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ANTIOXIDANT ACTIVITY OF PEA PROTEIN HYDROLYSATES PRODUCED BY BATCH FERMENTATION WITH LACTIC ACID BACTERIA

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Abstract: Nine *Lactobacillus* strains known for surface proteinase activity were chosen from our collection and tested for their ability to grow in pea seed protein-based medium, and to hydrolyze purified pea proteins in order to produce peptides with antioxidant (AO) activity. Two strains, *Lactobacillus rhamnosus* BGT10 and *Lactobacillus zeae* LMG17315, exhibited strong proteolytic activity against pea proteins. The AO activity of the pea hydrolysate fraction, MW <10 kDa, obtained by the fermentation of purified pea proteins with *Lactobacillus rhamnosus* BGT10, was tested by standard spectrophotometric assays (DPPH, ABTS, Fe^{3+}-reducing capacity) and the recently developed direct current (DC) polarographic assay. The low molecular weight fraction of the obtained hydrolysate was separated using ion exchange chromatography, while the AO activity of eluted fractions was determined by means of a sensitive DC polarographic assay without previous concentration of samples. Results revealed that the fraction present in low abundance that contained basic peptides possessed the highest antioxidant activity. Based on the obtained results, it can be concluded that *Lactobacillus rhamnosus* BGT10 should be further investigated as a candidate strain for large-scale production of bioactive peptides from legume proteins.

Key words: *Pisum sativum* L.; pea protein hydrolysate; *Lactobacillus*; antioxidant peptides; batch fermentation.

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INTRODUCTION

Pea seeds are a rich source of highly digestible proteins comparable to other commonly used legumes (Gausseres et al., 1997; Barac et al., 2010; Kotlartz et al., 2011). Besides nutritional value, pea seed-derived proteins can also serve as source of peptides with a wide spectrum of bioactive properties. Small peptides, containing 3-20 amino acids are in an inactive form, encrypted in the primary structure of the protein, prior to their release by enzymatic hydrolysis. The released peptides can exhibit local and systemic effects, as they have been shown to possess antimicrobial, antioxidant (AO), antihypertensive, immunomodulatory and anticancerogenic activities (Shahidi and Zhong, 2008; Kim and Wijesekara, 2010). The antioxidant and antihypertensive properties of enzymatic pea protein hydrolysates have been established in several studies (Li and Aluko, 2010; Pownall et al., 2010). According to the literature (Schaffner and Beuchat, 1986; Oboh et al., 2008; Oyarekua et al., 2011), fermentation of legume seeds in the presence of lactic acid bacteria can lead to an improvement in texture, organoleptic properties, flavor and AO activity of the final product. In recent years, interest in the utilization of different *Lactobacillus* strains in the production of bioactive peptides from soy whey has been steadily growing (Fung and Liong, 2010). Virtanen et al. (2007) reported the application of lactic acid bacteria in improving antioxidant activity in milk whey. Since the significance of using *Lactobacillus*

strains in the production of antioxidant peptides has been determined in the aforementioned studies, the screening of numerous strains in search for candidates that can be used for effective large-scale production of AO peptides is an important scientific challenge. Hydrolysis of plant proteins using lactic acid bacteria yields complex mixtures of peptides that must be further analyzed in order to detect fractions with the strongest AO activities. Common techniques for AO activity analysis of small peptide fractions produced by protein hydrolysis usually include gel filtration, solid phase extraction, ion exchange chromatography, chromatography of hydrophobic interactions (HIC) or a reverse-phase high-performance liquid chromatography (RP-HPLC) for resolving peptide fractions. These separation techniques are usually followed by lyophilization of the obtained fractions prior to classic AO assays, which are both material and time consuming (Pownall et al., 2010; Tang et al., 2010). On the other hand, the recently developed highly sensitive direct current (DC) polarographic assay (Sužnjević et al., 2011), which has been validated using complex mixtures containing peptides such as beer, honey and herbal infusions, can be effectively applied for direct estimation of AO activity measurement in highly diluted peptide samples without previous concentration.

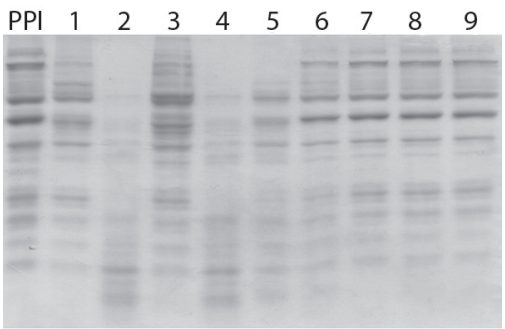

Fig. 1. Hydrolysis of pea proteins by whole cells of selected lactobacilli after 24 h of incubation. PPI – pea protein isolate, 1 *Lb. casei* ATCC393, 2 – *Lb. rhamnosus* BGT10, 3 – *Lb. paracasei* BGHN14, 4 – *Lb. zeae* LMG17315, 5 – *Lb. plantarum* LMG9208, 6 – *Lb. plantarum* BGBUK2-5, 7 – *Lb. plantarum* PV2-45a, 8 – *Lb. plantarum* BGGA8, 9 – *Lb. plantarum* BGHO10

The production of antioxidant peptides from pea proteins using batch fermentation with lactic acid bacteria has not been reported to date, to the best of our knowledge. The aim of our study was to screen the collection of lactic acid bacteria for their ability to grow efficiently in pea protein media and produce peptides with antioxidant activity. For this reason, the low molecular weight fraction, MW <10 kDa, obtained by the fermentation of pea proteins with selected strains, was tested by standard common spectrophotometric AO assays. Additionally, the DC polarographic assay was used for determination of AO activity in the highly diluted fraction of peptides obtained by preparative ion exchange chromatography.

MATERIALS AND METHODS

Chemicals and materials

All chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA). Agar was purchased from Torlak (Belgrade, Serbia). Dried seeds of green pea (*Pisum sativum*) cultivar "Mraz" were obtained from the collection of the Department of Forage Crops – Institute of Field and Vegetable Crops, Novi Sad, Serbia.

Bacterial strains

Bacterial strains used in the study were: *Lactobacillus casei* ATCC393 – dairy isolate, ATCC collection; *Lb. zeae* LMG17315 – isolate from corn steep liquor, BCCM/LMG collection; *Lb. paracasei* BGHN14 – isolate from homemade cheese, Prt^+ (Kojic et al., 1991); *Lb. rhamnosus* BGT10 – human vaginal isolate, Prt^+ (Pastar et al., 2003); *Lb. plantarum* LMG9208 – sauerkraut, Prt^+, BCCM/LMG collection; *Lb. plantarum* BGBUK2-5 – isolate from homemade cheese, Prt^+ (Strahinic et al., 2010); *Lb. plantarum* PV2-45a – natural isolate from homemade cheese, Prt^+ (Strahinic et al., 2010); *Lb. plantarum* BGGA8 – natural isolate from homemade cheese, Prt^+ (Strahinic et al., 2010); *Lb. plantarum*

BGHO10 – human oral isolate, Prt$^+$(Strahinic et al., 2010). Stocks of cultures were kept at -80°C in MRS broth containing 15% (v/v) glycerol.

Preparation of pea protein isolate (PPI)

Dry pea seeds were dehulled manually to minimize the presence of phenolic compounds, which are mainly located in seed coat (Troszynska and Ciska, 2002). Cotyledons were ground in liquid nitrogen using a mortar and pestle to obtain seed flour. Afterwards the seed flour was defatted according to the previously described method (Du et al., 2012) and extracted three times with a tenfold volume of acetone/water/acetic acid mixture (70:29:1) in order to eliminate residual phenolic compounds that could interfere with the AO assays (Xu and Chang, 2007). To extract proteins, flour was air dried for 24 h at room temperature, mixed with deionized water (1:10, w/v) and 2% of insoluble polyvinylpolypyrrolidone (PVPP) to eliminate remaining water-soluble phenolic compounds (Xu and Diosady, 2000). The pH of the dispersion was adjusted to 10.0 with 1 M NaOH. The mixture was gently stirred for 2 h at room temperature, and centrifuged at 5,000 g for 15 min. The extracting procedure was repeated two more times to extract additional proteins from the residue. The proteins from combined supernatants were isoelectrically precipitated at pH 4.5 with 1 M HCl and kept at 4°C for 1 h. The precipitate was recovered by centrifugation at 5,000 g for 15 min, washed with deionized water and the pH value was adjusted to 7.0, freeze-dried to obtain PPI, and stored at 4°C.

Proteinase activity assay

In order to select the *Lactobacillus* strain that could produce AO peptides, the nine strains from our collection were first tested for their ability to hydrolyze pea proteins. The proteolytic activity of the different *Lactobacillus* strains was assayed according to the previously described method (Kojic et al., 1991). After the strains were grown on MCA plates for two days at 30°C, cells were collected and resuspended in sodium phosphate buffer (100 mM, pH 6.8). For analysis of the whole cells' activity, a cell suspension containing ~ 10^9 cells/mL was mixed with pea protein substrate (PPI 5 mg/mL^{-1} in the same buffer) in a 1:1 volume ratio and incubated for 24 h at 30°C. Subsequently, the reaction mixtures were centrifuged (13,000 g, 10 min), and aliquots of supernatants prepared for SDS-PAGE.

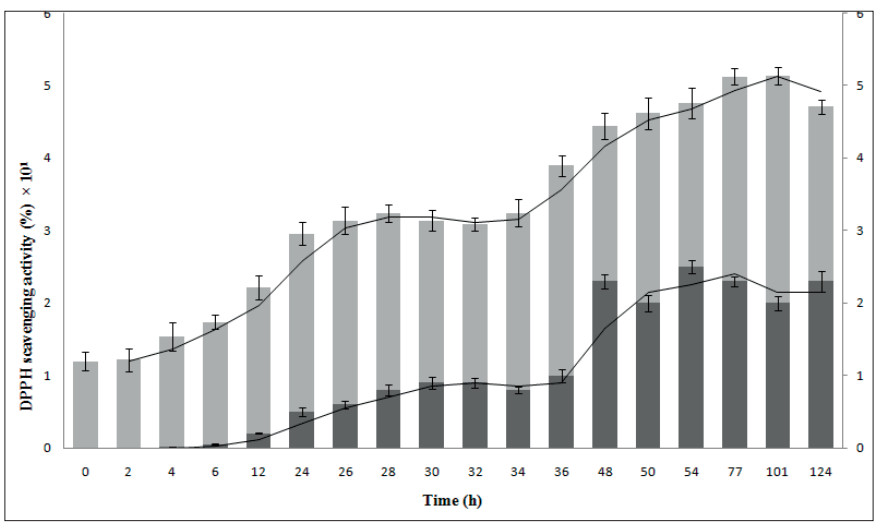

Fig. 2. Growth curve of *Lb. rhamnosus* BGT10 in pea protein based (NIG) liquid medium and changes of DPPH scavenging activity in filtered medium during growth. Black bars represent changes in cell number, and gray bars represent DPPH radical scavenging activity of filtrated medium (10 kDa cut-off membrane) during growth. Values represent mean ± standard error, n=3.

Preparation of pea protein-based cultivation medium (NIG)

The strain found to be the most potent in the hydrolysis of purified pea proteins was tested for its ability to grow in a specially designed medium containing pea-seed flour as the only source of protein. The purpose of this test was to establish whether the strain exhibiting the highest proteolytic activity could be grown on a large scale in pea protein-based medium with a simultaneous production of peptides with AO activity. The broth (NIG) was prepared according to the method of Pathak and Martirosyan (2012) with modifications. Ten g seed powder, 20 g glucose, 1 g Tween-80, 2 g K_2HPO_4, 5 g Na-acetate, 2 g ammonium citrate, 0.2 g $MgSO_4 \times 7H_2O$ and 0.05 g $MnSO_4 \times H_2O$, were resuspended in 800 mL of distilled water without pH adjustment and sterilized at 117°C for 15 min. The obtained NIG broth was further used for assessment of bacterial growth and the monitoring of changes in AO capacity during bacterial growth, using the classic spectrophotometric DPPH radical scavenging assay.

Determination of bacterial growth in NIG medium

The selected strain (*Lb. rhamnosus* BGT10) was grown in NIG broth at 30°C for 124 h. Bacterial inoculum (1%) was made from pelleted overnight culture. Samples (1 mL) were taken from the culture during growth at different time points, 100 µL was diluted for colony forming unit (CFU) determination, the rest was filtrated through an ultrafiltration unit with 10 kDa cut-off membrane and the filtrate was used for DPPH assay.

Production of pea protein hydrolysate (PH)

Pea protein hydrolysates (PH) were obtained by fermentation with whole cells of *Lactobacillus rhamnosus* BGT10. For preparation of PH, a cell suspension containing ~10^9 cells/mL in sodium phosphate buffer (100 mM, pH 6.8) was mixed with pea protein substrate (PPI 5 mg/mL^{-1} in the same buffer) at a 1:1 volume ratio, and incubated for 16 h at 30°C. Subsequently the mixture was filtered through a 10-kDa ultra filtration unit to produce the PH fraction MW <10 kDa; this was used for ion exchange chromatography or freeze-dried and kept at 4°C for AO assays.

Ion exchange chromatography

Filtrate of the PH fraction MW <10 kDa was separated by ion exchange chromatography on a DEAE Sepharose fast flow column (linear gradient of NaCl from 0 to 0.5 M, flow rate 1 ml/min). Elutes were collected in 5 mL fractions, and absorbance was measured at 214 nm to determine the peptide separation profile. Elutes were combined into eleven fractions (F1-F11).

Antioxidant activity assays

A 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay was carried out according to the previously described method (Pownall et al., 2010). Briefly, 500 µL of 100 µM DPPH solution in methanol was mixed with 100 µL of the sample (filtrate of cultured NIG broth MW <10 kDa, PH filtrate MW <10 kDa 0.1 mg/mL or glutathione 0.1 mg/mL) and 400 µL of 50 mM phosphate buffer, pH 7.0. The control consisted of 500 µL of phosphate buffer and 500 µL of DPPH solution. The mixture was incubated at room temperature in the dark for 30 min, and absorbance was measured at 517 nm. DPPH radical scavenging activity (%) was calculated as $\{(A_{517c}-A_{517s})/A_{517c}\} \times 100$, where c and s represent control DPPH and sample, respectively.

The reducing power of filtrates was measured according to the method of Pownall et al. (2010), with slight modifications. An aliquot of the sample 250 µL was added to 250 µL of 100 mM phosphate buffer, pH 6.6, and mixed with 250 µL of 1% potassium ferricyanide solution. The reaction mixture was incubated at 50°C for 20 min. After incubation, 250 µL of 10% trichloroacetic acid was mixed with 250 µL of the incubated mixture, 50 µL of 0.1%

ferric chloride and 250 µL of distilled water. Prior to absorbance measurement at 700 nm, the sample was additionally incubated for 10 min at room temperature. Absorbance at 700 nm was a measure of reducing capacity.

The capacity of pea protein hydrolysates to chelate Fe^{2+} ions was determined according to the method of El and Karakaya (2004), with some modifications. Sample aliquots (200 µL) were mixed with 740 µL of deionized water and 20 µL of 2 mM $FeCl_2$ solution, and incubated for 30 min at room temperature. After incubation, 200 µL of 5 mM ferrozine was added and the mixture was incubated for an additional 10 min in the same conditions. The absorbance was determined at 562 nm. Distilled water was used as control instead of the sample. Metal-chelating capacity was calculated as Fe^{2+} chelating capacity (%) = $\{(A_{562c}-A_{562s})/A_{562c}\} \times 100$, where c and s represent control and sample, respectively.

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid $(ABTS)^{\bullet+}$ scavenging activity was determined according to the previously described method (Re et al., 1999), with slight modifications. $ABTS^{\bullet+}$ solution, prepared 12 h before use, contained 7 mM $ABTS^{\bullet+}$ and 2.5 mM potassium persulfate. The obtained solution was then diluted with 200 mM phosphate buffer pH 7.4 to an absorbance of 0.7±0.02 at 734 nm, and 4 mL of diluted solution was mixed with 40 mL of peptide solution 0.1 mg/mL. Absorbance at 734 nm was read after 10 min using water instead of sample as a control. $ABTS^{\bullet+}$ scavenging activity was calculated according to formula: $ABTS^{\bullet+}$ scavenging (%) = $\{(A_{734c}-A_{734s})/A_{734c}\} \times 100$, where c and s represent control and sample, respectively.

For determination of superoxide scavenging activity, a pyrogallol autooxidation method was used (Udenigwe et al., 2009). An aliquot of peptide sample (800 µL) PH fraction MW <10 kDa 0.1 mg/mL was dissolved in 50 mM Tris-HCl buffer pH 8.2 with 1 mM EDTA and mixed with the same volume of buffer in a quartz cuvette and 400 µL of 1.5 mM pyrogallol in 10 mM HCl. The mixture was shaken vigorously. Measurement of absorbance at 420 nm was started

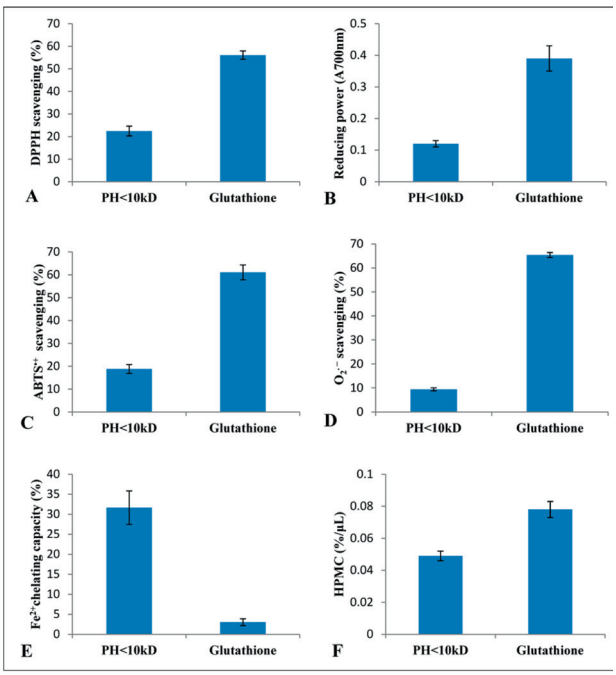

Fig. 3. Comparison between antioxidant activity of fraction PH<10kDa (0.1mg/ml) and reduced glutathione (0.1mg/ml) measured by conventional *in vitro* assays. A - DPPH radical scavenging assay, B – reducing power assay, C – ABTS radical scavenging assay, D – $O_2^{\bullet-}$ scavenging assay, E – Fe^{2+} chelating capacity assay, F – DC-polarographic assay. Values represent mean ± standard error, n = 3.

immediately and lasted for 4 min, using buffer as control. The scavenging activity was calculated according to formula: O_2^- scavenging (%)=$\{[(\Delta A/min)_c(\Delta A/min)_s]/(\Delta A/min)_c\} \times 100$, where c and s represent control and sample, respectively.

DC polarographic assay for AO activity determination is based on the decrease of anodic current obtained by dropping mercury electrode (DME) in an alkaline solution of hydrogen peroxide upon the addition of AOs (Sužnjević et al., 2011). The anodic current originated from Hg (II) hydroxo-perhydroxyl mixed complex (HPMC). The starting solution was 5 mM hydrogen peroxide in Clark and Lubs buffer, pH 9.8. Sample solutions were added in several equal aliquots. The slope of the starting linear part of the plot of the dependence of percentage on a decrease in anodic current on the volume of added sample solu-

tion (HPMC% μL^{-1}) is the measure of AO activity. Clark and Lubs buffer (pH 9.8) was prepared by mixing 25 mL of 0.4 M H_3BO_3, 25 mL of 0.4 M KCl and 40.8 mL of 0.2 M NaOH. The three electrode polarographic cells with a saturated calomel electrode (SCE) and an auxiliary platinum foil electrode were used. Before each measurement, the solution was deaerated with a stream of pure nitrogen, and inert atmosphere was maintained during recording. The polarographic curves were recorded using the polarographic analyzer PAR (Princeton Applied Research), model 174A, equipped with the Houston Omnigraphic 2000 X-Y recorder.

Statistical analysis

Descriptive statistical analysis for calculation of means and standard errors of the mean were conducted using Microsoft Excel software (Microsoft Office 2010). All the results are expressed as mean ± standard deviation. One-way ANOVA and post hoc Tukey's HSD, $p < 0.05$ level, was conducted using the statistic software SPSS 20.

RESULTS

Proteinase activity assay, bacterial growth and antioxidant activity changes during growth

Nine different *Lactobacillus* strains with extracellular proteinases were tested for the ability to hydrolyze pea proteins by applying a proteinase activity assay. Two strains (*Lb. rhamnosus* BGT10 and *Lb. zeae* LMG17315) hydrolyzed pea proteins efficiently, considering that almost no visible protein bands remained after overnight digestion (Fig. 1, lanes 2 and 4). Since the proteolytic system of *Lb. rhamnosus* BGT10 has been characterized previously (Pastar et al., 2003), this strain was selected for further experiments to monitor the bacterial growth of *Lb. rhamnosus* BGT10 in NIG broth and in a parallel test of AO activity of the filtered growth medium (MW<10 kDa) during 124 h using the DPPH scavenging activity assay. A rise in AO activity was observed already after 6 h, and the trend of AO activity increase was kept during incubation, reaching the maximum at 77 h (Fig. 2). Changes in AO activity showed a positive correlation with the increase in bacterial cell number ($r=0.98$), confirming the ability of the *Lb. rhamnosus* BGT10 strain to significantly increase the antioxidant activity of pea seed-based medium during fermentation. Based on obtained results, this strain was chosen for further experiments.

Antioxidant activity of pea protein hydrolysate

In order to investigate the effect of *Lb. rhamnosus* BGT10 proteolytic enzymes on the production of peptides with AO activity, purified pea protein isolate (PPI) was used as a substrate for hydrolysis with whole cells of *Lb. rhamnosus* BGT10 to produce the protein hydrolysate (PH). PH was a more convenient system for the analysis of proteolytic cleavage, purification of obtained products and assessment of their AO activity since the interactions with other antioxidants present in the seeds were minimized by multiple purification steps. As shown in Fig. 3A, the PH fraction MW<10 kDa (0.1 mg/mL) possessed significant DPPH scavenging activity, only 2.5 times lower than reduced glutathione at the same concentration. Similar results were obtained for $ABTS^{\cdot+}$ radical scavenging (Fig. 3C), which was shown to be about 3 times lower for the PH fraction MW<10 kDa than for reduced glutathione. The same fraction exhibited moderate superoxide radical scavenging compared to reduced glutathione – only 9.44% (Fig. 3D). As shown in Fig. 3E, the PH fraction MW<10 kDa showed significant chelating capacity on the ferrous ion at a concentration of 0.1 mg/mL – approximately 10 times higher than reduced glutathione at the same concentration. The reducing power assay showed that the PH fraction MW<10 kDa also exhibited a significant reduction of the Fe^{3+}/ferricyanide complex (Fig. 3B), indicating that fermentation of pea proteins with whole bacteria yielded a peptide mixture with considerable reducing power. Antioxidant activity measurement of concentrated peptide samples conducted by DC polarography (Fig. 3F) correlated well with other

AO assays, with the exception of the ferrous ion chelating assay.

Separation of hydrolysate fractions and DC polarographic assessment of AO activity

The AO activity of diluted peptide samples was successfully measured by the newly developed DC polarographic assay, which relies on the sensitivity of the anodic current originating from hydrogen peroxide complex formation (Gorjanović et al., 2012). Firstly, pea protein hydrolysates (PH fraction MW<10 kDa) obtained by fermentation with *Lb. rhamnosus* BGT10 were ultrafiltrated, separated using DEAE-Sepharose into eleven fractions (F1-F11) (Fig. 4A). Since the column used was a weak anion exchanger, results indicated that fractions F1-F3 were unbound, neutral or weak basic peptides, while F4-F11 represented acidic peptide fractions. AO activities measured directly in highly diluted fractions by DC polarographic assay were normalized on absorbance at 214 nm to obtain the most accurate results possible (Fig. 4B). Considering the AO activity of the hydrolysate fractions determined by DC polarographic assay, the unbound fraction F3 was estimated to be the most potent (Fig. 4B). Generally, it could be concluded that a group of basic peptide fractions had the highest AO activities, which is in accordance with previously published data (Ren et al., 2008; Zhang et al., 2009), although the tightly bound acidic peptide fractions F7 and F8 also possessed significant AO activities.

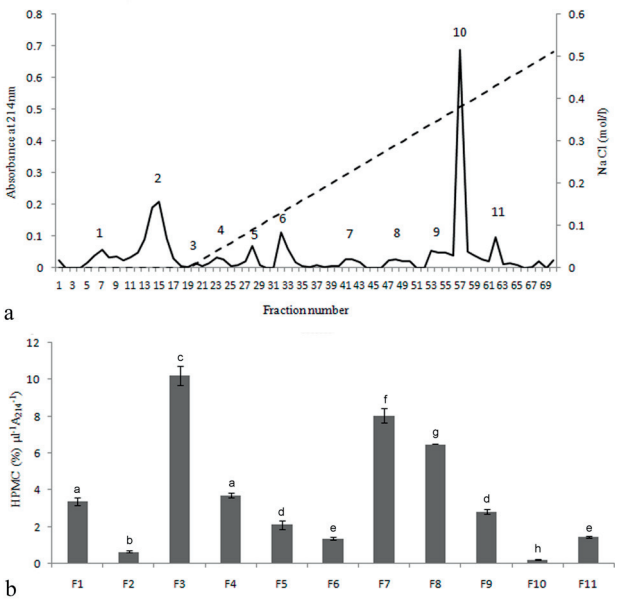

Fig. 4. (A) Fractionation of PH<10 kDa by ion-exange chromatography using DEAE Sepharose. **(B)** AO activity of resulting fractions determined by DC polarography. F1 – F11 refer to pooled peptide fractions. AO activity is expressed in HPMC % per µL of pooled fraction, devided by absorbance at 214 nm. Values represent mean ± standard error, n = 3. "a-h" values with the same letter are not statistically different at the $p < 0.05$ level (Tukey's HSD test).

DISCUSSION

Lb. rhamnosus BGT10 exhibited high proteolytic activity towards purified pea proteins, and showed excellent growth in pea protein-based NIG medium, considering that the cell number reached its theoretical maximum (2×10^9cells/mL). The growth of *Lb. rhamnosus* BGT10 was accompanied by an increment in antioxidant activity in the low molecular weight fraction (less than 10 kDa) of the growth medium. To evaluate the antioxidant potential of pea protein-derived peptides produced by *Lb. rhamnosus* BGT10, products obtained in the hydrolysis of the purified pea protein isolate were examined. The activity of the low molecular weight fraction of the hydrolysate was compared with reduced glutathione at the same concentration, using conventional spectrophotometric antioxidant activity assays and the recently developed DC polarographic assay. The analyzed hydrolysate (PH fraction MW<10 kDa) exhibited moderately high antioxidant DPPH scavenging activity. Our results are similar to those of Pownall et al. (2010) who showed that pea protein hydrolysates obtained by Thermolysine® (a commercial proteinase) exhibited approximately 1.5 times lower DPPH scavenging activity than reduced glutathione at the same concentration. $ABTS^{\bullet+}$ radical scavenging activity correlated well with the results of DPPH assay. The O_2^- scavenging activity measured in our assay is comparable to literature data, although caution should be taken in the interpretation of the results, since various

methods have been used to test superoxide scavenging activity. Our hydrolysate showed very moderate O_2^- scavenging activity, far lower than glutathione at the same concentration. Similar results were recorded by Pownall et al. (2010), who showed that pea protein hydrolysates fraction MW<3 kDa possessed moderate O_2^- scavenging activity compared to purified fractions of the same hydrolysate or pure glutathione at the same concentration. The differences in radical scavenging between the PH fraction MW<10 kDa and reduced glutathione observed in various assays could be explained by the fact that the radical system used in AO determination may severely influence the experimental results due to the steric accessibility of AOs to different radicals and different reaction kinetics (Magalhaes et al., 2008). The reducing power of our hydrolysate was about 3 times lower compared to reduced glutathione, indicating the importance of sulfur-containing groups as reducing agents as previously noted (Pownall et al., 2010). This lead us to assume that the PH fraction (MW<10 kDa) contained a smaller number of sulfhydryl groups than glutathione at the same concentration. Nevertheless, it can be concluded that the obtained peptides possessed a good electron-donating ability, which is in accordance with the results of the DPPH scavenging assay. As regards the ferrous chelating capacity, the examined hydrolysate showed a far stronger chelating capacity than that of glutathione, which is not exceptional. Complex protein hydrolysates usually contain more different metal chelating peptides exhibiting strong metal chelating capacity (Aluko, 2008; Pownall et al., 2010). Certain studies (Chan et al., 1994; Pownall et al., 2010) reported that substances with high chelating capacity might function as secondary AOs due to their ability to stabilize the oxidized form of metal ions. It could also be presumed that the PH fraction MW<10 kDa contributes to hydroxyl radical scavenging since this mechanism is effective mainly via the chelating of metal ions that are implicated in the generation of hydroxyl radicals (Zhang et al., 2009).

It has been widely accepted that the electrochemical properties of peptides can be used for the evaluation of their reducing or AO activity since their electrochemical oxidation potential is in relation with antioxidant versatility. The reducing power of a sample can be analyzed through the intensity of the anodic current, where anodic wave in a complex matrix represents more than a single component (Magalhaes et al., 2008). Polarographic study of the antioxidant activity of amino acids and proteins based on the shift of the first wave of oxygen reduction potential has been reported by Bumber et al. (2001). This assay has been previously applied to complex food and biological samples such as beer (Gorjanović et al., 2012; Gorjanović et al., 2013). The DC polarographic assessment of antioxidant activity in our hydrolysate showed that the PH fraction MW<10 kDa exhibited significant AO activity. The most important fact determined is that the results obtained by applying DC-polarography correlated well with three widely used spectrophotometric assays (DPPH, ABTS and Fe^{3+} reducing capacity assay). This close agreement indicates its applicability for direct AO activity determination in highly dilute peptide fractions. This finding corroborates with the previously reported sensitivity of the assay (Sužnjević et al., 2011).

In order to investigate the antioxidant potential of individual fractions, we separated hydrolysate fraction MW <10 kDa by ion exchange chromatography. The establishment of the actual peptide concentration in an individual fraction is a critical step for the accurate determination of AO activity or other biological activity in obtained fractions (Kuipers and Gruppen, 2007). The preparation of peptide solution with precise concentration, based on the weight of the lyophilized peptide, is inaccurate because there may be a significant amount of bound water and salts. In our case, absorbance at 214 nm was used to estimate the relative amounts of peptides present in different fractions of the obtained hydrolysate. Even though quantification based on absorbance at 214 nm is not a common method because the complexity of absorbance at this wavelength is higher than at 280 nm, it was applied because of the lack of a good alternative, especially for the peptides that do not contain tryptophan or tyrosine (Kuipers and Gruppen, 2007). By applying this approach, we successfully determined the antioxidant activity in all highly diluted fractions obtained after separation, without time-consuming

concentration steps. The obtained results showed that the unbound fraction of the hydrolysate (MW <10kDa) exhibited the highest antioxidant activity, which is in accordance with previously published data (Ren et al., 2008; Zhang et al., 2009).

Our results indicate that *Lactobacillus rhamnosus* BGT10 can grow in pea protein-based medium, resulting in elevated antioxidant activity, which can be detected in a low molecular fraction of the medium. Peptides produced during bacterially induced hydrolysis possess an antioxidant activity comparable to those obtained using commercial enzymes. Considering these results, it can be concluded that *Lactobacillus rhamnosus* BGT10 should be further investigated as a candidate strain for the large-scale production of bioactive peptides from legume proteins. Our research provides a valuable contribution to the investigation of bioactive peptide production using lactic acid bacteria, which could be applied to the formulation of novel functional food products based on pea proteins.

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