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Antioxidant and antimicrobial activity of lecithin free egg yolk protein preparation hydrolysates obtained with digestive enzymes

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ABSTRACT:

Several biological activities have now been associated with egg protein- derived peptides, including antihypertensive, antimicrobial, immunomodulatory, anticancer and antioxidant activities, highlighting the importance of these biopeptides in human health, and disease prevention and treatment. Special attention has been given to peptides with antioxidant and antimicrobial activities as a new source of natural preservatives in food industry. In this study, the antioxidant properties of the egg-yolk protein by-product (YP) hydrolysates were evaluated based on their radical scavenging capacity (DPPH), Fe^{2+}chelating effect and ferric reducing power (FRAP). Furthermore, antimicrobial properties of obtained hydrolysates against Bacillus species were studied. The degrees (DHs) of hydrolysis for 4h hydrolysates were: 19.1%, 13.5% and 13.0%, for pepsin, chymotrypsin and trypsin, respectively. Pepsin was the most effective in producing the free amino groups (1410.3 μmolGly/g). The RP-HPLC profiles of the protein hydrolysates showed differences in the hydrophobicity of the generated peptides.

Trypsin hydrolysate obtained after 4h reaction demonstrated the strongest DPPH free radical scavenging activity (0.85 μmol $Trolox_{eq}$/mg). Trypsin and chymotrypsin hydrolysates obtained after 4h reaction exhibited 4 times higher ferric reducing capacity than those treated by pepsin. The hydrolysis products obtained from YP exhibited significant chelating activity. The 4h trypsin hydrolysate exhibited weak antimicrobial activity against *B. subtilis B3; B. cereus B512; B. cereus B 3p and B. laterosporum B6.*

Keywords: Antioxidative activity, DPPH, peptides, hydrolysis, egg-yolk protein

INTRODUCTION:

The oxidative stress caused by free radicals would damage lipid, protein and DNA that resulted in cell death. The accumulation of peroxidants is associated with cancer, cardiovascular disorders, diabetes and Alzheimer's disease [1,2].

Lipid oxidation is also a significant problem to the food industry. The development of undesirable off-flavors, discoloration and potentially toxic reaction products are unavoidable during this process and contribute to the quality deterioration of stored food and shortening of the shelf life [2,3]. Therefore the control of lipid oxidation in food products is desirable, and attempts to anticipate its occurrence via modeling or otherwise to ascertain the benefits of antioxidants in food storage is of great interest in research. Special attention has been given to natural antioxidants (for example peptides) from a new trend to avoid the use of synthetic food additives [4,5].

Food-derived peptides isolated from different sources, such as rapeseed muscle of conger eel or soy protein hydrolysates have been found to possess antioxidant activity [6,7].

A rich source of antioxidant peptides are egg white and yolk proteins [3,4,8,9]. Numerous studies reported the antioxidant activity of egg-yolk protein in a linoleic acid oxidation system [3,4]. The hydroxyl radical and DPPH scavenging activity and suppression of discoloration of β-carotene have been also observed. In food modeling systems, peptides derived from egg-yolk hydrolysates effectively inhibited lipid oxidation processes in beef and tuna muscle homogenates [3]. Two peptides obtained from lecithin-free egg yolk hydrolysate also exhibited antioxidant activity in a linoleic acid model system. These peptides, composed of 10 and 15 amino acid residues, both contain a leucine residue at their N- therminal positions [10]. Phosvitin, which has been recognized as an egg yolk antioxidant protein, is the main egg yolk protein precursor of antioxidant peptides [8]. Oligophosphopeptides derived from the partial tryptic hydrolysis of dephosphorylated phosvitin exhibited strong capability against the oxidation of linoleic acid and also radical scavenging activity on DPPH free radicals. These peptides are characterized by a high content of phosphorus and amino acids such as histidine, methionine and tyrosine [8].

It has been shown that hydrolysis products obtained from phosvitin, by inhibiting the iron-dependent reaction in the production of most reactive hydroxyl radicals, have protective properties towards DNA. Thus this protein and its peptides are considered as factors preventing the occurrence of oxidative stress-induced diseases such as colon cancer, Alzheimer's or Parkinson's disease [11].

Not only chemical reactions but also microbials cause offensive sensory changes in food. The presence of pathogens and their toxic products may be health hazard. The use of natural antimicrobials in food products is a particularly attractive approach, due to the increasing prevalence of microorganisms resistant [12].

Hen egg white proteins are a source of number antimicrobial peptides [12,13,14]. Peptides released from lysozyme, ovalbumin and ovotransferrin, during proteolytic action of enzymes, possessed antimicrobial activity against gram positive and gram negative bacteria [12,13,14]. In contrast, not much information is available about egg yolk proteins as precursors of antimicrobial peptides.

The objective of this investigation was to assess the antioxidative and antimicrobial activity of enzymatic hydrolysates derived from protein by-product left during the course of lecithin isolation from egg yolk.

MATERIALS AND METHODS:

Egg- yolk protein preparation (YP) was obtained in our laboratory as a byproduct of lecithin iso-

lation [15]. Porcine pepsin type A (EC 3.4.23.1), TPCK-treated bovine trypsin T-8003 (EC 3.4.21.4), bovine α-chymotrypsin type II C-4129 (3.4.21.1), 5% picrylsulfonic acid (TNBS), bovine serum albumin tryptic soy broth and tryptic soy agar were obtained from Sigma Chemicals Co., trichloroacetic acid was obtained from Ubichem, acetonitrile was obtained from Lab-Scan, and trifluoroacetic acid (TFA) was obtained from Fluka. *Bacillus* strains were obtained from Department of Biotechnology and Food Microbiology University of Environmental and Life Sciences in Wroclaw.

