PAPER

BIOLOGICAL ACTIVITY OF EGG-YOLK PROTEIN BY-PRODUCT HYDROLYSATES OBTAINED WITH THE USE OF NON-COMMERCIAL PLANT PROTEASE

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ABSTRACT

Enzymatic hydrolysis leads to improved functional and biological properties of protein by-products, which can be further used as nutraceuticals and protein ingredients for food applications. The present study evaluated ACE-inhibitory, antioxidant and immunostimulating activities in hydrolysates of egg-yolk protein by-product (YP), generated during industrial process of delipidation of yolk. The protein substrate was hydrolyzed using non-commercial protease from Asian pumpkin (*Cucurbita ficifolia*). The reaction was conducted in 0.1 M Tris-HCl buffer (pH 8.0) at temperature of 37°C for 4 hours using different enzyme doses (100-1000 U/mg of substrate). The protein degradation was monitored by the determination of the degree of hydrolysis (DH), release of free amino groups (FAG) and by RP-HPLC. In the obtained hydrolysates we also evaluated biological activities. It was shown that the highest DH of substrate (46.6%) was obtained after 4h of reaction at the highest amount of enzyme. This hydrolysate exhibited antioxidant activity, including ferric ion reducing (FRAP) (56.41 μg Fe^{2+}/mg), ferric ion chelating (695.76 μg Fe^{2+}/mg) and DPPH free radical scavenging (0.89 μmol $trolox_{eq}$/mg) as well as ACE-inhibitory (IC_{50}=837.75 μg/mL) activities. The research showed improved biological properties of enzymatically modified YP by-product.

- Keywords: egg yolk proteins, *Cucurbita ficifolia* protease, hydrolysis, antioxidant, ACE-inhibitory activity, immunostimulating activity -

INTRODUCTION

Nowadays, the identification of bioactive food components, which can provide health benefits is one of the objectives of scientific research worldwide. Special attention is given to bioactive peptides due to their role in the prevention of numerous diseases (SHARMA and RANA, 2011). These peptides, released via the enzymatic hydrolysis of food proteins reveal numerous biological activities: antioxidant, antihypertensive, antimicrobial, antidiabetic, opioid and immunostimulating. These may have positive effects the cardiovascular, nervous, immune or digestive systems of the body (MINE and KOVAKS-NOLAN, 2006; CHAY PAK TING *et al.*, 2011; YU *et al.*, 2011; POKORA *et al.*, 2014).

The egg is recognized as a very valuable source of proteins for human nutrition, as well as proteins, which may be precursors of peptides with biological activity (MINE and KOVAKS-NOLAN, 2006; YU *et al.*, 2011; ZHIPENG *et al.*, 2011). ACE-inhibitory peptides are one of the best characterized peptides derived from eggs. The hydrolysis of ovalbumin, the main protein of egg white, conducted by gastrointestinal enzymes, results in the release of several ACE-inhibitory peptides (MIGUEL *et al.*, 2004; MIGUEL *et al.*, 2007). The effectiveness of these peptides was validated in tests *in vivo* conducted on spontaneously hypertensive rats (MIGUEL *et al.*, 2007). Antihypertensive activity was also demonstrated by some peptides released from egg-white protein treated by alcalase (LIU *et al.*, 2010; ZHIPENG *et al.*, 2011). As a result of peptide purification from those hydrolysates, ACE-inhibitory peptides: RVPSL and QIGLF were obtained (LIU *et al.*, 2010; ZHIPENG *et al.*, 2011).

It was also shown that egg is a rich source of proteins in which sequence numerous antioxidant peptides are encrypted. Phosphopeptides derived from egg phosvitin having molecular masses 1-3 kDa exert a strong ability to inhibit the oxidation of linoleic acid, to scavenge DPPH free radicals and to chelate iron ions (II) (XU *et al.*, 2007). Egg-yolk hydrolysates composed of peptides with a molecular weight lower than 1 kDa obtained with the use of proteinase from Bacillus ssp., also exhibited antioxidant capacities. Superoxide-scavenging activity and suppression of discoloration by β-carotene have also been observed (SAKANAKA and TACHIBANA, 2006). Egg yolk peptides obtained during alkalase and protease N digestion of delipidated egg yolk proteins were found to boost the systemic antioxidant status in the blood by increasing the GSH concentration in red blood cells (Young, Fan and Mine, 2010). It has been demonstrated that the consumption of egg yolk protein hydrolysates with antioxidative properties leads to the inhibition of tumor cell proliferation in the colon (ISHIKAWA *et al.*, 2009).

