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APPLICATION OF MICROBIAL PROTEASES TO OBTAIN EGG YOLK PROTEIN HYDROLYSATES WITH ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY

S u m m a r y

Natural antioxidants with high safety and long-term effect are subject of many studies because they are an alternative to chemical antioxidants which, in larger quantities, can be toxic. One type of natural antioxidants may be food- derived peptides.

The aim of this study was to obtain antioxidant and antimicrobial peptides from egg yolk phosvitin and immunoglobulin Y (IgY) with participation of microbial proteinases from *Bacillus amyloliquefaciens* (neutrase), *B. thermoproteolyticus Rokko* (thermolysin), *Streptomyces griseus* (pronase) and *Aspergillus melleus*. The progress of hydrolysis was monitored by the degree of hydrolysis (DH) and free amino groups concentration measurement. The resulting hydrolysates were subjected to an assessment of their ability to reduce the oxidation state of metal ions, scavenging of 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) free radicals and chelating iron ions.

The highest degree of hydrolysis of both proteins was obtained during the reaction with proteinase from *S. griseus*. The highest level of reduction in the oxidation state of iron ions was observed in IgY 24-hour hydrolysates obtained with the participation of enzyme from *B. thermoproteolyticus Rokko* (409.7 μg Fe^{2+}/mg). However, the 24-hour hydrolysates of IgY obtained after degradation with the proteinase from *A. melleus*, possessed the highest free radical scavenging activity equal to1.46 μM trolox/mg. The highest activity of chelating iron ions, equal to 891.64 μg Fe^{2+}/mg, was observed for products obtained during the 24-hour hydrolysis of phosvitin with the participation of protease from *B. thermoproteolyticus Rokko*.

Słowa kluczowe: egg-yolk proteins, hydrolysis, peptides, microbial proteases, activity

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Introduction

Egg yolk is extremely rich in active substances, for example phosvitin, IgY, phospholipids or vitamins which are used in various industries, including pharmaceutical, cosmetics and food industry [24]. Phosvitin, representing 25 % of high-density lipoprotein (HDL) in granular fraction and IgY - the main protein of plasma λ-livetin fractions in egg yolk - has particularly interesting biological properties [24]. Phosvitin, due to the unique amino acid composition (more than 55 % of the amino-acids are serine residues) and high content of phosphorus, is capable to complex numerous metal ions, such as P^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{3+} [16]. Owing to this chelating property, phosvitin shows strong antioxidant and antimicrobial activities [2]. Phosvitin is also considered a factor in preventing diseases caused by oxidative stress, such as colon cancer or Alzheimer's disease [2, 16]. The main task of IgY, present in amounts 8-20 mg/ml in egg yolk, is to provide effective humoral immunity in the offspring against the most common avian pathogens until the iremergence in their own immune system. Furthermore, IgY allows the treatment of certain bacterial and viral diseases in farm animals when conventional treatment fails [28].

Recently, a significant development of studies on enzymatic hydrolysis (*in vitro*) causing release of bioactive peptides from food proteins can be reported [16, 25]. Most enzymatic modifications of dietary proteins are carried out with the participation of enzymes of animal, plant and microbial origin. Microbial proteases are preferred due to their high activity, broad spectrum of activity and low price [23]. Bioactive peptides exert diverse biological activities, such as immunostimulating, antimicrobial, antioxidant, opioid, antihypertensive and anticancer [5]. As natural components of food or nutraceuticals, peptides gain more interest, because they can modulate the physiological functions of organism and prevent or treat diseases [10, 29].

The most chronic changes and the pathological conditions of the body (cardiovascular diseases, degenerative changes) result from the activity of free radicals [5]. Furthermore, the constantly increasing concentration of free radicals results in accelerated aging of the body.

Therefore, antioxidants, especially of natural origin, for example food-derived peptides are greatly appreciated at present. Antioxidant peptides may be released from numerous plant and animal origin proteins, such as whey protein, peanut kernels, rice bran or milk casein, mackerel, egg yolk and white [20]. It has been shown that peptides derived from egg white obtained after the isolation of lysozyme and cystatin exhibit antioxidant activity against free radicals, 2,2 - diphenyl-1-picrylhydrazyl (DPPH) [4]. It has been reported that egg-yolk hydrolysates exhibit antioxidant capacities in a linoleic acid oxidation system [18]. DPPH scavenging activity and suppression of discoloration of β-carotene have also been observed [18, 19]. The hydrolysis of egg yolk protein phosvitin with trypsin also leads to obtain a peptide fraction with an ability to in-

hibit the oxidation of linoleic acid, DPPH free radical scavenging and chelating iron ions (II) [27].