Pepsin activity was determined in the reaction with 2% acid-denaturized hemoglobin as a substrate [16]. The 0.2M phosphate-citrate buffer pH 3.0 (650 µl) was preincubated at 37 °C for 5 min. Then 100 µl of enzyme solution (2-20 µg) was added. The reaction was started by adding 250 µl of hemoglobin. After 10 min the reaction was stopped by the addition of 1500 µl of 10 % trichloroacetic acid. Then the tubes were centrifuged (5500 rpm, 15 min, 20°C). The absorbance of the supernatants was measured at 280 nm. One unit of enzymatic activity of pepsin (U) was defined as the amount of enzyme producing an increase in absorbance at 280 nm of 0.1 under reaction conditions.

Trypsin and α-chymotrypsin activities were determined in reaction with 1% casein as an substrate at pH 8.0 [17]. Each enzyme was diluted in 20 mM HCl with 80 mM $CaCl_2$ to a final protein concentration of from 4 to 20 µg/ ml. One ml of 1% casein (in 0.2 mM Tris-HCl buffer, pH 8.0) was preincubated at 37 °C for 5 min and the reaction was started by adding 1 ml of enzyme solution. After 10 min the reaction was stopped by the addition of 3 ml of 5 % trichloroacetic acid. Then the tubes were centrifuged 4.500 x g, 15 min, 20°C). The absorbance of the supernatants was measured at 280 nm. One unit of enzymatic activity of these enzymes (U) corresponded to that amount of enzyme which is capable of hydrolyzing 1% casein under reaction conditions and giving an increase in absorbance at 280 nm of 0.1.

The protein concentration was determined according to the method of Lowry et al.[18]. A standard curve was prepared for bovine serum albumin (BSA).

Preparation of egg yolk protein. YP was obtained as by-product of egg lecithin extraction [15]. Eggs were laid by Lohman Brown line hens. Egg yolks were homogenized, then diluted in bidistilled water 1:3 (v/v) and acidified to pH 5.0 with 1.0 M citric acid. The mixture was fractionated to plasma and granules by centrifugation (30 min. at 12 000 x g). Granules were frozen and kept for 7 days. After defrosting, granules were diluted in 1.7M NaCl (1:1.5 m/v) and kept for 24 h at 4°C. The mixture was centrifuged for 30 min. at 8 000 x g, and solid fraction was then lyophilized. Liophilisate was mixed with ethanol (96%) (1:3 m/v) and centrifuged for 30 min at 26 000 x g, and this process was repeated another two times. The solid fraction – egg yolk protein by-product (YP) was lyophilized.

Alkalaine dephosphorylation [8]. YP (50 mg) was dissolved in 2 ml of 0.1M NaOH and incubated at 37 ºC for 3h, after which the pH was reduced to 7.0 by the addition of 1M HCl. Following overnight dialysis against Milli-Q water the YP was lyophilized.

Enzymatic hydrolysis according to Graszkiewicz et al [19] method with modifications. The hydrolysis of YP (concentration of 5.0 mg/ml) with trypsin and chymotrypsin was carried out in 0.2M Tris- HCl buffer, pH 8.3 at 37^{o}C using a dose of enzyme of 10 U/mg of hydrolyzed protein. Hydrolysis with pepsin was conducted in 0.2M Gly- HCl buffer at pH 3.5. Hydrolysis was continued for 4 h and the hydrolysate samples were taken out at different time intervals (0.5, 1, 2, 3 and 4h). The reaction was terminated by heating at 100 ºC for 15 min. The hydrolysates were cooled and centrifuged (3145 g, 10 min, 10°C). The supernatants were lyophilized and stored at 4 ºC until use.

Degree of hydrolysis (DH) (%) was expressed as the percentage ratio of protein soluble in 10 % trichloroacetic acid (TCA) to total protein [20]. TCA (10 %) was added to the hydrolysates (1:1 (v:v)). After 1h of incubation at 4°C the samples were centrifuged (5500 rpm, 15 min, 20°C). The concentration of the trichloroacetic acid-soluble product in the supernatant was measured spectrophotometrically at 280 nm. The protein concentration was 1.0 mg/ml when the sample giving an absorbance = 1.0 at 280 nm.

$$DH\ (\%) = [(C_h - C_s)/C] \times 100$$

Where C_h is the concentration of protein soluble in 10% TCA after hydrolysis;

C_s is the concentration of protein soluble in 10% TCA before hydrolysis;

C is protein concentration of substrate solution before hydrolysis.

The concentration of the free amino groups was determined according to Kuchroo et al. [21]. The hydrolysate was diluted with 0.1M borate buffer to the final volume 2 ml and mixed with 50 µl of TNBS reagent (0.03 M). The samples were incubated in the dark for 2 h at room temperature. The reaction was stopped by adding 2 ml of 0.1 M sodium phosphate containing 1.5 mM sodium sulfate and the absorbance was measured at 420 nm. A blank was prepared with water instead of hydrolysate. The results were expressed as µmol Gly/g by reference to a standard curve prepared with defined concentrations of glycine.

Reversed - phase high performance liquid chromatography (RP-HPLC). Peptide separation was performed on Agilent system 1100 and 1200 equipped with diode-array detector (DAD) using a Zorbax XDB-C_{18} Agilent column (4.6 x 250mm) equilibrated with 0.1% TFA in water (trifluoroacetic acid) (phase A). The samples were diluted in phase A, applied on the column and eluted after 5 min analysis with an increasing percentage (2%/min) of phase B (0.1% TFA in acetonitrile). The operative conditions were: injection volume 100 µl; analysis time 55 min; T=30°C; flow rate 1 ml/min. The absorbance of the eluents was monitored at 230 nm.