Some peptides derived from egg proteins can act as immune modulators and may be used as nutraceuticals for the prevention or treatment of lifestyle dependent diseases. Immunomodulatory peptides may exhibit anti-inflammatory activity by decreasing the production of pro-inflammatory cytokines (MATTSBY-BALTZER *et al.*, 1996; CROSS and GILL, 2000; MINE and KOVACS NOLAN, 2006). Egg yolk peptides significantly reduce pro-inflammatory cytokine, IL-8, in the Caco-2 cell line (YOUNG and MINE, 2010). Furthermore, immunostimulatory activity, assayed as the ability to enhance the capacity of phagocytic cells in mice, was present in ovalbumin hydrolysates prepared by gastrointestinal enzymes (BIZIULEVIČIUS *et al.*, 2005).

Bioactive peptides can be also released from protein by-products generated during isolation of biologically active substances naturally occurring in egg. One such protein waste is a by-product of lysozyme and cystatin extraction from hen egg white by ethanol method (SOKOŁOWSKA *et al.*, 2007). Our previous studies showed that this by-product, which itself exhibits poor functional properties, can be a rich source of ACE-inhibitory and antioxidative peptides (POKORA *et al.*, 2013; 2014; ZAMBROWICZ *et al.*, 2013). Attention is also drawn to egg yolk as a source of substances, which may find wide application in the prevention and treatment of various medical conditions. Egg yolk is mainly used for the extraction of valuable phospholipids such as lecithin, which is more valuable than plant-derived lecithin due to the specific chemical composition. The main by-products of this process are partially denatured and defatted egg yolk proteins in the form of insoluble granule fractions (SIEPKA *et al.*, 2010).

The preparation of bioactive peptides by enzymatic hydrolysis of proteinaceous by-products could become an interesting method of waste disposal if the process was cost- effective. Therefore cheap and effective enzymes for this process are preferred. Plant serine protease isolated from *Cucurbita ficifolia* pulp used in this study exhibits strong proteolytic properties and is a relatively cheap proteolytic enzyme (ILLANES *et al.*, 1985; CUROTTO *et al.*, 1988).

The aim of this study was the enzymatic hydrolysis of a by-product of egg yolk phospholipid isolation, in order to obtain hydrolysates with antioxidant, ACE-inhibitory and immunostimulatory activities.

MATERIALS AND METHODS

Substrate

Eggs from 40-45 weeks old Lohman brown laying hens (housed in a bedding system) were stored at 4°C for 1 week. The eggs were automatically broken and their macroscopic parts were separated on an industrial scale. Phospholipids were extracted from the egg yolks (Siepka *et al.*,

2010). Defatted granules, a by-product of phospholipid extraction from the egg yolk, were lyophilized and stored frozen until used.

Enzyme

Non-commercially available protease from *C. ficifolia* was isolated according to the procedure described by DRYJAŃSKI and WILUSZ (1990). Serine protease was obtained by extraction of the homogenized pumpkin pulp separated from the solids by centrifugation (5000 G, 20 min, 4°C). To the supernatant, ammonium sulfate was added to 50% saturation, and allowed to stand for 24 hours, and then centrifuged (9600 rev/min, 30 min). The resulting precipitate (the enzyme preparation) was desalted by dialysis for 12 hours using distilled water (4°C), and then 0.02 M of phosphate buffer at pH 6.0.

Determination of proteolytic activity of protease from *C. ficifolia*

Proteolytic activity was determined by reaction with 1% casein as a substrate (BDH, Ltd., England) at pH 8.3 (KUNITZ, 1945). The substrate with the enzyme was incubated for 10 min at 37°C. The reaction was stopped by the addition of 5% trichloroacetic acid (TCA). The samples were then centrifuged, and the absorbance of supernatants were measured at λ=280 nm. One unit of enzymatic activity (U) was defined as the amount of enzyme giving an increase in absorbance of 0.1 at 280 nm under reaction conditions.

Determination of protein content

Total protein content (N x 6.25) in insoluble substrate was determined using the Kjeldahl method. Protein content in hydrolysates and peptide fractions was determined by the method of LOWRY *et al.* (1951).

Enzymatic hydrolysis

YP hydrolysis was carried out according to a modified method of Zambrowicz *et al.* (2013a). 1% substrate suspension in 0.1 M Tris-HCl buffer (pH 8.0) was hydrolyzed at 37° C for 4 hours using *C. ficifolia* protease at doses of 100, 200, 400 and 1000 U of active enzyme applied on 1 mg of YP substrate. The reaction was ended by heating the mixture at 100°C for 15 min. The hydrolysates were cooled, centrifuged (5500 G, 10 min, 10°C), then the supernatants were lyophilized and stored at 4°C until used.

The degree of hydrolysis

The degree of hydrolysis (DH %) was determined as the percentage ratio of protein soluble in 10% trichloroacetic acid (TCA) to total protein (SPELLMAN, 2003). TCA was added to the hydrolysates (1:1) and after 1 h of incubation at 4°C the samples were centrifuged (4500 G, 15 min, 20°C). The concentration of the trichloroacetic acid-soluble product in the supernatant was measured spectrophotometrically and calculated from the following equation:

$$DH (\%) = (\text{mg soluble protein after hydrolysis / mL} \div \text{mg soluble protein before hydrolysis/mL}) \times 100\%$$

The content of free amino acid groups

The content of free amino acid groups (FAG) (μmol/g) was determined by using trinitrobenzene sulfonic acid (TNBS, Sigma) according to a modified method by Kuchroo *et al.* (1983).

Reversed-phase high-performance liquid chromatography

Peptide profiles of hydrolysates were monitored by reversed-phase high-performance liquid chromatography (RP-HPLC). Separation was performed using a Zorbax XDB-C 18 Agilent column (1.8 mm × 50 mm). The operation conditions were as follows:
injection volume: 50 μL; mobile phase A – 0.1% TFA in water; mobile phase B – 0.1% TFA in acetonitrile, column temperature: 30°C. Flow rate: 1mL/ min. Analysis time and gradient conditions can be found in drawings. The absorbance of eluent was monitored at λ=230 nm.

Determination of ACE-inhibitory activity

ACE (EC 3.4.15.1) inhibitory activity was measured spectrophotometrically according to the method described by MIGUEL *et al.* (2004) with some modifications. A hydrolysate solution (40 μL) mixed with a Hippuryl-His-Leu (HHL) substrate solution (5 mmol/L in 100 mmol/L potassium phosphate containing 300 mmol/L sodium chloride, pH 8.3) was preincubated at 37°C for 5 min, and the reaction was initiated by adding 20 μL (2 mU) of ACE solution, and then incubated for 30 min at the same temperature. The enzymatic reaction was terminated by the addition of 150 μL of 1 M HCl. The liberated hippuric acid was extracted using 1 mL of ethyl acetate and vigorously shaking, 750 μL of the upper layer was transferred into a test tube and evaporated under vacuum. The hippuric acid left in the tubes was re-dissolved in 800 μl of distilled water. The content of hippuric acid was determined spectrophotometrically at λ=228 nm.

All samples were tested in 3 replications. Inhibition activity was calculated using the following equation:

$$\text{Inhibitory activity } (\%) = ((Ac - As) / (Ac - Ab)) \times 100$$

where Ac is the absorbance of the buffer (control), As is the absorbance of the reaction mixture (sample), Ab is the absorbance when the stop solution was added before the reaction occurred (blank).

The IC_{50} value was defined as the concentration of peptides in μg/mL required to reduce 50% of ACE activity, which was determined by analysis of ACE inhibition (%) versus peptide concentration.

Determination of antioxidant activity as the ability to scavenge of DPPH free radicals

Antioxidant activity was determined by a modified method of Yen and Chen (1995) as the ability to scavenge of DPPH (2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl) free radicals in an aqueous solution of peptides. Absorbance measurements were made at λ=517 nm after 30 min incubation. The antioxidant activity of the analyzed peptides was determined on the basis of the standard curve prepared for trolox equivalent.

Determination of antioxidant activity by FRAP method

Antioxidant activity was determined as the ability to reduce the oxidation of iron Fe(III) to Fe(II) ions in a reaction with TPTZ (2,3,5-triphenyltetrazoliumchloride). The absorbance was measured at λ=593 nm. The concentration of Fe^{2+} ions was determined on the basis of the standard curve for known $FeSO_4$ solutions (BENZIE and STRAIN, 1996).

Determination of iron Fe(II) ion chelation

Chelation of iron ions was determined by colorimetric measurement of the quantity of Fe(II) not bound to the peptides in a reaction mixture with ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate) (Xu *et al.*, 2007). Absorbance measurement was made at λ=562 nm. The ability to chelate iron ions was determined on the basis of the standard curve for a $FeCl_2$ solution.

Determination of immunostimulatory activity

Immunostimulatory activity of the cytokine secretion in human whole blood was determined at the Department of Immunochemistry, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław (Poland). Cytokine secretion was induced according to the procedure described by INGLOT *et al.* (1996). Blood samples from at least 10 donors were collected in syringes containing sodium heparin. Within 1 h after collection, the blood was diluted 10-times with RPMI 1640 medium supplemented with penicillin/streptomycin, L-glutamine and 2% fetal bovine serum. 1 ml portions of the cell suspension were distributed in two 24-well flat-bottomed tissue culture plates. To the cell suspension of whole human blood (1 mL sample) the hydrolysates were added at 1.0, 10 and 100 μg. As a reference, the positive lipopolysaccharide inducer of *E. coli* at a concentration of 4 mg/mL was used. Control wells containing non-treated blood cell samples were used to measure the spontaneous production of cytokines (negative control). The plates were incubated for 22 h at 37°C in a 5% CO_2 atmosphere. After incubation, the plates were centrifuged at 200 G for 15 min at room temperature. The supernatants were collected and used for determination of the cytokines. IL-6 and IL-10 were determined by microplate enzyme-linked immunosorbent assay using commercially available sets from Becton Dickinson (Franklin Lakes, NJ, USA) according to the procedure recommended by the manufacturer.

Statistical analysis

All experiments were carried out in triplicates. The data obtained were subjected to multi-factor variance analysis (ANOVA), followed by the Duncan's multiple range test to determine the significant difference between sample at $p<0.05$ level using the Statistica v.9.0.

The results of immunostimulating activity were considered significant by a non-parametric Wilcoxon test at $p \leq 0.05$ (*) and $0.05 \leq p \leq 0.1$ (**) versus control (untreated cells).

RESULTS AND DISCUSSION

Enzymatic hydrolysis

Egg yolk protein preparation (YP), as a by-product of lecithin extraction, was treated by a non-commercial serine protease isolated from *C. ficifolia* in order to evaluate antioxidant, ACE-inhibitory and immunostimulatory properties.

The progress of hydrolysis was monitored by determining the degree of hydrolysis (DH) (%) (Fig. 1), the increase in the concentration of free amino groups (FAG) (Fig. 2), and by RP-HPLC peptide profile analysis (Fig. 3). DH depended on the enzyme dose and reaction time. DH increased slowly in the first 0.5 h, followed by a faster rate of increase up to 4 h, indicating that the maximum cleavage of proteins occurred in the last hour of hydrolysis. Dissimilar kinetics of protein substrate degradation with various proteolytic enzymes has been observed by other authors (OTTE *et al.*, 1998; ZAMBROWICZ *et al.*, 2012). Typically, enzymatic hydrolysis is most extensive during the first 30 minutes and then slows down, indicating a maximum of protein degradation in the first hour of hydrolysis.

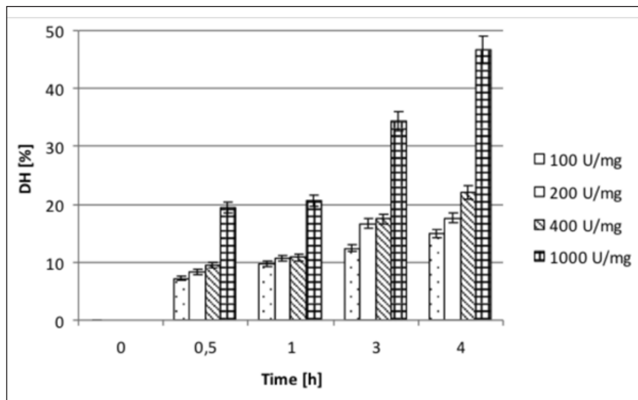

Fig. 1 - Effect of time on the degree of hydrolysis (DH) during hydrolysis of YP by protease from *C. ficifolia*. a-c means followed by the same letter do not differ significantly (p≤0.05).

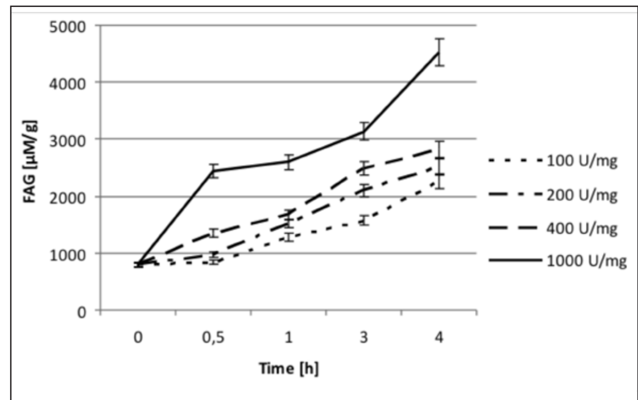

Fig. 2 - Effect of time on the free amino groups contents (FAG) during hydrolysis of YP by protease from *C. ficifolia.*

