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ANTIOXIDATIVE CAPACITY OF HYDROLYSATES OF HEN EGG PROTEINS

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The antioxidant capacity as free radicals of 1,1 diphenyl-2-picryhydrazyl (DPPH) scavenger of egg white protein hydrolysate was investigated. Egg white protein precipitate obtained as a by-product in cystatin and lysozyme isolation was hydrolysed with bovine trypsin and then separated by means of RP-HPLC. Of ten fractions collected only no. 2 (0.195 μmol Trolox /mg) and no. 5 (0.186 μmol Trolox /mg) displayed a considerable free radical-scavenging capacity. The rechromatography of these fractions yielded four products of raised antioxidant activity: no. 2E (obtained from fraction no. 2) and no. 5E, 5F, 5H (obtained from fraction no. 5) which amounted to 0.482 μmol Trolox /mg and 0.584, 1.375, 1.200 μmol Trolox /mg, respectively.

INTRODUCTION

Free radicals are atoms or groups of atoms with release electrons aiming at stability which may be achieved by their coupling. They are highly reactive and harmful compounds. Free radicals may react with proteins, lipids and DNA, which may affect health quality of food products [Oszmiański & Zarawska, 1992; Ismail *et al*., 2004].

Antioxidants are chemical compounds, capable of inhibiting oxidative reactions and protecting human organism and food ingredients against the destructive activity of free radicals [Molyneux, 2003].

The most common preservatives used in the food industry are very efficient, synthetic antioxidants: butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). However they have been reported to exhibit a number of side effects [Park *et al*., 2005; Bo Li *et al*., 2007]. That is why it is advisable to replace them with substances safer for human organism. Many antioxidative substances have been isolated from natural materials including food [Sakanaka & Tachibana, 2006], especially from protein hydrolysates like those of soybean [Gibbs *et al*., 2004], milk [Sakanaka *et al*., 2004], myofibrillar [Saiga *et al*., 2003] and egg proteins [Davalos *et al*., 2004]. Therefore, peptides with these properties could be considered as alternative substances to the synthetic preservatives.

Attractive sources of antioxidant peptides seem to be both hen egg yolk and egg white protein hydrolysates [Tsuge *et al*., 1991; Davalos *et al*., 2004; Sakanaka *et al*., 2004, 2006]. It has been reported that egg-yolk hydrolysates exhibit antioxidant capacity in a linoleic acid oxidation system [Sakanaka *et al*., 2004]. The DPPH and hydroxyl radical-scavenging activity and suppression of discoloration of β-carotene have been observed as well. In food model systems egg-yolk protein hydrolysates effectively inhibited lipid oxidation processes in beef and tuna muscle homogenates [Sakanaka *et al*., 2006].

High antioxidant capacity has also been observed in egg-white protein hydrolysates derived in the course of the digestion process. Ovalbumin-derived peptide Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu, which had strong ACE inhibitory activity, also possessed high radical scavenging activity and delayed the low-density lipoprotein lipid oxidation induced by Cu^{2+} ions [Davalos *et. al*., 2004].

The aim of the present study was to obtain hydrolysates of egg-white proteins left during the course of isolation of cystatin and lysozyme [Sokołowska *et al*., 2007] and to determine their antioxidative properties.

MATERIAL AND METHODS

Materials. Egg-white protein precipitate was obtained in our laboratory as a by-product in lysozyme and cystatin isolation according to Sokołowska *et al.* [2007]. Bovine trypsin was received from the Faculty of Biotechnology, University of Wrocław. 1,1 Diphenyl-2-picryhydrazyl (DPPH) was purchased from Fluka, N-benzoylo-DL-arginine p-nitroanilide (BAPNA), molecular mass standard, picrylsulfonic acid 5% (TNBS), chloric acid VII acetonitrile, and trifluoroacetic acid (TFA) were bought in Sigma Chemicals Co.

Enzymatic activity. Trypsin activity was determined in a reaction with a synthetic substrate N-benzoylo-DL-arginine p-nitroanilide (BAPNA) according to Erlanger [1961]. One unit of enzymatic activity of trypsin (U) was defined as the amount of enzyme giving an increase in absorbance at 410 nm of 1.0 under reaction conditions.

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Protein assay. Protein concentration in a solution of pure protein was determined spectrophotometrically at $A_{280.}$ For a mixture of protein use was made of the formula $(A_{235}-A_{280})/2.51$ (mg/mL) [Whitaker & Granum, 1980]. For a mixture of peptides the formula $(A_{215}-A_{225})$ x 144 (μg/mL) was applied [Wolf, 1983].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [1970] in 10% (w/v) polyacrylamide gel. Gel was stained using 0.1% Coomassie Brilliant Blue R-250 for 6 h. Afterwards, the gel was distained with a solution containing 40% methanol in 10% acetic acid.

Enzymatic hydrolysis. The egg-white precipitate was dissolved in a reaction buffer (0.1 mol/L Tris-HCl with 0.2 mol/L $CaCl_2$, pH 8.3) to the final protein concentration of 1.5 mg/L. The hydrolysis was started by adding trypsin (1 U per 1 mg of protein) and the reaction was carried out at 30°C for 72 h. It was stopped by adding an equal volume of 0.3 mol/L chloric acid VII. Then the hydrolysate was centrifuged (4.500xg, 15 min, 4°C), supernatant was lyophilized and stored at –18°C.

Degree of hydrolysis (DH). DH was defined as the ratio of protein soluble in 0.3 mol/L chloric acid VII to total protein [Silvestre, 1997].

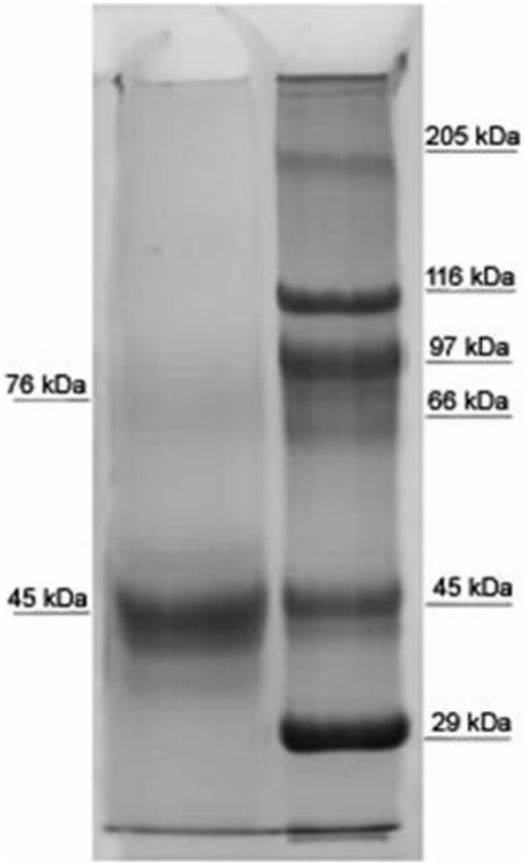

FIGURE 1. SDS-PAGE of precipitate of egg white proteins (60 μg of proteins was analysed in 10% polyacrylamide gel; the gel was stained using 0.1% Coomassie Brilliant Blue R-250).

Determination of free amino groups content. The concentration of free amino groups was determined in a reaction with trinitrobenzenesulfonic acid (TNBS) according to Kuchrro & Ramilly [1983] in modification by Chrzanowska [1993].

Peptides purification by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). The chromatography was performed on Zorbax XDB–C_{18} (4.6×250 mm) column. Operation conditions were as follow: temperature 30°C, flow rate 1 mL/min, mobile phases: (A) 1 mL of trifluoroacetic acid (TFA) per liter in miliQ water, (B) 1 mL of trifluoroacetic acid (TFA) per liter in acetonitrile. The absorption of eluent was monitored at 230 nm. The conditions of chromatography are described under Figure 4. The 5 mL fractions were collected and lyopholified.

Rechromatography of fractions no. 2 and no. 5. Both fractions were dissolved in 1 mL of mobile phase A and applied again on the same column. The conditions of chromatography are described under Figure 4.

Antioxidant capacity as scavenging of free radical 1,1diphenyl-2-picrylhydrazyl (DPPH). The antioxidant capacity was measured according to Yen & Chen [1995] with slight modifications. The tested samples were dissolved in miliQ water to the 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. Radical scavenging activity of peptides was expressed as μmol/L Trolox/mg protein with reference to the standard curve.

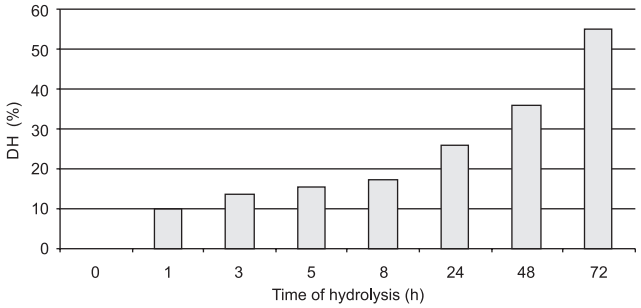

FIGURE 2. Degree of hydrolysis of egg white proteins precipitate.

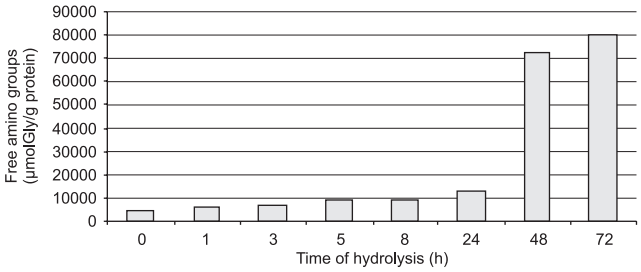

FIGURE 3. Concentration of free amino groups in hydrolysate of egg white proteins precipitate.

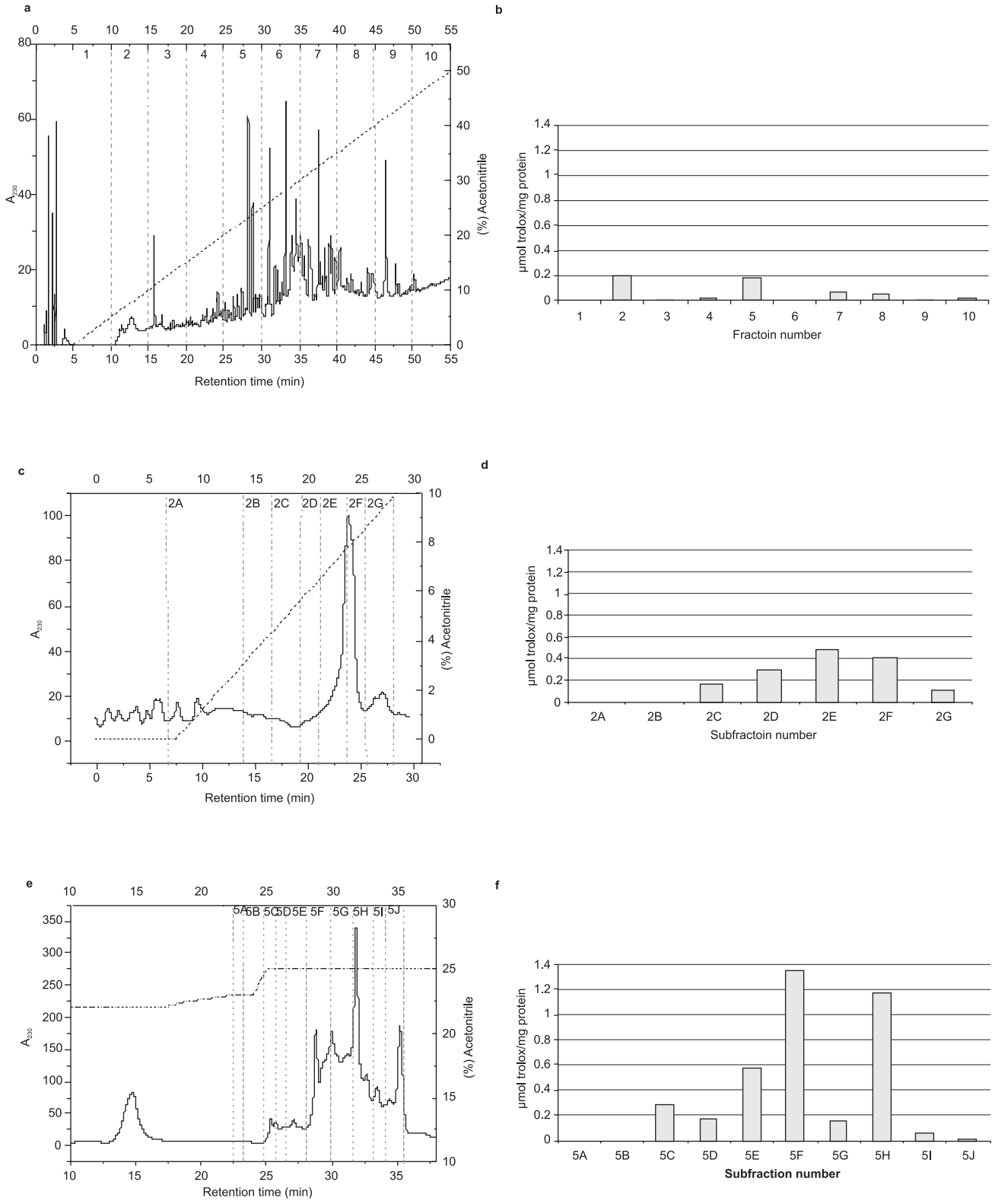

FIGURE. 4. Reversed phase chromatography on Zorbax XDB–C_{18} column and antioxidant capacityy of peptides fractions. (a) Elution profile of egg white precipitate hydrolysate; conditions: temp. 30°C, flow rate 1 mL/min, mobile phases: (A) 0.1% TFA in water, (B) 0.1% TFA in acetonitrile; elution gradient of B presented at the figure. (b) Free radical DPPH scavenging activity (μmol trolox/mg protein) of fractions obtained in RP-HPLC of egg white precipitate hydrolysate. (c) Rechromatography of fraction no. 2; conditions: temp. 30°C, flow rate 0.4 mL/min, mobile phases like in (a); elution gradient of B presented at the figure. (d) Free radical DPPH scavenging activity (μmol trolox/mg protein) of peptides of fraction no. 2. (e) Rechromatography of fraction no. 5; conditions: temp. 30°C, flow rate 0.4 mL/min, mobile phases like in (a); elution gradient of B presented at the figure. (f) Free radical DPPH scavenging activity (μmol trolox/mg protein) of peptides of fraction no. 5.

RESULTS AND DISCUSSION

The research material was a precipitate of egg white proteins, left in the course of cystatin and lysozyme isolation according to Sokołowska *et al.* [2007].

The investigated material was analysed in polyacrylamide gel electrophoresis with SDS. The electropherogram showed the presence of two proteins with molecular masses corresponding to ovalbumin (45 kDa) and ovotransferrin (76 kDa) (Figure 1). However the ovalbumin was a quantitatively predominant protein.

Enzymatic hydrolysis of the proteins was performed using bovine trypsin. The progress of hydrolysis was monitored by determining DH and concentration of free amine groups (Figures 2 and 3). The degree of hydrolysis of the protein is an important parameter in enzymatic modification of proteins and might be a factor controlling the composition and properties of the modified proteins [Ge & Zhang, 1993].

During 72 h of hydrolysis an increasing amount of peptides soluble in 0.3 mol/L choric acid VII was observed. The DH after 24, 48 and 72 h amounted to 25.6%, 36% and 55%, respectively. Over the entire process of protein degradation the concentration of free amino groups increased continuously reaching 13056, 72800 and 79557 μmol Gly/g after 24, 48 and 72 h., respectively.

Our results confirmed observations of other authors that trypsin is an effective enzyme in egg white protein degradation. Miguel *et al*. [2004, 2006] have used trypsin, chymotrypsin and pepsin to obtain crude egg-white and pure ovalbumin hydrolysates. They have noticed also that peptides obtained during crude egg-white hydrolysis were derived mainly from ovalbumin, whereas ovotransferrin was much more susceptible to proteolysis.

The mixture of peptides was resolved by means of RP-HPLC (Figure 4a). Ten fractions were collected and the free radical of DPPH scavenging activity was monitored. The level of determined antioxidant capacity varied considerably between the fractions (Figure 4b). Significantly higher antioxidant capacity was found in fraction no. 2 and fraction no. 5, *i.e.* 0.195 μmol Trolox /mg and 0.186 μmol Trolox /mg, respectively. Fraction no. 7 exhibited 2.8 times lower capability to scavenge free radicals than fraction no. 2. The activity of other fractions was insignificant. No activity was observed in fraction no. 6.

The fractions no. 2 and no. 5 were further investigated. The rechromatographies of these fractions were performed under modified conditions (Figure 4c,e).

One peak of retention time 22.5 to 24.5 min (10% B) was observed in elution profile of fraction no. 2. The material was collected in seven subfractions (Figure 4c). labelled 2A, 2B, 2C, 2D, 2E, 2F, 2G. The antioxidative capacity was determined in each of them (Figure 4d). The highest level was observed in subfraction 2E, corresponding to the peak fraction of the peptide profile. Its antioxidant capacity amounted to 0.482 μmol Trolox /mg and was 2.45 times higher than in the initial fraction (no. 2).

In elution profile of fraction no. 5 several peptide peaks were observed (Figure 4e) and subfractions labelled 5A, 5B, 5C, 5D, 5E, 5F, 5G, 5H, 5I, 5J were collected. The highest levels of antioxidant capacity were estimated in subfractions 5F and 5H (Figure 4f) and approximated 1.375 and 1.200 μmol Trolox /mg, respectively. These levels were 7.4 and 6.4 times higher, respectively, than those reported in fraction no. 5. The lower free radical scavenging activity (0.584 μmol and Trolox/mg) was observed in subfraction 5E. The other subfractions showed lower antioxidant capacities.

The antioxidant capacity in egg-white proteins hydrolysates was also reported by other authors [Davalos *et al.,* 2003; Tsuge *et al*., 1991]. Davalos *et al.* [2003] demonstrated free radical of AAPH (2,2'-azobis (2-methylpropionamidine) dihydrochloride) scavenger activity of peptides produced by enzymatic hydrolysis of crude egg white with pepsin. They isolated and characterised few peptides exhibiting antioxidant activity the amino acid sequence of which corresponded to ovalbumin fragments.

CONCLUSIONS

A protein by-product left in the course of lysozyme and cystatin isolation can be exploited as a source for natural antioxidant substances. Enzymatic hydrolysis of egg-white protein provides that peptides exhibit free radical scavenging activity. Bovine trypsin was an effective enzyme in egg proteins degradation.

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AKTYWNOŚĆ PRZECIWUTLENIAJĄCA W HYDROLIZATACH BIAŁEK JAJA KURZEGO

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Celem badań było otrzymanie hydrolizatów z białek jaja kurzego będących produktem ubocznym procesu izolacji lizozymu i cystatyny oraz oznaczenie aktywności przeciwutleniającej powstałych peptydów.

Frakcję białek jaja kurzego, wolną od lizozymu i cystatyny, przeanalizowano elektroforetycznie (SDS-PAGE), stwierdzając w niej obecność dominującego ilościowo białka o masie 45 kDa, odpowiadającego masie albuminy jaja, oraz śladowych ilości białka o masie 76 kDa, odpowiadającego masie konalbuminy (rys. 1). Białka te poddano hydrolizie przy użyciu trypsyny bydlęcej. Przebieg reakcji degradacji białek monitorowano poprzez oznaczenie stopnia hydrolizy (DH) oraz przyrostu wolnych grup aminowych (rys. 2, 3). Po 72 godz. hydrolizy DH osiągnął poziom 55%, natomiast stężenie wolnych grup aminowych wynosiło 79557 μmol Gly/g. Otrzymaną mieszaninę peptydów rozdzielano techniką RP-HPLC (rys. 4a). W otrzymanych frakcjach peptydów oznaczono aktywność przeciwutleniającą wyrażoną jako zdolność zmiatania wolnych rodników 1,1 difenylo-2-pikrylohydrazylu (DPPH). Spośród dziesięciu badanych frakcji dwie, oznaczone jako nr 2 i nr 5, wykazywały znacząco wysoką aktywność osiągającą wartości odpowiednio 0,195 μmol Trolox /mg i 0,186 μmol Trolox/mg (rys. 4b). Rechromatografie tych frakcje pozwoliły na ich dalsze oczyszczenie, czego efektem był wzrost poziomu badanej aktywności przeciwutleniającej (rys. 4c,e). Najwyższe poziomy tej aktywności odnotowano w subfrakcjach: 2E, 5E, 5F, 5H i wynosiły one odpowiednio: 0,482; 1,385; 1,200; 0,584 μmol Trolox /mg (rys.4d,f).