Scavenging of free radicals activity. Antioxidant activity of obtained hydrolysates was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity according to Yen and Chen [22], with modifications. The tested samples were diluted in water to a final volume of 1 mL and mixed with 1 mL of ethanol (98%). The reaction was started by adding 0.5 mL of 0.3 µmol/L DPPH in ethanol. The mixtures were

left for 30 min at room temperature and the absorbance of the resulting solution was measured at 517 nm. Aqueous solutions of known Trolox concentration ranged from 2 to 20 μg (able to scavenge 500 μL of 0.3 mmol/L DPPH radical solution) was used for calibration. Radical scavenging activity of the peptides was expressed as μmol $Trolox_{eq}$/mg protein.

FRAP activity. The Ferric Reducing Antioxidant Power (FRAP) method was used to determine the antioxidative capacity in the hydrolysates according to Benzie and Strain [23]. 3 mL of FRAP working solution (300 mmol/L acetate buffer pH 3.6; 10 mmol/L 2,4,6,tripyridyl-s-triazine (TPTZ); 20 mmol/L $FeCl_3$ x 6 H_2O (10:1:1 v/v)) was mixed with a 1 mL sample. After 10 min of reaction, the absorbance was measured at 593 nm. Aqueous solution of known Fe (II) concentration was used for calibration (in a arrange of 100 to 1000 μg). Results were expressed as μg Fe^{2+}/mg protein.

Determination of Fe (II) ion chelation. Chelation of iron ions by hydrolysates was estimated by the method of Xu et al.[8], with modifications. A 250 μL sample was mixed with 1250 μL H_2O and 110 μL 1mmol/L $FeCl_2$. After 2 min, 1 mL of 500 μmol/L ferrozine aqueous solution was added and the mixture was allowed to react for 10 min. The absorbance of ferrous iron-ferrozine complex was measured spectrophotometrically at 562 nm. A known concentration of $FeCl_2$ (0-20 μg) was used as a standard curve and the ability to chelate iron ions was expressed as μg Fe^{2+}/mg protein.

Antimicrobial activity. The strains *B. subtilis B172, B. subtilis B3; B. cereus B512; B. cereus B 3p and B. laterosporum B6* were grown in tryptic soy broth (TSB) medium at 37°C for 18 h. The inocula were diluted to a final concentration of approximately $1x10^5$ CFU/ml inn the nutrient plates with TSB. Each bacterial suspension was spread over the surface of tryptic soy agar (TSA) as a thin film. Then the sterile of 1-cm-diameter wells were placed and loaded with 50 μl of hydrolysate with a concentration of 2 mg/ml. The plates were incubated at 37°C for 24 h and the growth inhibition zones were observed [24].

Data 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 Statistica v. 9.0.

RESULTS AND DISCUSSION:

The YP was obtained as the by-product of egg lecithin extraction [15]. YP was composed of about 10% lipid and 60% protein (mainly phosvitin). Egg yolk phosvitin has been recognized to have strongly antioxidant protein by chelating iron ions, owning to its highly phosphorylated form [8,12]. Therefore alkaline dephosphorylation of YP was performed until hydrolysis. YP after dephosphorylation didn't exert antioxidant and antimicrobial activity (data not shown). To evaluate biological activity, YP obtained during the course of lecithin isolation, was hydrolyzed with digestive enzymes. The progress of hydrolysis was monitored by determining the degree of hydrolysis (DH, %) (Fig.1) and the concentration of free amino-groups (Fig.2). The RP-HPLC peptide profiles of hydrolysates were also performed (Fig.3). Degree of hydrolysis is an im-

portant parameter in the enzymatic modification of proteins and might be a factor controlling the composition of the modified proteins [25]. In many cases a relationship between the DH and the biological activity of protein hydrolysates was observed [26]. The antioxidant activity of the protein hydrolysates also depends on the protein substrate and the specifity of the enzyme. Therefore YP hydrolysis was performed using trypsin, chymotrypsin and pepsin.

As shown in Figure 1, the degree of hydrolysis (DH) for trypsin and chymotrypsin increased rapidly in the first 0.5 h, followed by a slower rate increase up to 4h.

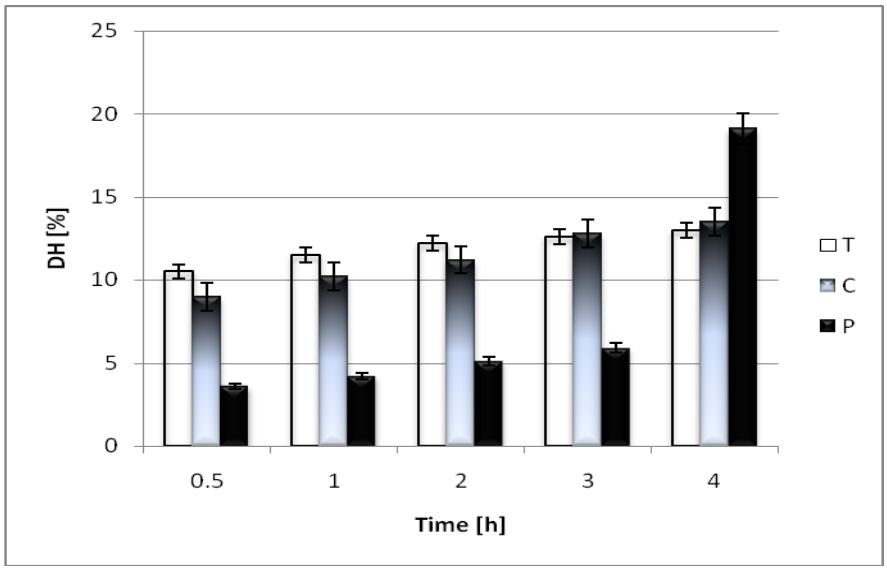

Figure 1. The degree of hydrolysis (DH) of YP treated by trypsin (T), chymotrypsin (C) and pepsin (P)

In our case, pepsin was the most effective in the degradation of YP. After 4h hydrolysis DH reached of: 19.1 %. While YP was more resistant for trypsin (13.0 %) and chymotrypsin (13.5 %) than for pepsin. The greatest increase in free amino group concentration was also observed for pepsin. The level of free amino groups determined in the 4h hydrolysate was: 1410.3 μ molGly/g. The final concentrations of free amino groups in chymotryptic and tryptic hydrolysates reached of 1252.0 and 1105.1 μmol Gly/g, respectively (Figure 2).

The more extensive hydrolysis of YP with pepsin may be attributed to its broad specificity to produce small- size peptides and free amino acids. Whereas trypsin and chymotrypsin cleave peptide bonds at the interior of the polypeptides chains.

Chymotrypsin hydrolyzes peptide bonds with aromatic amino-acid residues at the P1 position with high efficiency as well as peptide bonds with Leu and Met at P1, but at much lower efficiency. Trypsin exhibits affinity only to basic amino-acid residues such as Lys and Arg [28].

The RP-HPLC chromatograms of the enzymatic hydrolysates of YP after 0.5h of hydrolysis were characterized by numerous peaks eluted from 5 to 25 minutes of analysis (Fig. 3). Whereas peaks weren't found at indicated retention time in the chromatogram of substrate. With prolonged hydrolysis time, peaks eluted from 5 to 25 minutes of analysis, were slightly enhanced and confirmed slow incensement of the degree of hydrolysis at the time above 0.5 h. The

different peptide profiles of the degraded YP resulted from the unique specify of the investigated proteolytic enzymes.

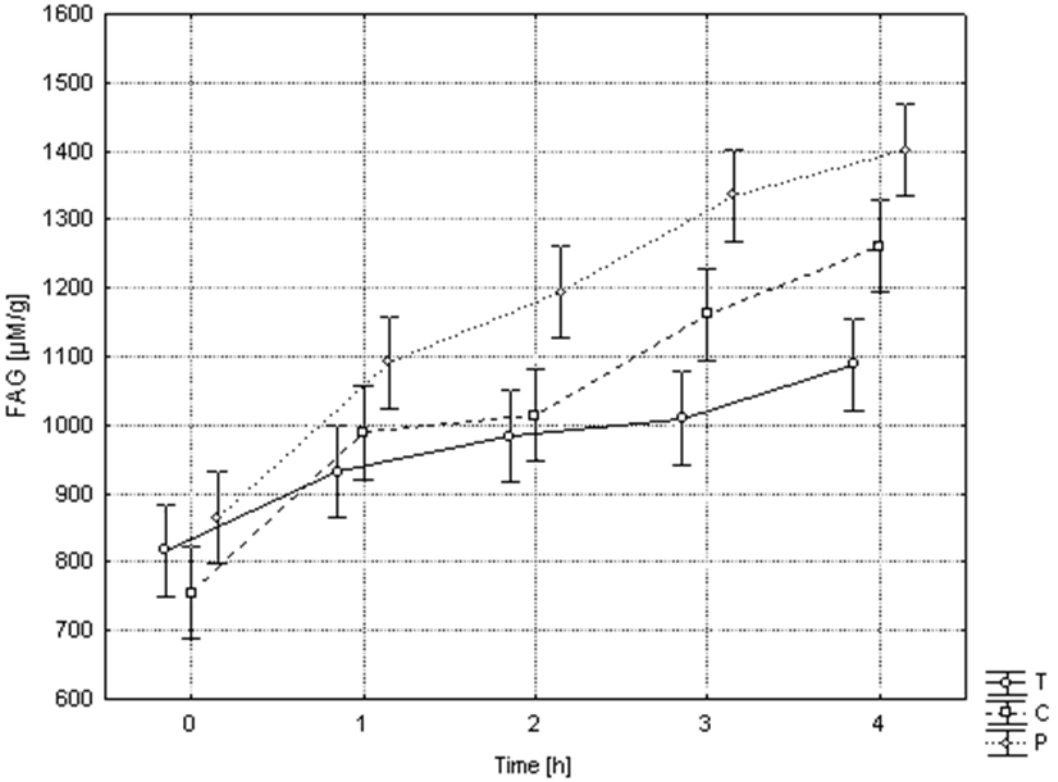

Figure 2. The content of free amino acid groups (FAG) of YP hydrolysates treated by trypsin (T), chymotrypsin (C), pepsin (P)

The peptides released from egg-yolk proteins by action of different proteases (trypsin, orientase and proteases from *Bacillus ssp* and *Aspergillus melleus*) have been demonstrated to exhibit *in vitro* antioxidant activity [3,4,8, 29]. The antioxidants may have different properties such as reactive oxygen species scavenging, inhibition of the generation of free radicals, chain breaking activity and metal chelation [1]. Therefore the antioxidant properties of the YP hydrolysates were evaluated based on their free radical (DPPH) scavenging activity (Fig. 4.), ferric reducing power (FRAP) (Fig. 5) and chelating activity on iron (II) (Fig. 6).

Long-time (4h) hydrolysis with each enzymes lead to obtain higher free radical scavenging activity than partial hydrolysis of YP. The hydrolysate treated by trypsin showed the highest level of free radical DPPH scavenging activity, amounting to 0.85 µmol $Trolox_{eq}$/mg during the 4h hydrolysis. Whereas chymotryptic and peptic hydrolysates showed a lower free radical DPPH scavenging activity than tryptic hydrolysate, reaching of 0.70 and 0.30 µmol $Trolox_{eq}$/mg, respectively (Fig.4).

Free radical DPPH scavenging activity of tryptic and chymotryptic hydrolysates increased obviously with increasing hydrolysis time up to 1h of reaction, and then leveled off during the next 3h. On the contrary, free radical DPPH scavenging activity of peptic hydrolysates was increased significantly during the last hour of hydrolysis, reaching of: 0.32 µmol $Trolox_{eq}$/mg.