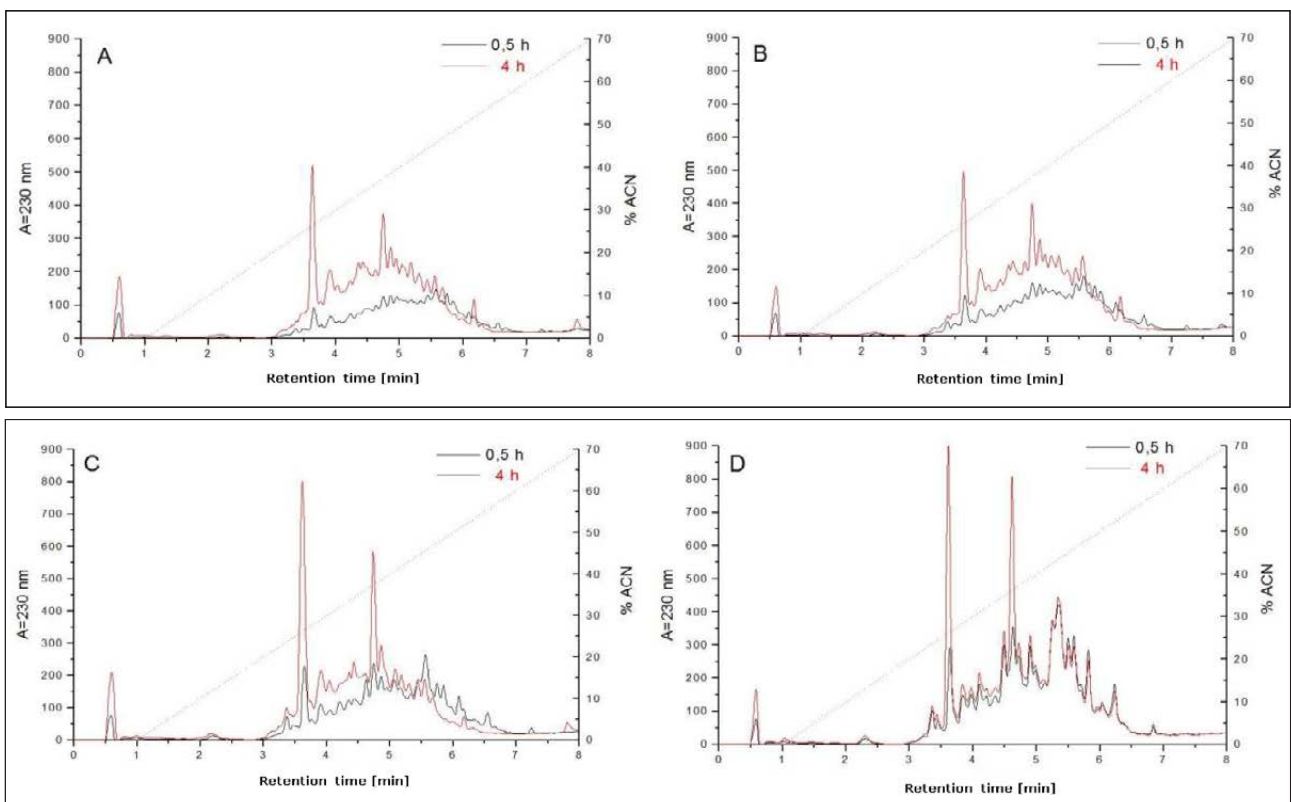

Fig. 3 - Peptide profiles (RP-HPLC) of YP protein hydrolysates obtained after using: A. 100 U/mg, B. 200 U/mg, C. 400 U/mg and D. 1000 U/mg of protease from *C. ficifolia.*

Probably, the hydrolysis process with the use of protease from *C. ficifolia* proceeded according to the "one-by-one" mechanism. During the initial stage of the reaction (determining the overall level of hydrolysis) it is necessary to partially unfold native protein molecules. As a result, the protein loses its stability, more peptide bonds are exposed on the outside of the molecule (intermediate products) and the enzyme has access to the hydrolyzed peptide bond. In a further step, intermediate products are very rapidly degraded to small peptides (KUNST, 2003).

The use of the lowest dose of enzyme (100 U/mg) resulted in a nearly 15% DH of YP. The increase of the dose of *C. ficifolia* proteinase to 400 U/mg did not exert any significant impact on DH rate, whereas the addition of the enzyme at 1000 U/mg resulted in a DH value of more than 46% after 4 hours of digestion. Analysis of the FAG concentration of the obtained hydrolysates confirmed the above results. The greatest increase in concentration was observed during the long time of hydrolysis. The application of enzyme at doses from 100 to 400 U/mg resulted in similar levels of increases of FAG from 2255.20 to 2325.74 µM Gly/g. The most intensive increase of FAG (4525.1 µM Gly/g) occurred during the 4-hour reaction using 1000 U/mg of protease.

Protein-peptide profiles obtained by RP-HPLC technique demonstrate the extent of YP hydrolysis (Fig. 3). They were identified with both a longer retention time (3-7 min) specific for the more hydrophobic peptides, and peaks with a short retention time (0.5-1 min) typical for hydrophilic peptides. The wide distribution of degradation products indicates that each of the hydrolysates is composed of peptides with different hydrophobic properties, which may impact the biological activity of the obtained hydrolysates. The results described above indicate that serine protease from *C. ficifolia* is an effective enzyme in hydrolyzing egg yolk protein by-product. Previously, the proteolytic properties of this enzyme had been tested on casein, a protein from corn gluten, and egg white proteins (ILLANES *et al.*, 1985; CUROTTO *et al.*, 1989; POKORA *et al.*, 2014).

