F and C = 1.54; Krab – R and F = 2.09, P and C = 1.81.

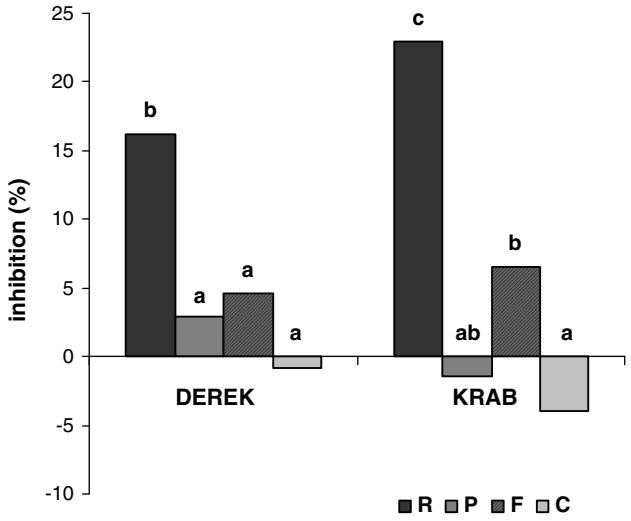

Fig. 4. TAA of buffer extracts. R–raw seeds, P–seeds prepared for inoculation, F–fermented seeds (tempeh), C-cooked seeds. Statistical analysis within varieties – values with different letters differ significantly ($p < 0.05$). SEM: Derek – R, P, C = 3.62 and F = 4.04; Krab – R and F = 2.86, P and C = 2.56.

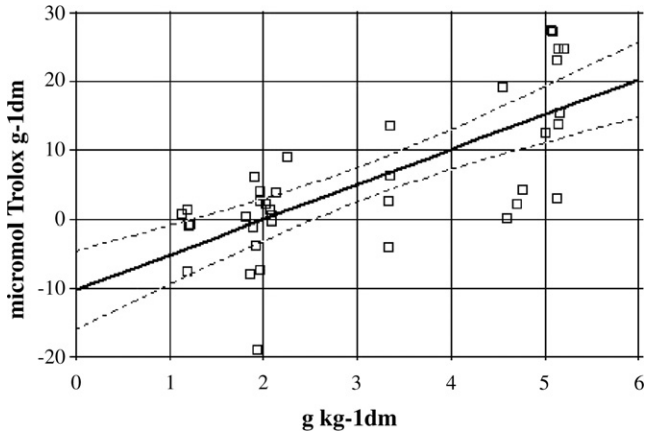

Fig. 5. Correlation between the TAA values and the level of phenols. Model $y = a + bx$, correlation coefficient = 0.72, standard error of prediction = 7.53.

The TAA values obtained in the experiment correlated linearly with content of phenols in corresponding extracts from raw, pretreated to inoculation, fermented and cooked seeds of both grass pea cultivars (correlation coefficient 0.72) (Fig. 5).

4. Discussion

4.1. The influence of treatments prior to inoculation with Rhizopus oligosporus and subsequent fermentation

4.1.1. Radical scavenging activity (DPPH· and ABTS·+ assays) and phenols content

Among the compounds with antiradical activity against DPPH· and ABTS·+ radicals measured in our experiment, the most important ones were soluble phenols, as their content strongly correlated with RSA activity (Figs. 1 and 2). It is believed that phenolic compounds are major components of antiradical activity measured in tested solutions (Moure et al., 2001). Our data are similar to observations by Zieliński et al. (1998), who showed that the scavenging activity against ABTS·+ radical measured in phosphate buffer extracts from cereal grains was strongly linked with the level of phenolic compounds. However, the Folin–Ciocalteu reagent is able to react not only with phenols but also with other reductive compounds having an aromatic ring within molecule e.g. aromatic amino acids, peptides and polypeptides. These compounds could be antioxidants reacting with the ABTS·+ radical but not capable of scavenging the DPPH· radical or were not present in ethanol:glycerin:water extracts. The last hypothesis may be confirmed by higher content of compounds reacting with the Folin–Ciocalteu reagent in buffer extracts than in ethanol:glycerin:water extracts (Tables 2 and 1).

As observed in our study, treatments prior to inoculation with *R. oligosporus* (soaking, dehulling, and subsequent cooking) resulted in a decrease of antiradical activity, accompanied by a loss of a major part of soluble phenols, as compared to raw seeds (Tables 1 and 2). It is known that phenols are present in seeds mainly within the hulls to protect the seeds from invasive diseases development and from consumption by insects (Liyana-Pathirana & Shahidi, 2006).