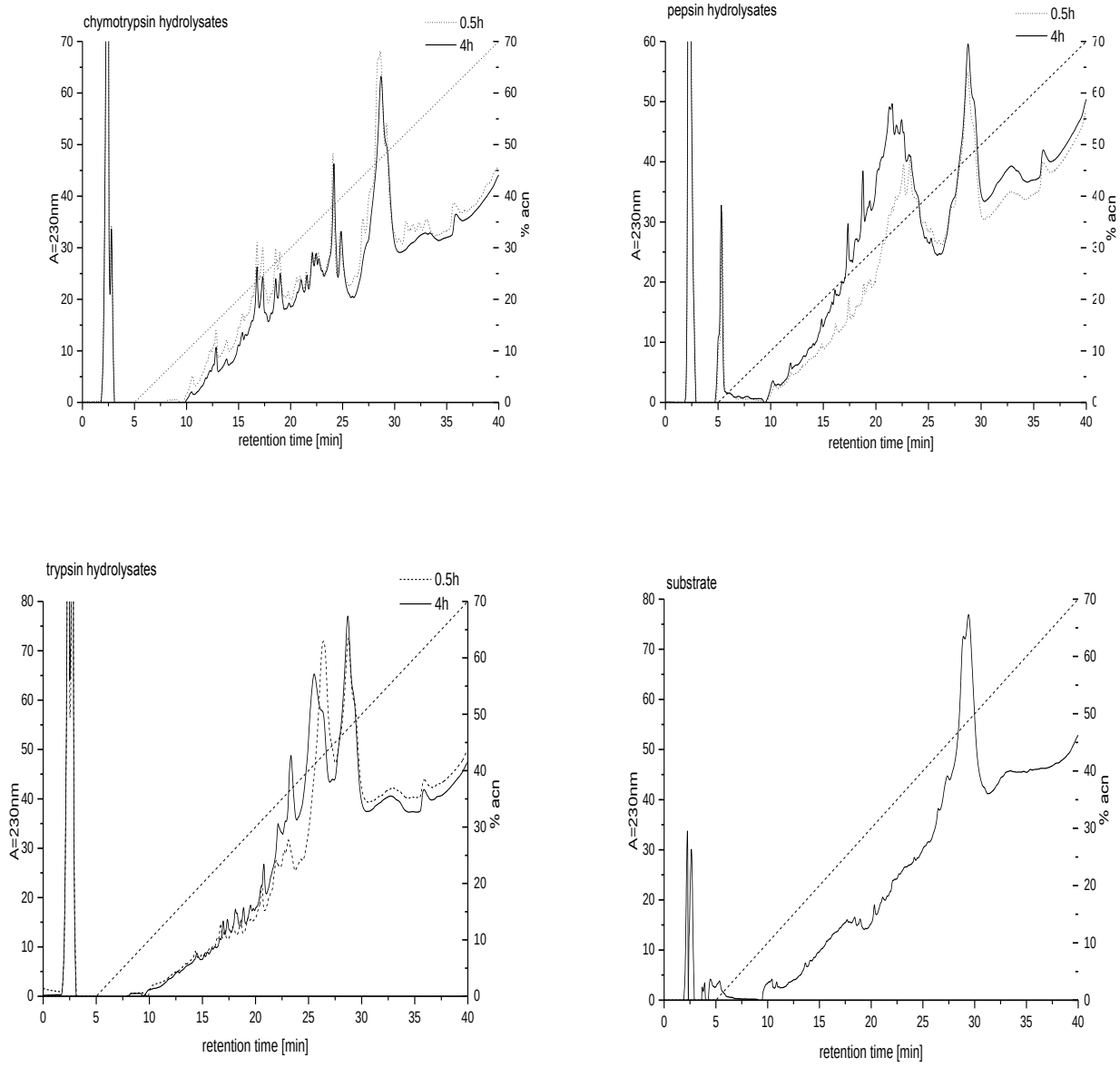

Figure 3.RP-HPLC chromatogram of YP hydrolysates after 0.5 and 4h of hydrolysis treated by trypsin, chymotrypsin and pepsin.

Our results confirmed observations that trypsin is an effective enzyme in producing antioxidant peptides able to scavenge free radicals. In a previous study we showed that the degradation of egg white protein precipitate with trypsin generates peptides which possessed stronger free radical (DPPH) scavenging activity than hydrolysates of these proteins treated by chymotrypsin and elastase. The free radical DPPH scavenging activity of trypsin, chymotrypsin and elastase hydrolysates reached of: 3.8, 3.3 and 2.1 μmol $Trolox_{eq} \times 10^{-2}$/mg, respectively [19]. Sakanaka and Tachibana (2006) reported that hydrolysates prepared by sequential hydrolysis of egg yolk conducted with first orientase and then with protease from *Bacillus* ssp. showed strong antioxidative activity. At 0.5% of the hydrolysates, DPPH and hydroxyl radical scavenging activities were: 74.2% and 91.7%, respectively.