The biological activities of YP enzymatic hydrolysates

The antioxidant activity of YP hydrolysates was studied in terms of the scavenging effect on DPPH radicals, ferric reducing power (FRAP), and iron chelating activity (Table 1).

The enzymatic treatment of YP leads to an increase in DPPH free radical scavenging activity in the hydrolysates. ELIAS *et al.* (2008) explained that the antioxidant activity of the hydrolysates/peptides is the result of the proteolytic action of the enzyme. The specific amino-acid sequence of peptides and their changed physical properties allow exposing the amino acid residues and their action as electron donors. As a result of these reactions, the peptides combine with radicals and form stable complexes, which inhibit oxidation processes. The various YP hydrolysates showed different potencies in scavenging DPPH radicals. The results indicated no direct relationship between DH and the values of DPPH free radical scavenging activity. The highest DPPH scavenging potency was shown by the hydrolysate obtained after 4 hours degradation with an enzyme dose of 1000 U/mg (0.89 μmol $trolox_{eq}$/mg) (Table 1). The significant level of DPPH free radical scavenging activity (0.63 μmol $trolox_{eq}$/mg) was also observed for the hydrolysate obtained with the use of 200 U/mg protease after 0.5 hour digestion. In previous works, YP protein by-product was treated with pepsin and neutrase leading to final hydrolysates with DH values: 45.3% and 27.6%, respectively (ZAMBROWICZ *et al.*, 2014; POKORA *et al.*, 2013). Peptic hydrolysate and hydrolysate obtained by neutrase showed DPPH free radical scavenging activity values: 0.5 troloxeq/mg and 0.44 μM $trolox_{eq}$/mg, respectively (ZAMBROWICZ *et al.*, 2014; POKORA *et al.*, 2013). It may be explained that the potency of YP-hydrolysates to scavenge DPPH free radical depends more on the specificity of the enzyme than on the degree of hydrolysis (DH). Our results also indicate that YP is a better source for peptides exhibiting DPPH scavenging activity than other protein waste, such as by-products of lysozyme and cystatin isolation from egg white. Egg white protein by-product hydrolysates obtained with trypsin and neutrase exhibited free radical scavenging activity up to 0.21 and 0.17 μmol $trolox_{eq}$/mg, respectively (ZAMBROWICZ *et al.*, 2013).

Previously, serine protease *C. ficifolia* was used by DĄBROWSKA *et al.* (2013) to evaluate the antioxidant activity of bovine casein. Casein hydrolysates possessed a different ability to scavenge of DPPH radicals (from 0.06 to 2.21 μmol $trolox_{eq}$/mg), depending on enzyme dose and reaction time. However, the DPPH scavenging potency of many of them was at the same level as the YP hydrolysates. In most of the hydrolysates obtained with different doses of protease, the ferric reducing ability was increasing gradually with the time of hydrolysis. The only exception was the 0.5 h hydrolysate obtained with the enzyme dose of 200 U/mg, which possessed significantly higher ferric reducing activity than the products obtained during long time of degradation (more than 30 minutes). A maximum value of this activity reached 56.41 μg Fe^{2+}/mg, for the 4 h hydrolysate obtained with the participation of 1000 U/mg of the enzyme (Table 1). The ferric reducing activity of the 4h hydrolysate increased with the increased doses of the enzyme. The application of protease at 100, 400 and 1000 U/mg resulted in hydrolysates 3.44, 5.0 and 5.47 times more potent than YP, respectively. On the other hand, hydrolysates exerted more than 3 times lower ferric reducing activity in comparison to the YP hydrolysate prepared with neutrase (177.35 μg Fe^{2+}/mg) (Pokora *et al.*, 2013). This results gives an indication that unconventional protease from *C. ficifolia* is characterized by a lesser ability to release peptides with ferric reducing activity from YP than commercially available neutrase. An increase in chelating activity was also observed as a result of progress in hydrolysis. The highest chelating activity was obtained in hydrolysates with DH above 35 %. YP degraded with participation of 1000 U/mg protease during 3 and 4 hour reactions exhibited ferrous ion chelating activity at 692.49 and 695.76 μg Fe^{2+}/mg, respectively (Table 1). Significant ferric chelating power was also shown by hydrolysates in which DH ranged from 15% to 20%. Similar results were obtained by Torres-Fuentes *et al.* (2011), who analyzed the antioxidant properties of plant protein hydrolysates in terms of their ability to complex iron ions.