The fermentation of seeds with *R. oligosporus* caused a significant increase in the antiradical activity of extracts. The average activity against DPPH· radical obtained in our experiment in tempeh extracts (Table 1) was much lower than the activity measured by Sheih et al. (2000) in soybean fermented with different *Rhizopus* strains–12 μmol Trolox g^{-1} dm and 150–280 μmol Trolox g^{-1} dm, respectively. However, soybean is one of the richest natural sources of antiradical constituents mainly due to high concentration of specific isoflavones like genistein and daidzein (Sheih et al., 2000). Moreover, it has been confirmed that the DPPH· scavenging ability of extracts from legume seeds fermented with filamentous fungi may differ depending on species/strain of microorganism and incubation conditions, mainly time and temperature (Lin, Wei, Yu, & Chou, 2006). The results obtained in our study are comparable to the data determined by Stratil, Klejdus, and Kuban (2006). In that work, green and red paprika, salad and pumpkin had antioxidant activities similar to the Derek cultivar, whereas cauliflower and broccoli similar to values found in the Krab cultivar of grass pea.

The pronounced increase in the RSA values as a result of fermentation observed in the present experiment (Tables 1 and 2) is in agreement with the data given by Vattem, Lin, Labbe, and Shetty (2003) who found that fermentation of cranberry pomace by *R. oligosporus* resulted in an increase of its antiradical effect against DPPH·.

The rise of phenol contents corresponding with antiradical activity of extracts as a result of fungal fermentation of

pretreated seeds observed in our study is consistent with data presented by other authors (Lin et al., 2006; Randhir et al., 2004; Vattem et al., 2003). It has been suggested that during fermentation at least a partial cleavage or change in the phenol glycosides takes place as a result of enzymatic breakdown of plant cell walls by fungi and as a consequence the aglycones are liberated. Phenols aglycones exhibit stronger antiradical potential than corresponding glycosides (Finotti & Di Majo, 2003). The fermented grass pea seeds showed higher activity against DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ radicals than the raw ones, while the content of phenols did not change (ethanol:glycerin:water extracts) or was even lower (buffer extracts) in fermented seeds, as compared with raw ones (Tables 1 and 2). Thus, we assume that the qualitative composition of total soluble phenols, obtained in extracts from fermented and raw seeds, could differ substantially. During fermentation, the qualitative changes in the profile of phenolic compounds in seeds may occur as a result of fungi action. Horii, McCue, and Shetty (2003) found that there was positive correlation between activities of α-, β-glucosidase and laccase of *Lentinus edodes* and the increase in total soluble phenols in fermented soy powder.

Apart from phenols, compounds that could add to RSA values (DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ assays) in tempeh samples measured in our study might be products of seed proteins hydrolysis by *R. oligosporus*. It has been postulated that during tempeh fermentation the antiradical ability of the product may correlate with the activity of fungus protease (Sheih et al., 2000). Sheih et al. (2000) considered peptides, liberated by the activity of the enzyme, as important antiradical compounds of soy bean tempeh.

4.1.2. Total antioxidant activity (TAA)

4.1.2.1. Methanol extracts. Methanol extracts from the fermented grass pea seeds of the Krab and the Derek cultivars inhibited oxidation of linoleic acid (Fig. 3). It has been confirmed that fermented products can act as antioxidative agents slowing down lipid oxidation in model systems. During tempeh fermentation the compounds capable of inhibiting lipid peroxidation may be synthesized, e.g. due to transformation of substrate by the mold (Esaki, Onozaki, Kawakishi, & Osawa, 1996). However, in the experiment we did not observe significant differences between TAA values of methanol extracts from the raw and fermented grass pea seeds. It has been shown that fermentation of legume seeds does not necessarily enrich the product in compounds of antioxidant properties. According to Doblado et al. (2005), lactic acid fermentation of *Vigna sinensis* resulted in decreasing the pool of reduced glutathione, a compound capable of binding intermediate products of lipid oxidation such as peroxides (Bartosz, 2004).