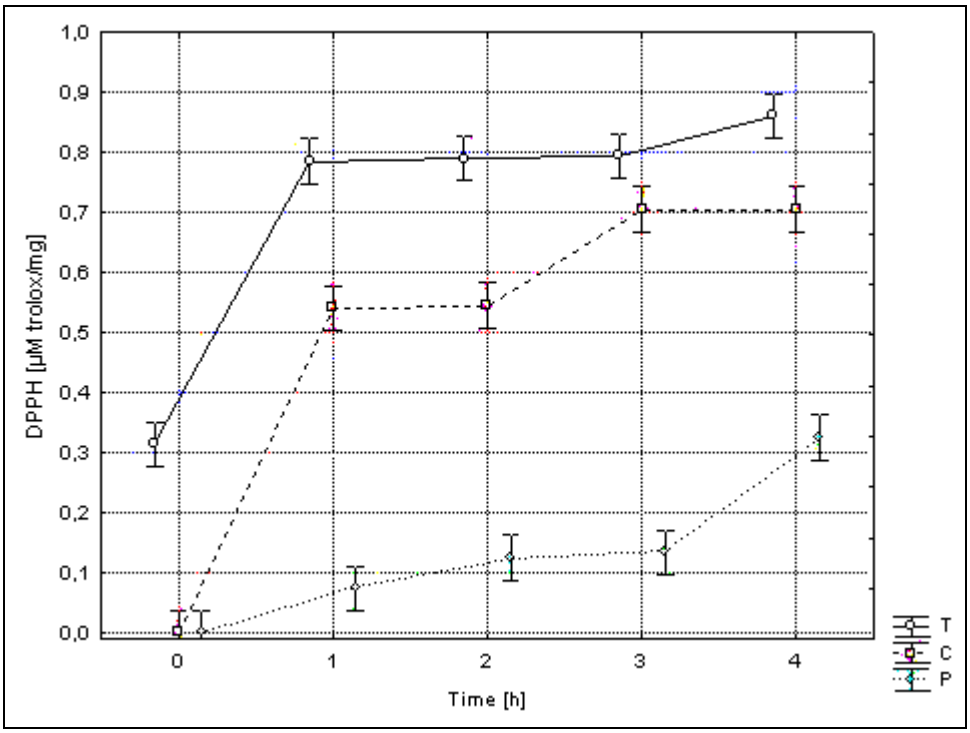

Figure 4. DPPH radical scavenging activity of YP hydrolysates treated by trypsin (T), chymotrypsin (C) and pepsin (P).

As shown in Fig. 5, hydrolysates of YP treated by chymotrypsin possessed significantly higher reducing power than tryptic and peptic hydrolysates. No significant relationship between DH and the levels of ferric reducing and chelating activities was observed. Reducing power of chymotryptic hydrolysates increased slightly (6.7-6.8 µg Fe^{2+}/mg) with increasing hydrolysis time up to 1 h of reaction, and then decreased during the next 1h. Up to 4h incubation the reducing power of chymotryptic hydrolysate increased, reaching: 7.5 µg Fe^{2+}/mg. The same trend was also observed for tryptic hydrolysate. In opposite, ferric reducing power of peptic hydrolysate was reduced significantly (4.4- 3.1 µg Fe^{2+}/mg) from 0.5h to 1h of hydrolysis and then increased slightly afterwards. However, ferric reducing power of peptic hydrolysate was stronger (4.4 µg Fe^{2+}/mg) during 0.5 h of hydrolysis than ferric reducing power of the final (4 h) peptic hysrolysate (3.8 µg Fe^{2+}/mg). (Fig.5). Similar results obtained Xia et al.[30], who evaluated the antioxidant properties of the barley glutelin hydrolysates. The increase or decrease in ferric reducing power for protein hydrolysates may be related to the exposure of electron –dense amino acid side chain groups, such as polar or charged moieties during hydrolysis [30]. In our previous study, hydrolysates from phosvitin protein preparation treated by trypsin and protease from *A. melleus* exhibited much greater ferric reducing power with values of 24.0 and 31.3 µg Fe^{2+}/mg, respectively [29].

The antioxidant properties of the YP hydrolysates were evaluated based also on their iron ions chelation effect. Egg yolk phosvitin has been recognized to have strongly antioxidant protein by chelating iron ions, owning to its highly phosphorylated form [8,12]. Therefore alkaline dephosphorylation of YP was performed until use.

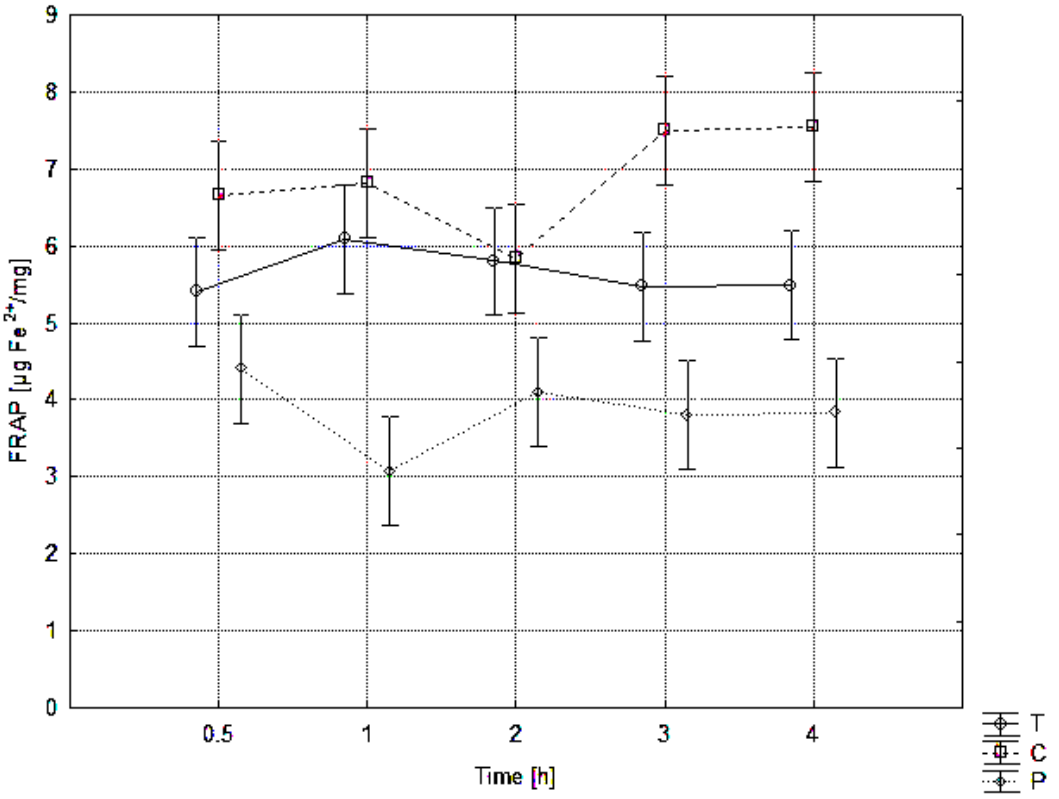

Figure 5. Ferric reducing power of YP hydrolysates treated by trypsin (T), chymotrypsin (C) and pepsin (P)

In Fig. 6., the iron ions chelating capacity of peptic hydrolysates was low (5.0- 12.0 µg Fe^{2+}/mg) and influenced by hydrolysis time.

