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Research Article

Systematic Investigation of Antioxidant Activity of Egg White Protein Hydrolysates Obtained by Pepsin

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Abstract: Antioxidative activity of protein hydrolysates from egg white hydrolyzed by Pepsin with different Degrees of Hydrolysis (DHs) was investigated. As the DH increased from 6.47 to 18.22%, the antioxidative activity of Egg White Protein Hydrolysates (EWPHs) first increased and then decreased, except for the reducing power of EWPHs. The EWPHs with DH 16.93% showed higher DPPH radical scavenging activity (96.07±3.84%), hydroxyl radical scavenging activity (36.82±1.46%), superoxide anion scavenging activity (67.72±2.51%) and inhibitory activity of tea oil autoxidation (62.68±2.32%) compared to other EWPHs. At DH 18.22%, the EWPHs exhibited the strongest reducing power (0.34±0.014). The results revealed that antioxidative activity of protein hydrolysates from egg white was determined by the DH. According to the experimental results, the EWPHs have potential for use as a natural antioxidant for food preservation.

Keywords: Antioxidative activity, egg white, egg white protein hydrolysates, hydrolysis

INTRODUCTION

It is well known that functional properties of protein can be improved by enzymatic hydrolysis under suitable conditions. Hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolysates. The characteristics of hydrolysate directly influence the functional properties and the uses as a valuable ingredient for the food, pharmaceutical and cosmetic industries. Recently, some protein hydrolysates have been reported to exhibit antioxidant activity (Sakanaka and Tachibana, 2006; Klompong *et al*., 2007; Sun *et al*., 2010). In the past several years, many researchers reported that both the type of protease and the degree of hydrolysis can affect the antioxidative activity of protein hydrolysate (Saiga *et al*., 2003; Hou and Zhao, 2011). Thiansilakul *et al*. (2007) suggested that muscle protein hydrolysates produced with flavourzyme showed a higher DPPH radical scavenging activity and reducing power, but a lower Fe_2+ chelating ability than the hydrolysates prepared with alcalase. You *et al*. (2009) found that as DH increased from 18 to 33%, the hydroxyl radical scavenging activity of loach protein hydrolysates increased first and then decreased. Different types of protease and different DHs contribute to the molecular weight and amino acid sequence of peptides, which may affect antioxidant activities of protein hydrolysates.

Eggs are an important source of high quality protein. Protein hydrolysates from egg white and egg yolk have been found to possess antioxidant activity (Sakanaka *et al*., 2004; Lin *et al*., 2012). The molecular weight and amino acid sequence of peptides were reported to determine the antioxidant activities of protein hydrolysates (You *et al*., 2009). However, there is a little information regarding their antioxidative activity as affected by DH. Therefore, this study aimed to produce a protein hydrolysate from egg white with different DHs using pepsin and to study systematically their antioxidative activities.

MATERIALS AND METHODS

Materials and chemicals: Fresh eggs were obtained from the farmer's market of Huazhong Agricultural University (Wuhan, Hubei, China). The egg white was freeze-dried after the separation of egg white and egg yolk. Pepsin was purchased from Novozyme Co. (Bagsvaerd, Denmark). All radical testing chemicals including 1.10-Phenanthroline, pyrogallic acid and 1.1-Diphenyl-2-Picrylhydrazyl (DPPH) were purchased

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from Sigma Chemical Co. (Sigma Chemical Co., St. Louis, MO, USA). Other chemicals used were of analytical grade.

Methods:

Preparation of egg white proteins hydrolysates: After the sample (hen's egg white) was diluted with distilled water to the final concentration of 3% (w/v) and heated at 90°C in a water bath for 15 min to denature the egg white protein before the mixed solution was cooled down to room temperature. Then, the pH of the mixed solution was adjusted to 2.0 with 1 M HCl. The mixed solution was hydrolyzed with pepsin at 37°C for 1, 3, 5 and 7 h, respectively, which achieved various DHs. The enzyme to substrate ratio (E/S) was 9000:1 (U/g). The pH of the mixed solution was maintained constant at 2.0 by continuous addition of 1 M HCl or 1 M NaOH. In order to reach complete enzyme inactivation, the samples were treated at 85°C for 30 min. The hydrolysates were centrifuged in a GL-21 M refrigerated centrifuge (Xiangyi Instrument Co. Ltd., Changsha, China) at 4000 g for 20 min and the supernatants were freeze-dried with a vacuum freeze dryer (Christ Alpha 1-2 LD, Bioblock Scientific, France) and stored at -18°C for further use.

Determination of the degree of hydrolysis: The Degree of Hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (h_{tot}), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis as given below (Adler-Nissen, 1986):

$$DH\ (\%) = h \times 100\% / h_{tot}$$

$$= B \times N_b \times 100\% / M_p \times a \times h_{tot} \quad (1)$$

where,

B = The amount of NaOH consumed (mL) to keep the pH constant during the reaction
N_b = The normality of the base
M_p = The mass (g) of protein (N×6.25)
a = The average degree of dissociation of the a-NH_2 groups released during hydrolysis expressed as:

$$a = 10^{(pH-pK)}/(1+10^{(pH-pK)}) \quad (2)$$

where, pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in an egg white protein concentrate was assumed to be 9.14 meq/g.

Determination of DPPH radical scavenging activity: The DPPH radical scavenging activity of the EWPHs was determined as described by Bersuder *et al*. (1998), with a small modification. A volume of 2 mL of sample (5 mg/mL) was mixed with 2 mL of 0.1 mM DPPH reagent in 99.5% (w/v) ethanol. The mixture was then kept in the dark at room temperature for 60 min and the absorbance of the mixture was measured at 517 nm using a UV-Visible spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd., China). The DPPH radical scavenging activity was calculated as follows:

$$\text{The DPPH radical scavenging activity (\%)} = (A_c - A_s) \times 100\% / A_c \quad (3)$$

A_c = Absorbance of control
A_s = Absorbance of sample

The control was conducted in the same manner except that distilled water was used instead of the sample. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. All the assays were carried out in triplicate.

Determination of hydroxyl radical scavenging assay: The hydroxyl radical scavenging activity was evaluated as described by Li *et al*. (2008), with some modifications. A mix of 600 μL of $FeSO_4$ (5.0 mM), 600 μL of 1,10-phenanthroline (5.0 mM) and 600 μL of Ethylene Diaminetetraacetic Acid (EDTA) (15 mM) were mixed with 400 μL of sodium phosphate buffer (0.2 M, pH 7.4). Then, 1 mL of the EWPHs solution (5 mg/mL) and 400 μL of H_2O_2 (0.01%) were added. The mixture was incubated at 37°C for 60 min and the absorbance was measured at 536 nm. The hydroxyl radical scavenging activity was calculated using the following equation:

$$\text{The hydroxyl radical scavenging activity (\%)} = (A_s - A_0) \times 100\% / (A_c - A_0) \quad (4)$$

where,

A_s = The absorbance of the sample mixture
A_0 = The absorbance of the blank solution using distilled water instead of sample mixture
A_c = The absorbance of a control solution in the absence of H_2O_2

All the assays were carried out in triplicate.

Determination of superoxide anion scavenging activity: The superoxide anion scavenging activity was assayed by measuring the inhibition of the autoxidation of pyrogallol using the method of Marklund and Marchland (1974), with a slightly modification. One mL of the sample solution (2 mg/mL) and 4.5 mL of 50 mM phosphate buffer (pH 8.24) were mixed with 0.1 mL of 3 mM pyrogallol solution (freshly prepared). Then, 100 μL of 0.2 M ascorbic acid was added

immediately to the mixture. After 4 min of incubation, the absorbance of the reaction mixture was measured at 325 nm against a blank. The superoxide anion scavenging activity was calculated as follows:

$$\text{The superoxide anion scavenging activity (\%)} = (\Delta A_1/\Delta t - \Delta A_2/\Delta t) / (\Delta A_1/\Delta t) \quad (5)$$

where, $\Delta A_2/\Delta t$ is the changes of absorbance of pyrogallol solution after the addition of the sample, which indicated the autoxidation rate of pyrogallol after the addition of the sample. $\Delta A_1/\Delta t$ is the changes of absorbance of pyrogallol solution after the addition of distilled water instead of sample, which showed the autoxidation rate of pyrogallol without the addition of the sample. All the assays were carried out in triplicate.

Determination of reducing power assay: The ability of the EWPHs to reduce iron (III) was determined by the method of Yildirim *et al*. (2001), with a small modification. Different concentrations of the EWPHs samples in 1 mL of distilled water were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide and then the mixture was incubated at 50°C for 30 min. Afterward, 2.5 mL of 10% (w/v) trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride solution. After a 10 min incubation time, the absorbance of the mixture was measured at 700 nm. A higher absorbance of the reaction mixture indicated greater reducing power. All the assays were carried out in triplicate.

Determination of Inhibition of linoleic acid autoxidation: The lipid peroxidation inhibition activity of the hydrolysates obtained from egg white was measured in the tea oil emulsion system according to the method of Osawa and Namiki (1985), with some modifications. Fifty mg of the hydrolysates was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0) and then mixed with 65 μL of 100% (w/v) tea tree oil and 10 mL of 99.5% (w/v) ethanol. Briefly, the reaction solution (0.1 mL) was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride solution in 3.5% HCl. The mixture solution was then incubated for 3 min at 40°C. The absorbance of the mixture was measured at 500 nm against a blank containing the same volume of distilled water. Fifty mM phosphate buffer (pH 7.0) was used as a control. The lipid peroxidation inhibition activity was expressed as follows:

$$\text{The lipid peroxidation inhibition activity (\%)} = (A'_c - A'_s) \times 100\% / A_c \quad (6)$$

A'_c = Absorbance of control
A'_s = Absorbance of sample

Statistical analysis: All the tests were conducted in triplicate. The results were expressed as a mean ±Standard Deviation (S.D.) and were subjected to one-way Analysis of Variance (ANOVA). The values $p<0.05$ were regarded as significant. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Preparation of EWPHs using pepsin: It has been demonstrated that the antioxidant of proteins can be increased through hydrolysis with certain enzymes and some peptides or fractions have a stronger antioxidant capacity than others (Chen *et al*., 1995). Furthermore, the antioxidant activity of protein hydrolysates depends on the protein substrate used, proteolysis conditions, the specificity of the enzyme used for the proteolysis and the DH. Protein hydrolysates with different DHs were obtained by treating egg white with pepsin. The hydrolysis curve of egg white proteins after 1 to 7 h of incubation was shown in Fig. 1; the results showed that the DH of the egg white protein increased rapidly within the first 2 h. The rate of enzymatic hydrolysis subsequently decreased and then the enzymatic reaction reached a steady-state phase in all enzymatic hydrolysis, in which no apparent further hydrolysis took place. A typical curve for this hydrolysis was also reported for hydrolysis of muscle proteins of smooth hound (Mustelus mustelus) (Bougatef *et al*., 2009); harp seal (Phoca groenlandica) (Shahidi *et al*., 1994) and yellow stripe trivially (Klompong *et al*., 2007). After 1, 3, 5 and 7 h of hydrolysis, the average DHs were 6.47, 13.37, 16.93 and 18.22%, respectively.

DPPH radical-scavenging activity: The DPPH• radical has a single electron and shows the maximum absorbance at 517 nm, the radical is widely used in model systems, to investigate the scavenging activity of some natural compounds such as phenolic compounds,

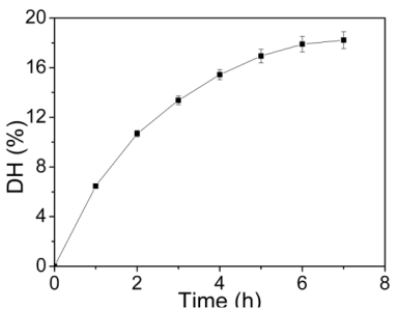

Fig. 1: The hydrolysis curve of egg white proteins hydrolyzed by using pepsin

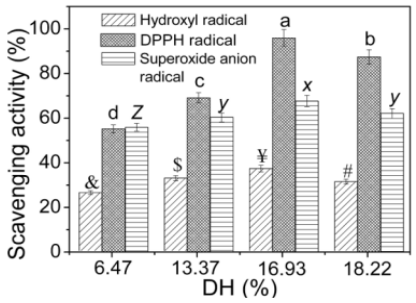

Fig. 2: DPPH, hydroxyl, and superoxide anion radical scavenging activity of the EWPHs with different DH

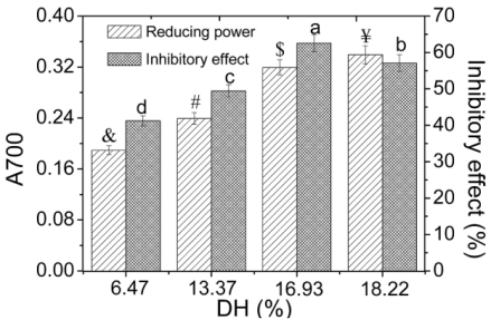

Fig. 3: Reducing power activity and lipid per oxidation inhibition activity of the EWPHs with different DH

anthocyanins and some protein hydrolysates. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance is reduced (Shimada *et al*., 1992). The DPPH radical-scavenging activities of the EWPHs were shown in Fig. 2, the results clearly indicated that the EWPHs with different DHs exhibited different scavenging activities against DPPH radicals. The protein hydrolysates with DH 16.93% showed the highest DPPH radical scavenging activity (96.07±3.84%) among four protein hydrolysates. The protein hydrolysates with DH 6.47% exhibited the lowest DPPH radical-scavenging activity, which could also reach 55.37±1.78%. The results indicated that the EWPHs acted as a good electron donor and could react with free radicals to terminate the radical chain reaction.

Hydroxyl radical scavenging activity: The hydroxyl radical is the most potent free radical derived from oxygen, it can easily react with biomolecules such as amino acids, proteins and DNA. Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases (Cacciuttoloa *et al*., 1993; Qian *et al*., 2008). The hydroxyl radical scavenging activities of the EWPHs were shown in Fig. 2, the results showed that the protein hydrolysates with DH 16.93% exhibited the highest free radical scavenging activities for hydroxyl radical, reaching 36.82±1.46%, while the hydroxyl radical scavenging activities of the EWPHs with DH 6.47, 13.37 and 18.22% were 26.13±0.84, 32.55±1.14 and 30.98±1.15%, respectively. The differences may be attributed to differences in type of amino acids and peptide fragments produced by pepsin hydrolysis. The results also indicated that the EWPHs were an effective electron donor for the reduction of hydroxyl radical.

Superoxide anion scavenging activity: Superoxide anion radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species, which can cause disease and damage to tissues. And thus investigation of the free radical scavenging activity is of great importance (Kanatt *et al*., 2007). Superoxide anion scavenging activity of the EWPHs with different DHs was presented in Fig. 2, the protein hydrolysates showed considerable scavenging abilities over superoxide anion. The EWPHs with DH 16.93% showed significant stronger superoxide anion scavenging activity (67.72±2.51%) than other egg protein hydrolysates ($p<0.05$).

Reducing power: Reducing power assay is often to evaluate the ability of natural antioxidant to donate electron or hydrogen (Yildirim *et al*., 2000). Many reports have revealed that there is a direct correlation between the antioxidative activities and the reducing power of bioactive compounds. For the reducing power assay, the presence of antioxidants in the tested samples caused the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power of the EWPHs with different DHs was showed in Fig. 3. As the DH increased from 6.47 to 18.22%, the reducing power of the EWPHs increased from 0.19±0.007 to 0.34±0.014. The EWPHs with DH 18.22% had the strongest reducing power. The difference might be attributed to the presence of more specific peptides or active amino acids in the EWPHs with high DH, which could react with free radicals to form more stable products.

Inhibition of tea oil autoxidation: Free radical-induced lipid peroxidation is a complex process that involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids (Bougatef *et al*. 2010). The inhibitory effects of the EWPHs as an antioxidant in prevention of tea oil autoxidation were investigated. As shown in Fig. 3, all the tested samples showed strong inhibitory effects, the inhibitory activity of the EWPHs with DH 16.93% (62.68±2.32%) was significantly higher than that of other EWPHs ($p<0.05$). The results indicated that limited hydrolysis of the egg white protein could lead to better antioxidant ability than extensive hydrolysis.

CONCLUSION

The antioxidant activities of EWPHs were related to DH. At DH 16.93%, the EWPHs exhibited the strongest antioxidant activity, including DPPH radical

scavenging activity (96.07±3.84%), hydroxyl radical scavenging activity (36.82±1.46%), superoxide anion scavenging activity (67.72±2.51%) and the inhibitory activity of tea oil autoxidation (62.68±2.32%). The EWPHs showed the strongest reducing power (0.34±0.014) at DH 18.22%. Therefore, the EWPHs can be used in food systems as a natural additive possessing antioxidative properties.

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REFERENCES

Adler-Nissen, J., 1986. A Review of Food Hydrolysis Specific Areas. In: Adler-Nissan, J. (Ed.), Enzyme Hydrolysis of Food Proteins. Elsevier Applied Science Publishers, Copenhagen, Denmark, pp: 57-109.

Bersuder, P., M. Hole and G. Smith, 1998. Antioxidants from a heated histidineglucose model system I: Investigation of the antioxidant role of histamine and isolation of antioxidants by high performance liquid chromatography. J. Am. Oil Chem. Food Agr., 75: 181-187.

Bougatef, A., M. Hajji, R. Balti, I. Lassoed, Y. Triki-Ellouz and M. Nasri, 2009. Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein Hydrolysates obtained by gastrointestinal proteases. Food Chem., 114: 1198-1205.

Bougatef, A., N. Nedjar-Arroume, L. Mani, R. Ravallec, A. Barkia, D. Guillochon and M. Nasri, 2010. Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinelle aureate*) by-products proteins. Food Chem., 118: 559-565.

Cacciuttoloa, M.A., L. Trina, J.A. Lumpkin and G. Rao, 1993. Hyperoxia induces DNA damage in mammalian cells. Free Radical Bio. Med., 14: 267-276.

Chen, H.M., K. Muramoto and F. Yamauchi, 1995. Structural analysis of ant oxidative peptides from soybean β-conglycinin. J. Agric. Food Chem., 43: 574-578.

Hou, Y. and X.H. Zhao, 2011. Limited hydrolysis of two soybean protein products with try sin or neutrals and the impacts on their solubility: Gelatin and fat absorption capacity. Biotechnology, 10: 190-196.

Kanatt, S.R., R. Chandler and A. Sharma, 2007. Antioxidant potential of mint (*Menthe spectra*, L.) radiation-processed lamb meat. Food Chem., 100: 451-458.

Klompong, V., S. Benjakul, D. Kantachote and F. Shahidi, 2007. Ant oxidative activity and functional properties of protein Hydrolysates of yellow stripe trivially (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem., 102: 1317-1327.

Li, Y.H., B. Jiang, T. Zhang, W.M. Mu and J. Liu, 2008. Antioxidant and free radical scavenging activities of Chickpea Protein Hydrolysates (CPH). Food Chem., 106: 444-450.

Lin, S., Y. Guo, Q. You, Y. Yin and J. Liu, 2012. Reparation of antioxidant peptide from egg white protein and improvement of its activities assisted by high-intensity pulsed electric field. J. Sci. Food Agr., 92: 1554-1561.

Marklund, S. and G. Marchland, 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and convenient assay for superoxide dismutase. Eu. J. Biochem., 47: 469-474.

Osawa, T. and M. Namiki, 1985. Natural antioxidant isolated from eucalyptus leaf waxes. J. Agr. Food Chem., 33: 777-780.

Qian, Z.J., W.K. Jung, H.G. Byun and S.K. Kim, 2008. Protective effect of an ant oxidative peptide purified from gastrointestinal digests of oyster, Crassostrea gigas against free radical induced DNA damage. Bioresource Technol., 99: 3365-3371.

Saiga, A., S. Tanabe and T. Nishimura, 2003. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. J. Agr. