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Effect of Inoculated Lactic Acid Fermentation on Antinutritional and Antiradical Properties of Grass Pea (*Lathyrus sativus* 'Krab') Flour

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Grass pea flour was subjected to fermentation induced with *Lactobacillus plantarum* (5% (v/v) and 10% (v/v) inoculum representing 10^9 cells/mL). Processing (24 h fermentation) resulted in reducing levels of β-*N*-oxalyl-L-α-β-diaminopropionic acid (β- ODAP) (by nearly 10%) and trypsin inhibitors (by about 30%) as compared to raw seeds. Effective elimination of inositol phosphates was observed, however, the products of phytate hydrolysis (myoinositol triphosphate – IP_3 and myoinositol tetraphosphate – IP_4) were not detected in fermented grass pea flour. On the contrary, the amount of total phenolics distinctly increased, by 100% (5% (v/v) inoculum) and 200% (10% (v/v) inoculum). Antiradical activity (DPPH$^\bullet$ assay) of flour was partially improved due to bacterial fermentation. The flour of better quality was obtained as a result of the fermentation with 5% (v/v) inoculum as compared to 10% (v/v) inoculum.

INTRODUCTION

Grass pea (*Lathyrus sativus)* is a legume plant widely consumed in Asia and Africa for its high nutritional value and little cultivation requirements. In Poland, its two cultivars, Krab and Derek, were selected in 1998 from the local forms grown in the Podlasie region [Milczak & Masłowski, 1993]. They are worth popularization since the participation of legume seeds in Eastern Europeans diet is still too low due to consumers' habits.

Among many processes applied to improve the nutritional value of legume seeds, fermentation is one of the most effective. The fermentation reduces antinutrients level, increases digestibility of the product and enriches it with valuable nutritional compounds *e.g.* vitamins. The seeds may be fermented as a whole during solid state fungal and bacterial/fungal mixed fermentations, *e.g.* tempeh type, or as a flour suspension in water during submerged bacterial processes. The most popular kind of submerged fermentation of legume seeds is lactic acid fermentation, spontaneous or induced with bacterial strains inoculum [Krishna, 2005; Frias *et al*., 1996].

The purpose of the experiment was to investigate if the induced lactic acid fermentation influences the levels of β-*N*-oxalyl-L-α-β-diaminopropionic acid (β- ODAP), trypsin inhibitors, inositol phosphates and phenols in the seeds of Krab cultivar. We also compared antiradical properties of processed and raw grass pea flours. Fermented grass pea flour of good antiradical properties and nutritional quality improved by reducing and/or eliminating antinutritional compounds level may be considered as a potential food additive.

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MATERIALS AND METHODS

Materials

The seeds of grass pea (*Lathyrus sativus* L.) Polish cultivar Krab were obtained from the company 'Spójnia Hodowla i Nasiennictwo Ogrodnicze' in Nochowo, Poland. Lyophilized preparation of *Lactobacillus plantarum* DSM 20174 culture was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ).

Raw seeds

Grass pea seeds were washed with tap water and rinsed three times with distilled water. After that, they were dried at 50°C for 6 h and ground in a seed mill (0.5 mm in mesh diameter).

Inoculum preparation

The starter culture was grown at 30°C for 24 h on autoclaved MRS broth and counted on MRS agar by a standard plate counting method.

Fermentation

Flour (32 g) was mixed with 80 mL of sterilized tap water with addition of 0.5 g/L potassium sorbate (to inhibit fungal growth) in a 250 mL glass container (7 cm in diameter). Then, it was aseptically inoculated with a 5% (v/v) and 10% (v/v) inoculum representing 10^9 cells/mL of *Lactobacillus plantarum*

(LP5% and LP10%). Every container was covered with perforated aluminum foil and incubated at 30°C. The fermentation was carried on until the pH of the suspensions decreased below 4.0 in order to preserve the product from the development of nondesirable microorganisms associated with spoilage and pathogenic ones [Frias *et al*., 1996]. The above was obtained after 6 (10% inoculum) and 7 (5% inoculum) hours of processing (sample II and I, respectively). The fermentation was also continued up to 24 h (sample III = 5% v/v inoculum, sample IV = 10% v/v inoculum). All processing treatments were completed in duplicates.

pH

Changes in the pH during the fermentation were monitored every hour with a Checker 1 pH meter.

Preparing material for analysis

The flour from raw seeds and the fermented suspensions were lyophilized as a whole and stored at 2°C in closed vessels until analyzed.

Reagents

The Folin-Ciocalteu reagent, *N*-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), dimethyl sulfoxide (DMSO), bovine trypsin (catalogue no. T-1426, 12,900 units/mg), diaminopropionic acid (DAP), o-phthalaldehyde (OPA), mercaptoethanol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), sodium phytate were obtained from Sigma. The tannic acid and other reagents were of analytical grade from Chempur or Przedsiębiorstwo Odczynniki Chemiczne (POCh), Poland.

N-oxalyl-L-α-β-diaminopropionic acid (ODAP)

The content of ODAP (α+β isomers) was estimated according to Briggs *et al*. [1983]. The method is based on the spectrophotometric assay of DAP, the product of ODAP hydrolysis. The results were expressed as mg ODAP/g DM.

Trypsin inhibitors

The method of Kakade *et al*. [1974] was used for determination of trypsin inhibitors activity. One trypsin inhibitors unit (TIU) was defined as a decrease in absorbance at 410 nm by 0.01, and data were expressed as TIU/g DM.

Inositol phosphates

The content of inositol phosphates (mg/g DM) was measured as described by Latta & Eskin [1980] in extracts prepared according to method given by Harland & Oberleas [1979].

The qualitative assay of inositol phosphates was obtained in raw grass pea flour and flour fermented with 5% v/v *Lactobacillus plantarum* inoculum by HPLC. Inositol phosphates were extracted with 0.5 mol/L HCl for 2 h at room temperature. Next, the extract was centrifuged (30 min at 1000 × *g*) and passed through a Bio-Rad AGI-X8 column. Inositol phosphates were washed out with 2 mol/L HCl, evaporated to the volume of 1.5 mL and filtered through a 0.45 μm nylon membrane. Inositol phosphates profile was obtained by HPLC (column: CarboPac PA-100 (250 x 4 mm)) with UV detection at 295 nm after derivatization with 1 g/L $Fe(NO_3)_3$ x 9 H_2O in 0.33 mol/L $HClO_4$. Inositol phosphates were eluted by gradient elution. The mobile phase consisted of 8% 0.5 mol/L HCl and 92% H_2O at the beginning and 100% 0.5 mol/L HCl at the end with a flow rate of 1 mL/min. A reference sample for the identification of peaks was prepared by thermal hydrolysis of phytic acid, dodecasodium salt hydrate (autoclaving 20 μmol/L solution, pH 4.0 for 40 min at 121°C).

Total phenolics

The extracts were prepared according to Tyczkowska [1977]. Lyophilized flour (0.31 g) was shaken with 11.25 mL of the extraction mixture (96% ethanol, glycerin and distilled water 1:1:1 v/v) for 2 h at 60°C. After centrifugation (15 min at 1000 × *g*) the supernatant was made up to the final volume of 50 mL with distilled water. The content of soluble phenols was measured by the method given by Swain & Hillis [1959], based on the reduction of Folin-Ciocalteu reagent by compounds present in samples. As a standard, 0.005% tannic acid was used. The results were expressed in mg tannic acid equivalent per gram of dry matter.

Antiradical activity (DPPH$^{\bullet}$ assay)

Extracts were prepared as described by Tyczkowska [1977]. Flour (3 g) was shaken with 45 mL of the extraction mixture (96% ethanol, glycerin and distilled water 1:1:1 v/v) for 2 h at 60°C. After centrifugation (15 min, 1000 × *g*), the supernatant was made up to the final volume of 50 mL with distilled water. The DPPH radical scavenging activity was measured according to Pekkarinen *et al*. [1999]. A 0.05 mL portion of the extract was mixed with 2.950 mL of DPPH$^{\bullet}$ radical (0.1 mmol/L in 80% methanol) and the absorbance was measured at 516 nm after 5 min against an 80% methanol blank. The results were calculated according to equation: 100 – [(absorbance of DPPH$^{\bullet}$ with extract – absorbance of DPPH$^{\bullet}$ blank)/100] and expressed as % of antiradical activity.

Statistic analysis

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

RESULTS AND DISCUSSION

ODAP

The concentration of ODAP obtained in raw seeds was about 2.4 mg/g DM (Table 1), which confirms that the Krab cultivar is low-toxic [Campbell *et al.,* 1994]. The obtained ODAP level is higher than values reported by Grela *et al*. [2001], by about 1 mg/g. However, it has been shown that the content of ODAP in seeds may differ according to environmental conditions occurring during plant growth [Campbell, 1997].

The induced fermentation of grass pea seeds resulted in a slight but significant decrease in ODAP content, by nearly 10%, as compared to raw seeds (Table 1). In case of 5% (v/v) inoculum, the reduction of ODAP concentration was obtained only after 24 h of incubation (sample III), whereas

TABLE 1. Antinutritional parameters and antiradical activity of raw and fermented seeds of *Lathyrus sativus* 'Krab'; sample I and II = flour fermented until the drop of pH < 4.0 with 5% and 10% inoculum of *L. plantarum*, respectively; sample III and IV = flour fermented for 24 hours with 5% and 10% inoculum of *L. plantarum*, respectively.

Parameters	Raw seeds	Sample I	Sample II	Sample III	Sample IV
ODAP (mg/g DM)	$2.38^{b\ 1,2}$	2.47^{b}	2.19^{a}	2.10^{a}	2.19^{a}
Trypsin inhibitors (TIU/g DM)	$21919^{e\ 1,2}$	16129^{c}	19786^{d}	13206^{a}	15828^{b}
Inositol phosphates[3] (mg/g DM)	$0.151^{b\ 1,2}$	0.060^{a}	0.073^{a}		
Phenols (mg/g DM)	$0.023^{a\ 1,2}$	0.028^{b}	0.043^{c}	0.047^{d}	0.073^{e}
Antiradical activity (%)	$7.21^{a\ 1,2}$	11.21^{c}	9.77^{b}	7.20^{a}	6.44^{a}

[1] statistical analysis within parameters; [2] statistical analysis within rows, values with different letters differ significantly ($p<0.05$);

[3] the level of inositol phosphates obtained only in raw seeds, sample I and II.

the 10% (v/v) inoculum was effective for both analytical points (sample II and IV). The fermented flour suspensions were lyophilised as a whole to avoid the effect of ODAP leaching from the plant tissue [Srivastava & Khokhar, 1996]. Thus, the observed changes could result directly from the activity of *Lactobacillus plantarum*. It has been shown that lactic acid bacteria, *e.g. L. plantarum* strains, are capable of lowering the level of some free protein amino acids in legumes seeds [Yigzaw *et al*., 2004]. Some species of bacteria may utilise ODAP as carbon and nitrogen source, as proved by Sachdev *et al*. [1995, in Akalu *et al*., 1998] with *Pseudomonas stutzeri* (soil bacteria). The results obtained in our experiment are different from data presented by Akalu *et al*. [1998], who did not find the decrease in the ODAP level after spontaneous lactic acid fermentation of grass pea seeds. However, it has been shown that tempeh fermentation with fungus *Rhizopus oligosporus* is effective in partial elimination of ODAP from grass pea seeds [Kuo *et al*., 1995].

Trypsin inhibitors

The activity of trypsin inhibitors obtained in raw grass pea seeds of the Krab cultivar was about 22,000 TIU/g DM (Table 1), which is slightly higher from previous findings [Grela *et al*., 2001]. Induced fermentation significantly decreased the activity of protein inhibitors. The 5% (v/v) inoculum was more effective than 10% (v/v) one and after 24 h resulted in decomposing 20% of the level obtained in raw seeds (sample III).

The trypsin inhibitors present in grass pea seeds belong mainly to the thermolabile Kunitz family [Studziński & Grela, 1997]. In our experiment, the observed changes could occur due to the activity of microorganisms introduced to flour suspensions since both raw and fermented materials were prepared from seeds previously subjected to drying at 50°C for 6 h.

The obtained results are in agreement with other findings concerning spontaneous [Granito *et al*., 2001] and induced with *L. plantarum* [Doblado *et al*., 2003; Ibrahim *et al*., 2002] fermentation of seeds of legume species. According to Ibrahim *et al*. [2002], the effectiveness of lactic acid bacteria towards trypsin inhibitors elimination may be comparable with the activity of fungus *Rhizopus oligosporus*.

Inositol phosphates

The level of inositol phosphates was obtained in the raw seeds and flours fermented with 5% (v/v) and 10% (v/v) *L. plantarum* inoculum until pH of the suspensions decreased below 4.0 (sample I and II).

Induced fermentation resulted in a significant decrease of inositol phosphates content, as compared to raw grass pea flour (Table 1). The most effective elimination was observed with 5% (v/v) inoculum, up to 50% of the value obtained in raw seeds. However, the products of phytate hydrolysis (IP_3 and IP_4) were not detected in raw nor in fermented flour (Figures 1 and 2). The decrease in inositol phosphates level due to lactic acid fermentation has been

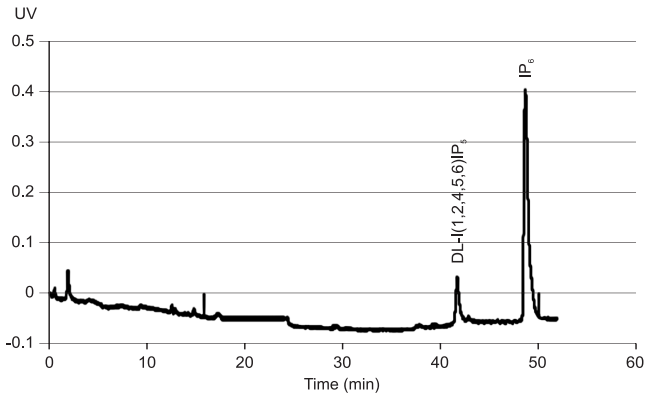

FIGURE 1. The profile of inositol phosphates in extracts of raw grass pea flour (IP_5 myoinositol pentaphosphate, IP_6 myoinositol hexaphosphate).

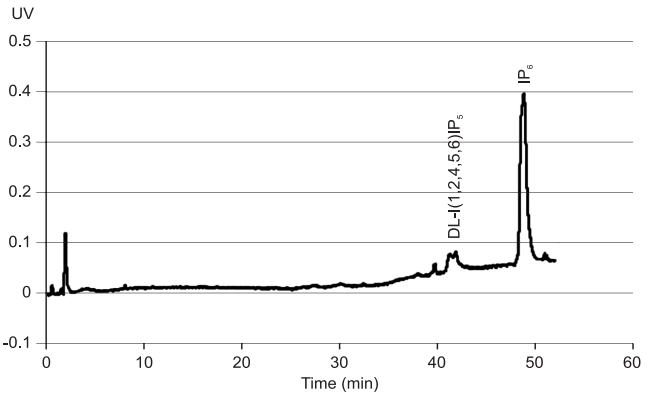

FIGURE 2. The profile of inositol phosphates in extracts of grass pea flour fermented with 5% v/v *Lactobacillus plantarum* inoculum (IP_5 myoinositol pentaphosphate, IP_6 myoinositol hexaphosphate).

previously observed for cowpea (*Vigna sinensis)* seeds processed with *L. plantarum* and *L. acidophilus* [Ibrahim *et al*., 2002]. It has also been shown that *Lactobacillus plantarum* strains may produce exogenous non-specific acid phosphatase [Zamudio *et al*., 2001]. Apart from the activity of bacteria, changes in inositol phosphates level observed in our experiment might partially result from endogenous plant phytase activity, as suggested by Doblado *et al*. [2003].

Total phenolics and antiradical activity

The level of total phenolics obtained in raw seeds was about 2 mg/g DM (Table 1), which is similar to data presented by Troszyńska *et al*. [1993] concerning Polish cultivars of grass pea.

In our experiment, the significant rise in total phenolics concentration was observed as a result of fermentation, higher in the case of 24 h incubation (sample III and IV). The more rapid increase was found when 10% (v/v) inoculum was used, as compared to 5% (v/v) inoculum.

The increase in the level of phenolic compounds during lactic acid fermentation might be connected with the activity of seed as well as microbial enzymes. According to McCue & Shetty [2005], during fermentation of soymilk with active yogurt cultures (*e.g. Lactobacillus plantarum*) the activity of laccase and peroxidase increased. Reactive oxygen species, generated due to laccase action might be the substrates for peroxidase that catalysed degradation of phenolic polymers. As a consequence, the liberation of soluble phenols took place [McCue & Shetty, 2005]. Moreover, the drop of pH occurring as a result of lactic acid bacteria fermentation could be the factor activating some enzymes participating in the hydrolysis of phenol glycosides, yielding soluble aglycones, as suggested by Dueñas *et al*. [2005]. Data obtained by other authors show both the increase [Ibrahim *et al*., 2002] and the drop of the total phenolics content in legumes seeds due to lactic acid fermentation, the latter explained by the rise of polyphenol oxydase activity in fermented tissues [Khetarpaul & Chauchan, 1991].

It should be noted that the rise in total phenolics level obtained in our experiment might be accompanied by qualitative changes in the profile of these compounds, as observed by Dueñas *et al*. [2005] for cowpea fermented with *Lactobacillus plantarum*. The quantitative and qualitative changes in phenolic compounds may be connected with a higher antioxidant activity of the product, in comparison with raw flour [Dueñas *et al*., 2005; Dolbado *et al*., 2003]. In our study, the antiradical activity against DPPH$^{\bullet}$ obtained in extracts used for the assay of phenols increased as a result of fermentation (sample I and II), as compared to raw seeds (Table 1). However, when the processing was prolonged up to 24 h (sample III and IV), the drop of radical scavenging activity occurred, to the level obtained in the control. Thus, the changes in the antiradical activity did not correlate with the quantitative changes in phenols content. Among all processed grass pea flours, the highest activity against DPPH$^{\bullet}$ and the lowest content of phenols were measured in sample I fermented with 5% v/v *Lactobacillus plantarum* until pH of the suspensions decreased below 4.0.

CONCLUSIONS

Fermentation of grass pea flour induced by *Lactobacillus plantarum* resulted in the significant lowering of ODAP, trypsin inhibitors and inositol phosphates contents, as compared to raw material. The effect was more pronounced when the fermentation was prolonged up to 24 h, with the exception of inositol phosphates.

As a result of bacterial fermentation of grass pea, the rapid accumulation of phenols occurred. The lowest level of phenols was however accompanied by the highest antiradical activity of the fermented flour.

The 5% v/v inoculum of *Lactobacillus plantarum* enabled better quality of fermented flours, as compared to 10% v/v inoculum. The exception was ODAP content.

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