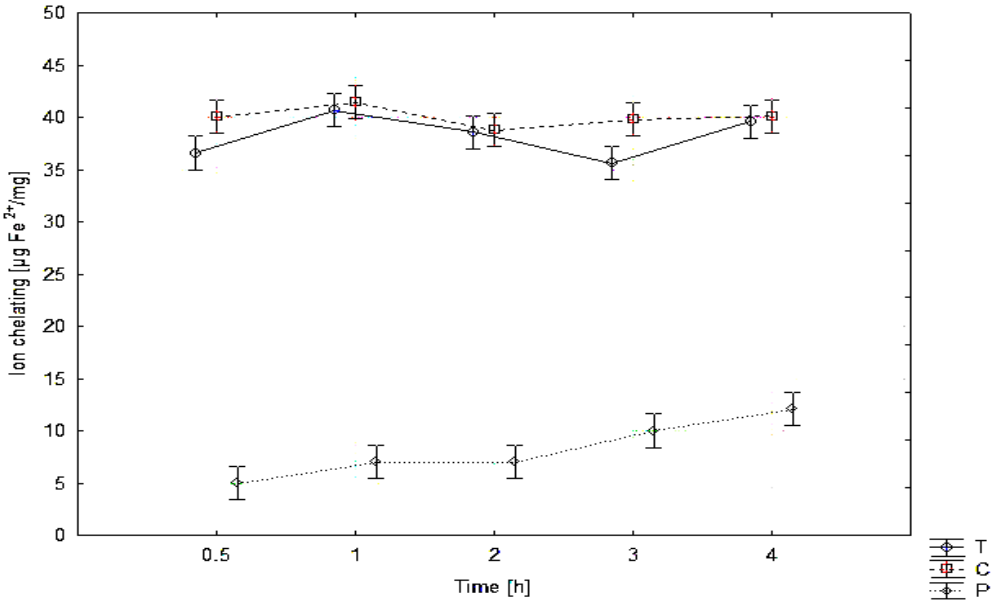

Figure 6. Ferrous ion chelating activity of YP hydrolysates treated by trypsin (T), chymotrypsin (C) and pepsin (P)

On the other hand iron ions chelating capacity of tryptic and chymotryptic hydrolysates increased dramatically up to 0.5 h of hydrolysis, then the level of iron ions chelating capacity was almost constant with prolonged hydrolysis time. The maximum iron ions chelating capacity were: 40.1 and 39.8μg Fe^{2+}/mg for chymotryptic and tryptic hydrolysates after 4 h of hydrolysis, respectively (Fig.6).

The above results demonstrated that the type of enzyme used is a key factor in determining antioxidant activity of YP hydrolysates. YP hydrolysates treated by alkalaine endopeptidases (trypsin and chymotrypsin) exhibited significantly higher antioxidant capacity than peptic hydrolysates in most of the selected assays. The antimicrobial activity was also determined for hydrolysates of YP treated by chymotrypsin, trypsin and pepsin (Tab.1).

Table 1. Antimicrobial activity of lecithin free egg yolk protein hydrolysates

Enzyme	Time [h]	Inhibition zones diameters[mm]				
		B. subtilis B 172	*B. subtilis B3*	*B. cereus B 3p*	*B.cereus B 512*	*B. laterosporum*
Trypsin	0.5	-	-	-	-	-
	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	1	1	1	1
Chymotrypsin	0.5	-	-	-	-	-
	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
Pepsin	0.5	-	-	-	-	-
	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-

The influence of enzymatic hydrolysates on microbial growth was estimated towards food-spoilage bacillus strains: *B. subtilis B172, B. subtilis B3; B. cereus B512; B. cereus B 3p and B. laterosporum B6.* The antimicrobial activity was noted when the 4-hour tryptic hydrolysate was applied against *Bacillus* species. It caused the inhibition growth zones reached 1 mm diameters. Only *B. subtilis B172* was resistant to the action of this hydrolysate. Although a number of egg-white derived peptides have demonstrated antimicrobial activity, there is no information about egg-yolk derived peptides with potentially antimicrobial activity [12].

CONCLUSION:

The free radical scavenging, ferric reducing and chelating on iron activities assays confirmed that hydrolysates of egg-yolk protein preparation obtained during the course of lecithin isolation may

be a interesting source of natural antioxidants. The hydrolysates of egg yolk protein preparation treated by trypsin and chymotrypsin demonstrated significantly stronger free radical DPPH scavenging activity and iron ions chelating capacity than peptic hydrolysates. The tryptic hydrolysate of YP showed the great free radical DPPH scavenging activity with an value of 0.85 μmol $Trolox_{eq}$/mg after 4 h of hydrolysis Short term hydrolysis (0.5h) of YP by trypsin and chymotrypsin lead to obtain hydrolysates demonstrated iron ion cheating activity with an value of: 37.1 and 40.1 for tryptic and chymotryptic hydrolysates, respectively. Furthermore hydrolysate of YP treated by trypsin possess weak antimicrobial activity after 4h of hydrolysis. The present study indicates that enzymatic hydrolysates of egg-yolk protein preparate could be used as food ingredients aiming to enhance the antioxidant properties of functional food and as natural preservatives inhibiting microbial growth.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: Dr. Zambrowicz, Miss Pokora and Miss Eckert are the principal investigators for the study, provided experiments and were involved in the writing of this manuscript. Dr Szołtysik and Dr Dąbrowska provided additional analysis. Prof. Chrzanowska and Prof. Trziszka were consultants in the field of conducted research.

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REFERENCES:

1. Adebiyi AP, Adebiyi AO, Ogawa T, Muramoto K. Purification and characterization of antioxidative peptides from unfractionated rice bran protein hydrolysates. *International Journal of Food Science and Technology* 2008:43:35-43.
2. Ranathunga S, Rajapakse N, Kim SK. Purification and characterization of antioxidative peptide derived from muscle of conger eel (*conger myriaster*). European Food Research and Technology 2006;222:310-315.
3. Sakanaka S, Tachibana Y. Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates. *Food Chemistry* 2006;95:243-249.
4. Sakanaka S, Tachibana Y, Ishihara N, Juneja LR. Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. *Food Chemistry* 2004;86:99-103.
5. Rossini K, Norena CPZ, Cladera-Olivera F, Brandelli A. Casein peptides with inhibitory activity on lipid oxidation in beef homogenates and mechanically debonded poultry meat. *LWT Food Science and Technology* 2009;42: 862-867.
6. Yoshie-Stark Y, Wada Y, Schott M, Wäsche A. Functional and bioactive properties of rapeseed protein concentrates and sensory analysis of food application with rapeseed protein concentrates. *Food Science and Technology* 2006;39:503–512.

7. Gibbs BF, Zougman A, Masse R, Mulligan C. Production and characterization of bioactive peptides from soy hydrolysate and soy–fermented food. *Food Research International* 2004; 37:123–131.
8. Xu X, Katayama S, Mine Y. Antioxidant activity of tryptic digests of hen egg yolk phosvitin. *Journal of the Science of Food and Agriculture* 2007;87:2604-2608.
9. Davalõs A, Miguel M, Bartolome B, López–Fandiño R. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection* 2004;67:1939-1944.
10. Park PJ, Jung WK, Nam KS, Shahidi F, Kim SK. Purification and characterization of antioxidative peptides from protein hydrolysate of lecithin-free egg yolk. *Journal of the American Oil Chemist's Society* 2001;78(6):651-656.
11. Matoba N, Usui H, Fujita H, Yoshikawa M. A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in a isolated SHR mesenteric artery. *FEBS Letters* 1999;452: 181-184.
12. Mine Y, Kovacs-Nolan J. New insights in biologically active proteins and peptides derived from hen egg. *World's Poultry Science Journal* 2006;62(1):87-95.
13. Ibrahim HR, Sugimoto Y, Aoki T. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochimica et Biophysica Acta* 2000;1523:196-205.
14. Pellegrini A, Thomas U, Wild P, Schraner E, von Fellenberg R. Effect of lysozyme or modified lysozyme fragments on DNA and RNA synthesis and membrane permeability of *Echerichia coli. Microbiological Researc*h 2000;155:69-77.
15. Siepka E, Bobak Ł, Trziszka T. Frakcjonowanie żółtka w celu pozyskiwania preparatów wzbogaconych w substancje biologicznie aktywne. *Żywność Nauka Technologia Jakość* 2010;6(73):158-167. (in polish).
16. Chrzanowska J, Kołaczkowska M: Production of extracellular proteolytic enzymes by *Beauveria bassiana*. *Acta Mycologica* 1998;33: 277-285.
17. Kakade ML, Swenson DH, Liener IE. Note on the determination of chymotrypsin and chymotrypsin inhibitor activity using casein. *Analytical Biochemistry* 1970;33:255–258.
18. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin-phenol reagent. *The Journal of Biological Chemistry* 1951;193:265-275.
19. Graszkiewicz A, Żelazko M, Trziszka T. Application of pancreatic enzymes in hydrolysis of egg-white proteins. *Polish Journal of Food Nutrition and Science* 2010;60(1):57-61.
20. Silvestre MPC. Review of methods for the analysis of protein hydrolysates. *Food Chemistry* 1997;60:263-271.
21. Kuchroo CN, Ramilly J, Fox PF. Assessment of proteolysis in cheese by reaction with trinitrobenzene sulphonic acid. *Irish Journal of Food Science and Technology* 1983;7:129-133.
22. Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagencity. *Journal of Agricultural and Food Chemistry* 1995;43:27-32.
23. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": the FRAP assay. *Analytical Biochemistry* 1996;293:70-76.
24. Liepke C, Zucht HD, Forssmann WG, Ständker L. Purification of novel peptide antibiot-

ics from human milk. *Journal of Chromatography B* 2001;752:369-377.

25. Ge SJ, Zhang LX. Control of hydrolysis of a protein modification with immobilized protease by the pH-Drop method. *Acta Biotechnologica* 1993;13(2):151-160.
26. Mullally MM, Meisel H, Fitz-Gerald RJ. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey protein. *International Dairy Journal* 1997;7:299-303.
27. Miguel M, Recio I, Gomez-Ruiz JA, Ramos M, Lopez-Fandino R. Angiotensin -I-converting enzyme inhibitory activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection* 2004;67,1914-1920.
28. Żelazko M, Chrzanowska J, Polanowski A. Pancreatic proteinases species diversity and the appending feeding and biotechnological implications. *Biotechnology* 2007;1:107-120.
29. Xia Y, Bamdad F, Gänzle M, Chen L. Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis. *Food Chemistry* 2012;134:1509-1518.
30. Zambrowicz A, Trziszka T. Aktywność przeciwutleniająca enzymatycznych hydrolizatów preparatu foswitynowego otrzymanego z jaj kurzych. *Acta Scientarum Polonorum Biotechnologia* 2010;9(3):27-36