Numerous antihypertensive peptides (eg. ovokinin, ovokinin 2-7, RADHP, YPI, DLIN) derived from egg white proteins by enzymatic hydrolysis have been characterized (MIGUEL *et al.*, 2004; LIU *et al.*, 2010). Interest has been aroused in ACE-inhibitory peptides generated from egg yolk proteins, because they have not been described as a

source of peptide inhibitors of ACE as much. The hydrolysates obtained in this work exhibited various abilities to inhibit the ACE enzyme (Table 1). The hydrolysates of DH lower than 10% did not exert any ACE inhibitory activity. The most active inhibitor of ACE (IC_{50}=467.5 μg/mL) was the hydrolysate obtained by an enzyme dose of 1000 U/mg after 4-hours digestion. Whole egg yolk in native form as a potential source of ACE-inhibitory peptides was tested by YOU and WU (2011). The level of this activity (IC_{50}) for hydrolysates prepared with the use of gastrointenstinal (pepsin, pancreatin) and microbial (thermolysin, alkalase) proteases ranged from 133.4 μg/mL to 210.2 μg/mL (YOU and WU, 2011). Such significant differences in the level of ACE-inhibitory activity may result from the fact that in the present study we used a by-product, denatured protein of the yolk granular fraction. Denaturation has a significant impact on the physico-chemical and biological properties of proteins.

The results indicate that protein by-product obtained from the isolation of phospholipids from hen egg yolk may be a better source of ACE-inhibitory peptides than other protein by-product from the isolation of cystatin and lysozyme from egg white. The peptic hydrolysate (DH: 38.3%) of this protein preparation exhibited an activity of IC_{50}=643.1 μg/mL (ZAMBROWICZ *et al.*, 2013). Recently, we indicated that serine protease from *C. ficifolia* may be effective in the conversion of this protein by-product to a value added product with ACE-inhibitory activity (POKORA *et al.*, 2014). The 50% inhibition of ACE was obtained with the presence of 9071.7 μg/mL of the hydrolysate.

Studies have shown a high linear correlation between DPPH free radical scavenging activity and immune activity with a positive correlation coefficient of 0.96 (HE *et al.*, 2014). Therefore hydrolysates with the highest DPPH free radical scavenging potency were also evaluated in terms of their immunostimulatory properties. It was assessed as the results of cytokines IL-10 and IL-6 induction by hydrolysates in whole human blood cell cultures (*ex vivo*) (Fig. 4). 4-hour hydrolysate obtained with the use of 1000 U/mg of *C. ficifolia* protease appeared to be slight inducer. The use of 100 μg/ mL of protease resulted in a low increase the concentration of IL-6, which reached the value: 3.05 ng/ mL (Fig. 4 A). However, the results were not statistically significant compared to the positive control (LP). Extremely different cytokine inducing activity was exerted by the yolkin, naturally occurring in egg yolk. Yolkin is a mixture consisting of several peptides of an apparent molecular weight of 1 to 35 kDa, produced as a result of vitellogenin II hydrolysis by cathepsins during the formation of an egg. Its constituent peptides were found to be efficient inducers of IL-1β, IL-6 and IL-10 secretion. A complex at a concentration of 100 μg/mL showed almost the same activity as the LPS-treated control in stimulating cytokine production. (POLANOWSKI *et al.*, 2013). The biological activity of enzymatic hydrolysates

Table 1 - Biological activities of YP hydrolysates obtained with using serine protease from *C. ficifolia*. All data were expressed as mean values (mean±SD, n=3). Values sharing the same letter at the same enzyme dose and test group are not significantly different at p<0.05.

Enzyme dose [U/mg]	Time of hydrolysis	DPPH scavenging activity [μM $Trolox_{eq}$/mg]	Ferric reducing ability (FRAP) [μg Fe^{2+}/mg]	Ferrous ion-chelating activity [μg Fe^{2+}/mg]	ACE inhibitory activity [IC_{50}] [μg/ml]
0	substrate	0.15±0.01[a]	10.3±0.16[a]	376.2±18.80[a]	nd[a]
100	0.5	0.28±0.03[a]	33.26±1.38[a]	497.10±8.22[b]	nd[c]
	1	0.32±0.03[b]	36.44±1.62[b]	466.26±3.89[c]	nd[c]
	3	0.18±0.02[c]	48.78±1.20[c]	474.25±4.01[d]	968.5±17.25[b]
	4	0.42±0.02[b]	35.40±0.67[d]	574.38±13.67[a]	837.75±15.25[a]
200	0.5	0.63±0.02[a]	46.14±1.33[a]	460.72±11.74[b]	nd[c]
	1	0.20±0.03[c]	35.53±1.21[a]	507.59±2.88[d]	nd[c]
	3	0.28±0.01[d]	37.11±0.73[b]	553.75±13.79[c]	890.75±11.25[b]
	4	0.27±0.01[b]	44.68±1.66[b]	626.16±6.56[a]	777.0±14.25[a]
400	0.5	0.20±0.03[ab]	38.91±1.41[a]	468.70±12.07[d]	nd[c]
	1	0.21±0.03[a]	36.77±1.20[a]	513.73±3.81[c]	nd[c]
	3	0.20±0.04[a]	35.44±1.30[ab]	573.56±3.77[b]	803.12±10.25[b]
	4	0.26±0.02[a]	51.46±0.78[ab]	625.86±7.11[a]	718.75±4.5[a]
1000	0.5	0.22±0.02[a]	21.71±1.37[d]	642.32±2.83[d]	657.75±4.5[a]
	1	0.25±0.02[c]	27.78±1.75[c]	654.79±5.78[b]	650.0±4.5[a]
	3	0.43±0.03[b]	43.33±1.06[b]	692.49±1.86[c]	554.75±7.75[a]
	4	0.89±0.03[a]	56.41±1.13[a]	695.76±14.91[a]	467.5±6.0[a]

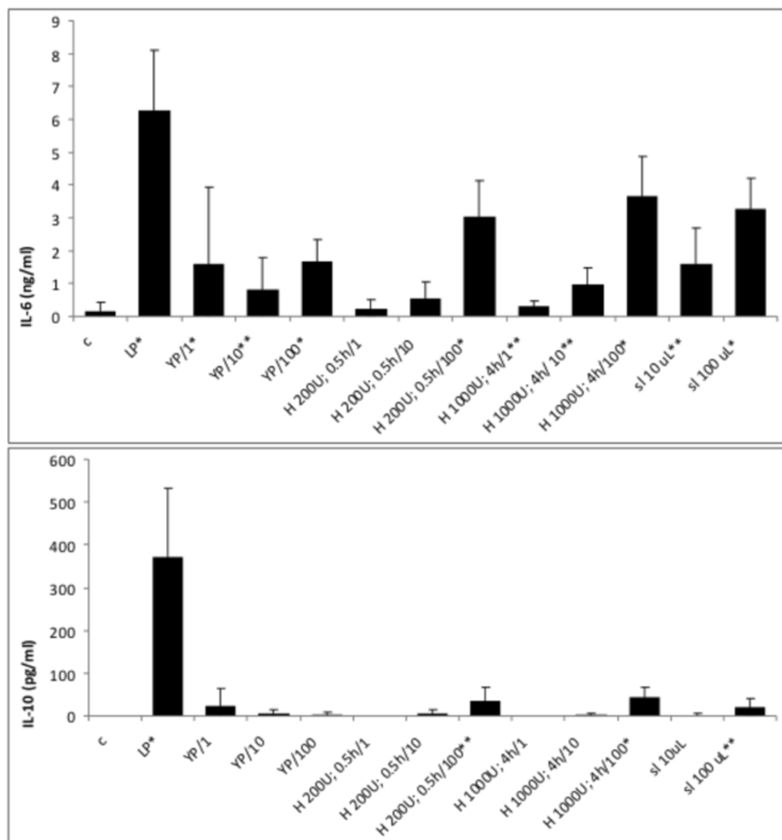

Fig. 4 - Induction of the cytokines A - interleukin-6 and B - interleukin-10 by YP and YP hydrolysates in whole blood cells cultures. C- control (untreated cells), LP -lipopolicascharide from *Escherichia coli* was used as positive control, sl- reagents used in enzymatic hydrolysis. YP and YP hydrolysates were applied at doses 1, 10 and 100 μg/mL. Data are presented as the median ± standard deviation. The results were considered significant by the non-parametric Wilcoxon test when $p \leq 0.05$ (*) and $0.05 \leq p \leq 0.1$ (**) versus control (untreated cells).

is determined by the protein sequence (type and location of amino acid residues) as well as by the specifity of the enzyme (CLEMENTE, 2000; PARK *et al.*, 2001). These two crucial factors are responsible for the disparate level of immunostimulatory activity of YP-hydrolysates.

CONCLUSIONS

The effect of enzymatic modification of an egg yolk protein preparation (YP), obtained as a by-product of phospholipid extraction, on its biological properties were evaluated. The hydrolysis process of YP was performed with the use of noncommercial serine protease from *C. ficifolia*. The most effective degradation of YP was noticed under the conditions: enzyme dose 1000 U/ mg and duration 4 h, when a significant degree of hydrolysis (46.6%) was obtained. Enzymatic hydrolysis of YP provided hydrolysates/ peptides exhibiting antioxidant and ACE-inhibitory activities. The YP hydrolysates showed significant antioxidant and degree of hydrolysis-dependent ACE-inhibitory activity. The 4-hour hydrolysate obtained with the highest amount of enzyme (1000 U/mg) showed the highest biological activity among the tested hydrolysates. It exhibited ferric ion reducing potential (FRAP) (56.41 μg Fe^{2+}/mg), ferric ion chelating activity (695.76 μg Fe^{2+}/mg), DPPH free radical scavenging activity (0.89 μmol $trolox_{eq}$/mg) and ACE- inhibitory (467.5 μg/mL) activity. Novel biological effects of egg-yolk protein by-product hydrolysates was shown.

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