4.1.2.2. Buffer extracts. Buffer extracts from the raw grass pea seeds of the Krab and the Derek cultivars showed much higher antioxidant activity than the methanol ones (Figs. 4 and 3). On the contrary, TAA values obtained in buffer and methanol extracts from the fermented seeds were similar and comparable to activity of pretreated seeds. Buffer extracts from the raw seeds contained higher level of phenols than the fermented ones (Table 2), which partially influenced the changes in antioxidant activity, as indicated by the correlation between TAA values and phenols content (Fig. 5). The antioxidant activity of extracts from grass pea seeds might result also from the presence of plant proteins with native conformation. Soluble proteins of legume seeds contain compounds of stronger antioxidant activity, e.g. isoflavones, which are effective peroxyl radical scavengers (Patel et al., 2001). Moreover, it has been shown that soluble proteins from plant seeds are capable of inhibiting lipid peroxidation in oil-in-water emulsions at pH 7.0.

4.2. The comparison of the fermented product and cooked grass pea seeds

4.2.1. Radical scavenging activity (DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ assays) and phenols content

Lower values of RSA (DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ assays) accompanied by the decrease in phenols content in the cooked grass pea seeds as compared to the fermented product (Tables 1 and 2) are consistent with data presented by Sheih et al. (2000) concerning the activity against the DPPH$^{\cdot}$ and the level of phenol aglycones of cooked soy seeds versus soy tempeh. In our experiment grass pea seeds were cooked without dehulling but nevertheless processing resulted mainly in about 2-fold lower antiradical activity than values obtained in the raw seeds (Tables 1 and 2). During cooking, approximately 50% of total seed phenols may bound to other compounds, resulting in formation of insoluble complexes, as shown by Fernandez-Orozco et al. (2003) in the case of lentil seeds. According to Siebert, Troukhanova, and Lynn (1996), such complexes might be formed between phenols and hydrophobic regions of proteins exposed as a result of denaturation.

Among other factors, the differences between RSA (DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ assay) of the cooked and fermented grass pea seeds obtained in our study could be connected with the content of peptides in ethanol:glycerin:water and buffer extracts. According to Sheih et al. (2000), soy tempeh contains many more peptides than cooked seeds. Data presented by Fernandez-Orozco et al. (2003) show that cooked lentil seeds had relatively low level of reduced glutathione.

4.2.2. TAA–methanol and buffer extracts

The noticeable differences between TAA values obtained in buffer extracts from the fermented and cooked grass pea seeds (Fig. 4) are in agreement with the previous findings. Tempeh, miso, natto and other fermented products are considered to be more effective in inhibition of lipid peroxidation than steamed unfermented soybeans, tempeh and miso being the most active (Esaki, Onozaki, & Osawa, 1994).

Interestingly, extracts from the cooked grass pea seeds stimulated the oxidation of linoleic acid, which most probably resulted mainly from the qualitative and/or quantitative changes in phenols profile caused by cooking. In our experiment, buffer extracts from the cooked seeds of the Krab and the Derek cultivars had significantly lower content of phenols than respective extracts from the fermented seeds (Table 2). It has been shown that the low level of phenols can be connected with the stimulation of the oxidation processes; a shift to antioxidant properties may occur along with the increase of the concentration of these compounds in tested solutions (Fukumoto & Mazza, 2000). Other factors that could add to the prooxidant activity of cooked seeds might be the qualitative changes in level of protein (buffer extracts) and peptides (buffer and methanol extracts), which possibly resulted in a different action of these compounds in the lipid peroxidation chain reactions. Moreover, buffer extracts from the cooked seeds should contain less soluble proteins than the raw or fermented ones due to denaturation processes.

5. Conclusions

Extracts from the raw, pretreated for inoculation and fermented seeds showed radical scavenging activity in investigated models, pronouncedly higher for the $ABTS^{\cdot+}$ assay. Fermentation with *R. oligosporus* caused significant increase in the antiradical properties of samples. Obtained RSA values ($DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assay) were strongly linked with the level of phenols. The correlation model observed for these parameters suggests that the qualitative composition of phenols could differ substantially in raw seeds and in fermented product.

Fermented grass pea seeds (methanol and buffer extracts) inhibited oxidation of linoleic acid. However, the highest TAA values were observed for the buffer extracts from the raw seeds of grass pea. Antioxidant activity of grass pea seeds could be correlated with presence of phenols (methanol and buffer extracts) and plant proteins (buffer extracts).

Extracts from cooked seeds of both grass pea cultivars showed significantly lower RSA values as compared to fermented ones. The observed differences were more pronounced for $DPPH^{\cdot}$ assay which may suggest that cooking strongly diminished the activity of constituents of high antiradical properties such as phenols.

Cooking of seeds resulted in prooxidant activity of methanol and buffer extracts, which we think may be connected with qualitative and quantitative changes in phenols and/or proteins profiles occurring during cooking.

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