Antioxidant properties of peptides often translate into a reduction of the risk of cancer. Ishikawa et al. [7], demonstrated that the consumption of egg yolk protein hydrolysates inhibits tumor cell proliferation in the colon. Studies have shown that this effect results primarily from an improvement of antioxidant protective systems in the mucosa of the colon. This can result from the fact that phosphooligopeptides from phosvitin have the ability to modulate the secretion of antioxidant enzymes such as catalase and glutathione reductase [7]. Moreover, it was demonstrated that these phosphooligopeptides have the ability to increase the activity of intracellular GSH and regulate the expression of γ-glutamylocysteine in intestinal epithelial cells, which catalyzes the synthesis of GSH [9].

Peptides showing a variety of properties are known as multifunctional [20]. For example, the study of Liu et al. [13] showed that peptides derived from hen egg lysozyme have the ability to neutralize reactive oxygen species (ROS) and inhibit the growth of *Bacillus* bacteria. Ovoalbumin, with antimicrobial properties, is one of the precursors of peptides. Hydrolysis of this protein with trypsin leads to the release of the penta-, hexa- and octapeptides exhibiting strong bactericidal activity against *B. subtilis* [17].

Peptides of antibacterial activity may act in different ways in bacterial cells through the cytoplasmic membrane disorder to the effect on their metabolism. This is thanks to the specific amphipathicity structure of the peptides and the presence of amino acids such as arginine, lysine or histidine in their structures [5]. Peptides with antimicrobial activity may find new applications, such as components of innovative pharmaceuticals, or a complement in conventional antibiotic therapy [17, 20]. Food protein hydrolysates and their multifunctional peptide fractions may also serve as useful components in the formulation of functional food and nutraceuticals.

The aim of this study was to compare the ability of various microbial proteases to generate hydrolysates of egg yolk phosvitin and IgY exhibiting antioxidant and antimicrobial activity.

Material and methods

Fresh eggs were obtained from hens of Lohmann Brown lines. Substrates for hydrolysis: phosvitin and IgY were isolated from egg yolks by the Siepka et al. [21] method. Next, phosvitin was dephosphorylated [27]. Both phosvitin and IgY were dialyzed, lyophilized and stored frozen until use.

Enzymes: neutrase from *B. amyloliquefaciens*, pronase from *S. griseus* type XIV, thermolysin from *B. thermoproteolyticus rokko* type X and protease from *A. melleus* type XXIII were obtained from Sigma-Aldrich. Trinitro-benzene sulfonic acid (TNBS)

was 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.

Proteolytic activity of enzymes was determined in reaction with 1 % casein as the substrate in 0.1 M buffer TRIS-HCl of pH 8.1 [8]. The absorbance of the supernatants was measured at 280 nm. One unit of enzymatic activity of proteases (U) corresponded to that amount of enzyme which under reaction conditions gave an increase in absorbance at 280 nm of 0.1.

Protein concentration was determined according to the method of Lowry et al. [14]. A standard curve was prepared for bovine serum albumin (BSA) obtained from Sigma-Aldrich.

Enzymatic hydrolysis. Phosvitin and IgY were dissolved in the reaction buffer (0.1 mol/L TrisHCl pH 8.3) to a final concentration of 10.0 mg/ml. Hydrolysis was started by applying enzymes (20 U per 1 mg of substrates) and the reaction was carried out at 37 °C for 24 h. Then it was stopped by heating at 100 °C for 15 min. The samples were centrifuged and the supernatants were lyophilized.

The degree of hydrolysis (DH %) was expressed as the percentage ratio of protein soluble in 10 % trichloroacetic acid (TCA) to total protein content [22]. The concentration of acid-soluble product in the supernatant was measured spectrophotometrically at $\lambda = 280$ nm.

The concentration of free amino groups was determined with trinitro-benzene sulfonic acid (TNBS) reagent according to Kuchroo et al. [11]. The results were expressed as μmol Gly/g by reference to a standard curve prepared with defined concentrations of glycine.

RP- HPLC peptide profiles. The samples of hydrolysates were dissolved in the mobile phase A (1:1) and applied to a Zorbax XDB-C_{18} column (4.6 × 250 mm, Agilent). The operation conditions were as follows: flow rate: 1 ml/min, gradient: 2 % B/min, mobile phase A: 1 ml of trifluororacetic acid (TFA) per liter in bi-distilled water, phase B: 1 ml of trifluoroacetic acid (TFA) per liter in acetonitrile, temperature 30 °C, and retention time 5 min. The absorbed peptides were eluted by gradient phase B. The absorption of the eluents was monitored at 230 nm.

The antioxidant activity was determined by a modified method 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 after 30 min. incubation at the $\lambda = 517$ nm. The antioxidant activity of the 1 mg ml^{-1} protein solution was determined on the basis of the standard curve prepared for Trolox – synthetic antioxidant [29].

Ferric reducing activity. The ability of the hydrolysate to reduce the oxidation of iron Fe(III) to Fe(II) ions in reaction with TPTZ (2,3,5-triphenyltetrazolium chloride)

was determined. Absorbance measurement was made at λ = 593 nm. The concentration of Fe^{2+} ions in 1 mg/ml the protein solution was determined on the basis of the standard curve for $FeSO_4$ solution [1].

Chelation on iron ions was determined by colorimetric measurement of the quantity of Fe(II) not bound with the hydrolysate in the reaction mixture with the participation of ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate) [27]. 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.

The antimicrobial activity was determined by a modified method based on the diffusion test with filter discs [26]. Five strains of Gram-positive bacteria of the genus *Bacillus* (*B. subtilis B172 and B. subtilis B3, B512 of B. cereus, B. cereus B3p, B. laterosporum B6*) were used in the test.

Statistical analysis. All data were studied by analysis of variance (ANOVA) followed by Duncan's multiple range test using Statistical Analysis System Software (SAS version 10.0). Significance of differences was defined at the 5 % level ($p < 0.05$).

Results and discussion

In order to obtain peptides with potential antioxidant and antimicrobial activity, hydrolysis of egg yolk phosvitin and IgY was carried out. The reaction was performed with the use of commercial proteases of bacterial origin (*S. griseus*, *B. amyloliquefaciens*, *B. thermoproteolyticus rokko*) and one isolated from mould (*A. melleus*).These enzymes are widely used in food processing. Thermolysin is used for the enzymatic synthesis of the precursor of artificial sweetener – aspartame [12]. Neutrase obtained from *B. amyloliquefaciens* has been successfully applied to the hydrolysis of soy protein and animal skins [25].

The progress of phosvitin and IgY enzymatic degradation was monitored by determining the degree of hydrolysis (DH, %) (Fig. 1), the concentration of free aminogroups (Fig. 2) and the designation of the peptide profiles of RP-HPLC (Fig. 3). In all cases, the IgY was more resistant to proteolytic action of enzymes than phosvitin. Our results confirmed the observation of other authors that IgY is relatively resistant to digestion with proteolytic enzymes like: trypsin, chymotrypsin and pepsin [6]. However, its degradation seems to be highly dependent on the pH of the reaction. At pH 5 or higher, IgY retains almost all the properties of agglutination and antigen binding. However, at pH 4.5 or below, both of these features are lost.

The hydrolysis of egg yolk proteins with the microbial proteases was the most extensive during the first 60 min and then slowed down, indicating that maximum cleavage of peptides occurred within the first hour of reaction.

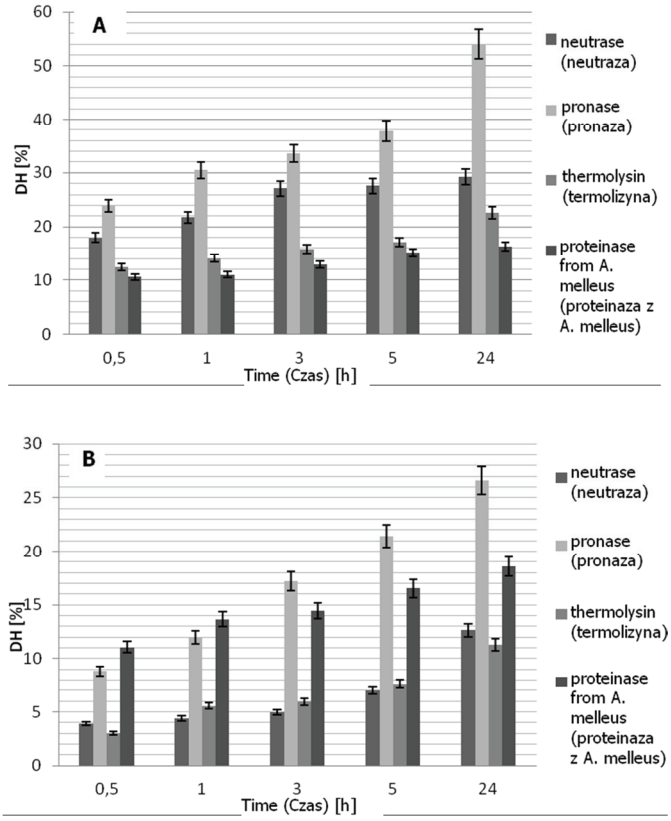

Fig. 1. Degree of hydrolysis DH [%] of phosvitin (A) and IgY (B), according to the reaction time.
Rys. 1. Stopień hydrolizy DH [%] foswityny (A) i IgY (B).

The most intensive degradation of phosvitin (DH = 54 % after 24 h) and Ig Y (DH = 26,6 % after 24 h) occurred using pronase from *S. griseus*. The highest ability of pronase to digest these proteins was also confirmed by the determination of free amino groups, the content of which, after 24 hours, amounted to 6131.96 μM/g and 2272.59 μM/g for phosvitin and IgY, respectively.

Pronase from *S. griseus* has an extremely broad substrate specificity. It is capable of hydrolyzing almost all peptide-bonds in protein until the majority of the amino acids constructing the protein are liberated as the respective free amino acids. Moreover, this enzyme may be able to hydrolyze not only the internal peptide-bonds of protein, but also the terminal peptide-bonds of oligopeptides, especially on the carboxyl side of substrates, including di- and tri-peptides [15].

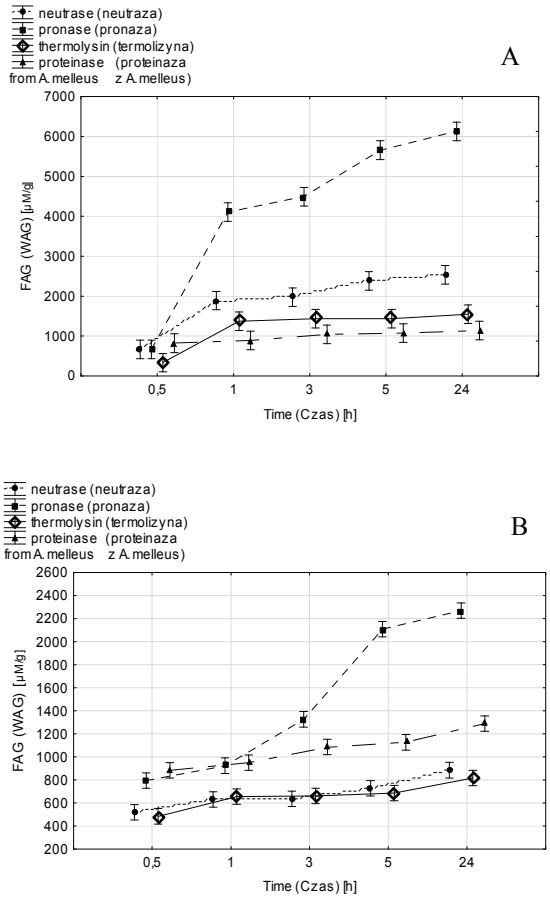

Fig. 2. Free amino groups content FAG [μM/g] in phosvitin (A) and IgY (B) hydrolysates according to the reaction time.

Rys. 2. Stężenie wolnych grup aminowych WAG [μM/g] w hydrolizatach foswityny (A) i IgY (B).

The lowest DHs of phosvitin and IgY were obtained during 24-hour degradation with the enzyme from *A. melleus* (16.24 %) and thermolysin from *B. thermoproteolyticus Rokko* (11.31 %), respectively (Fig. 1). The RP-HPLC peptide profiles of hydrolysates are shown on the graphs (Fig. 3). The differences in hydrophobicity of the enzymatic degradation products, which are strongly dependent on the substrate and the type of proteinase, were observed.

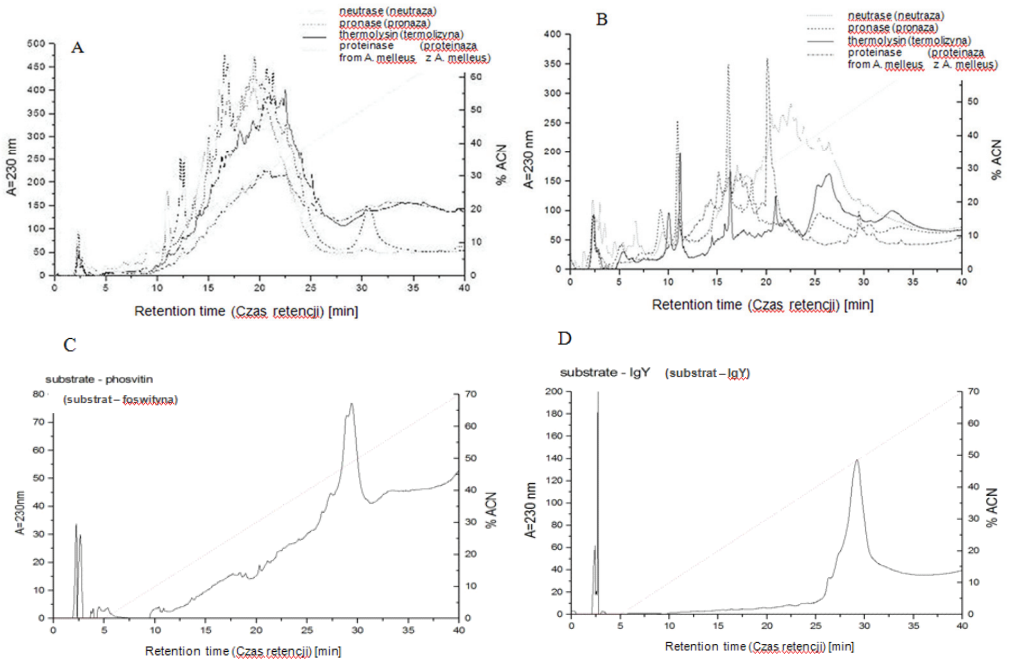

Fig. 3. The RP-HPLC peptide profiles of phosvitin (A) and IgY (B) hydrolysates after 24 h of reaction and substrates (C) phosvitin and (D) IgY.

Rys. 3. Profile peptydowe (RP-HPLC) 24-h hydrolizatów foswityny (A) i IgY (B) i substratów (C) foswityny i (D) IgY.

The antioxidant properties of hydrolysates were tested using three methods for identifying the free-radical scavenging activity with a stable DPPH radical (Fig. 4), ferric reducing activity (FRAP) (Fig. 5) and chelating activity on iron (II) (Fig. 6), because different mechanisms of action may be exercised by peptides. The relationship between the DH and the level of antioxidant activity in all variants of hydrolysates were observed. Both phosvitin and IgY hydrolysates obtained after 24 hour degradation exhibited higher antioxidant activity than these obtained after shorter time hydrolysis of that native proteins. Kong and Xiong [10] showed that the non-hydrolyzed proteins, due to their compact structure, have minimal antioxidant activity. Interactions of oxidants through the exposed active amino acids are only possible as a result of disorder of native protein structure by the action of the enzyme.

The highest free-radical scavenging activity was observed in 24-hour hydrolysates of IgY obtained with the participation of proteases from *B. amyloliquefaciens* (4.28 μM trolox/mg) and thermolysin from *B. thermoproteolyticusrokko* (4.20 μM trolox/mg)

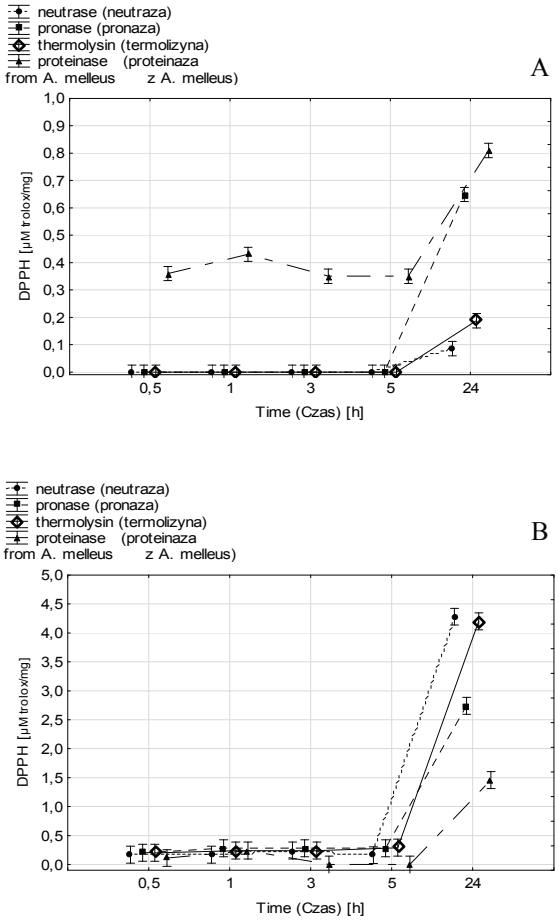

Fig. 4. Free-radical scavenging activity (DPPH) of phosvitin (A) and IgY (B) hydrolysates according to the reaction time.

Rys. 4. Zdolność do wygaszania wolnych rodników DPPH hydrolizatów foswityny (A) i IgY (B).

(Fig. 4). Phosvitin hydrolysates obtained during 5 hour reaction with bacterial proteases were unable to scavenge DPPH free radicals. The level of this activity of other degradation products ranged from 0.11 to 2.74 µM trolox/mg. Other authors suggested that DH may highly affect the antioxidant activity of the hydrolysates and that smaller peptides have a higher level of radical scavenging activity. Studies conducted by Sakanaka and Tachibana [19] also showed that hydrolysis of egg-yolk proteins with different proteases of bacterial origin produces hydrolysates exhibiting 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and hydroxyl radical-scavenging activity in food model systems [19].

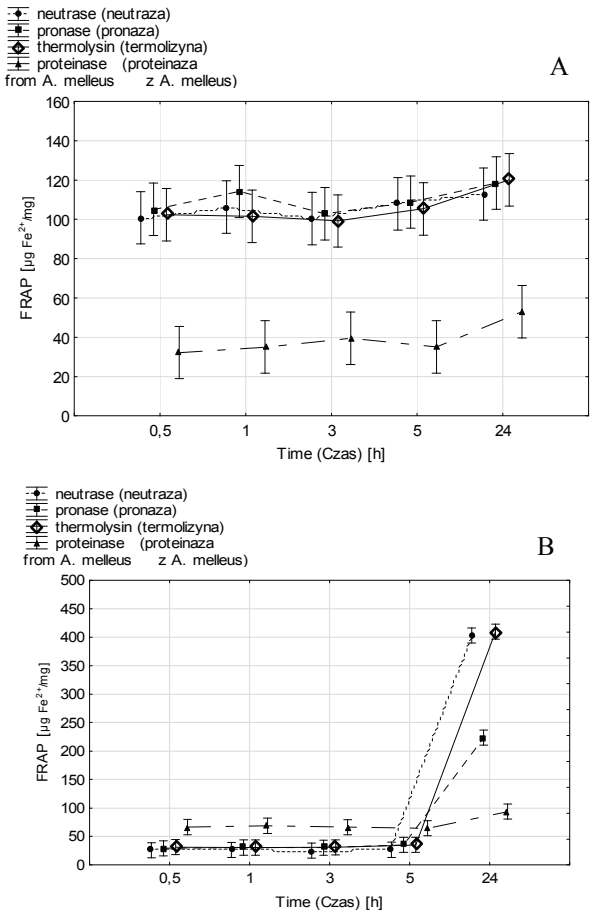

Fig. 5. Ferric reducing activity (FRAP) of phosvitin (A) and IgY (B) hydrolysates according to the reaction time.

Rys. 5. Zdolność do redukcji stopnia utlenienia jonów żelaza (FRAP) hydrolizatów foswityny (A) i IgY (B).

The ferric reducing activity of the obtained hydrolysates was also determined (Fig. 5). The highest level of this activity was exhibited by the most degraded IgY products obtained by the action of bacterial proteinases. In case of phosvitin, 24-hour hydrolysates obtained with the participation of proteases from *B. thermoproteolyticus rokko* were characterized by the highest ferric reducing activity (120 µg Fe^{2+}/mg).

This is in line with results obtained by other authors who reported that egg yolk proteins can be a source of peptides with antioxidant properties. In case of phosvitin hydrolysates, this effect is related to the high iron concentration, indicating that phosvitin and its peptides act as antioxidants by chelating iron ions [16]. In our study, high

ferrous ion chelating activity of phosvitin hydrolysates derived with the participation of thermolysin from *B. thermoproteolyticus rokko* (891.64 μg Fe^{2+}/mg) was observed. Similar results were obtained by Zambrowicz and Trziszka [30], who used the same enzyme to hydrolyze yolk protein preparation obtained as the by-product after isolation of lecithin.

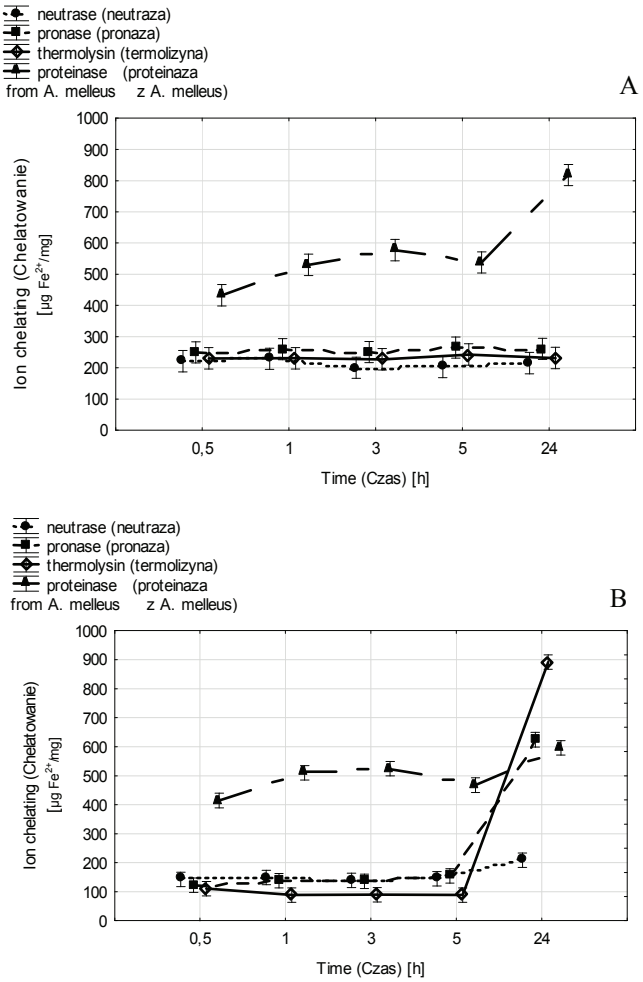

Fig. 6. Chelating activity on iron(II) ions of phosvitin (A) and IgY (B) hydrolysates according to the reaction time.

Rys. 6. Zdolność do chelatowania jonów żelaza(II) hydrolizatów foswityny (A) i IgY (B).

The results obtained by Xu et al. [27] also demonstrated that tryptic hydrolysis of phosvitin led to obtain peptides exhibited strong chelating iron ions activity. The high level of this activity was also observed in hydrolysates obtained after 24-hour degradation of IgY with proteinase from *A. melleus* (891.64 μg Fe^{2+}/mg) (Fig. 6). These studies

showed that IgY hydrolysates were even more effective antioxidants than phosvitin hydrolysates. However, so far there is no other information in the literature about the potential benefits resulting from hydrolysis of IgY.

IgY and phosvitin hydrolysate were also examined in terms of their antimicrobial activity. Studies confirm that the enzymatic hydrolysis of egg white proteins, such as ovalbumin, lysozyme or ovotransferrin increases their antimicrobial activity by exposing amino acids responsible for this activity [17]. IgY and phosvitin protect the developing embryo against potential pathogens in egg yolk [24]. For example, phosvitin exhibits antimicrobial activity against *B. subtilis* [17], which is the result of synergistic metal-chelating ability and high surface activity [2]. After oral administration, IgY antibody appeared effective against various enteric pathogens in animals and humans, such as rotaviruses, coronaviruses, *E. coli*, *Salmonella spp*, *Y. ruckeri*, *S. aureus* and *Pseudomonas* [28].

In our study, antimicrobial activity test was conducted against five strains of *Bacillus* bacteria. In food industry, this type of bacteria worsens the quality of many food products shortening their shelf life. It was also demonstrated that some of *B. cereus*, *B. subtilis* strains are responsible for food poisoning [3].

The results of our study have demonstrated that enzymatic degradation of both proteins leads to the loss of their antimicrobial activity. Other studies have also shown that IgY loses its activity as a result of hydrolysis with various proteases [6].

Conclusion

Hydrolysates of phosvitin and IgY obtained by enzymatic hydrolysis with four proteinases of microbial origin exhibited significant antioxidant activity. The greatest susceptibility to proteolysis was observed for phosvitin, but the most biologically active hydrolysates were obtained by enzymatic hydrolysis of IgY. The products of enzymatic degradation of both proteins can be used in food processing as natural preservatives, or as ingredients in functional food and nutraceuticals.

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ZASTOSOWANIE PROTEAZ POCHODZENIA MIKROBIOLOGICZNEGO DO OTRZYMYWANIA HYDROLIZATÓW O AKTYWNOŚCI PRZECIWUTLENIAJĄCEJ I PRZECIWDROBNOUSTROJOWEJ Z BIAŁEK ŻÓŁTKA JAJA

S t r e s z c z e n i e

Przeciwutleniacze naturalne są obiektem wielu badań ze względu na długotrwałą efektywność i bezpieczeństwo stosowania. Mogą one stanowić alternatywę syntetycznych przeciwutleniaczy, które są toksyczne dla organizmu. Do naturalnych przeciwutleniaczy zalicza się m.in. peptydy pochodzące z białek żywnościowych.

Celem pracy było otrzymanie peptydów o aktywności przeciwutleniającej i przeciwdrobnoustrojowej z foswityny i immunoglobuliny Y z wykorzystaniem enzymów proteolitycznych pochodzenia mikrobiologicznego, takich jak: proteaza z *Bacillus amyloliquefaciens* (neutraza), proteaza z *B. thermoproteolyticus Rokko* (termolizyna), proteaza ze *Streptomyces griseus* (pronaza) i proteaza z *Aspergillus melleus*. Postęp hydrolizy monitorowano przez oznaczenie stopnia hydrolizy (DH) i stężenia wolnych grup aminowych. Aktywność przeciwutleniającą otrzymanych hydrolizatów oznaczano jako zdolność do: redukcji stopnia utlenienia jonów żelaza, wygaszania wolnych rodników 2,2 difenylo-1-pikrylhydrazylowych i chelatowania jonów żelaza(II).

Zarówno foswityna, jak i IgY wykazały najwyższą podatność na działanie proteazy ze *S. griseus* (pronazy). Hydrolizat IgY otrzymany w wyniku 24-godzinnej reakcji z proteazą z *B. thermoproteolyticus Rokko* (neutrazą) charakteryzował się największą zdolnością redukującą (409,7 μg Fe^{2+}/mg). Natomiast najwyższy poziom zdolności wygaszania wolnych rodników DPPH, wynoszący: 1,46 μM trolox/mg uzyskano dla 24-godzinnego hydrolizatu IgY otrzymanego z udziałem proteazy z *A. melleus*. Największą zdolność do chelatowania jonów żelaza(II) wykazał hydrolizat uzyskany w wyniku 24-godzinnego działania proteazy z *B. thermoproteolyticus Rokko* na foswitynę (891,64 μg Fe^{2+}/mg).

Key words: białka żółtka, hydroliza, peptydy, proteazy pochodzenia mikrobiologicznego, aktywność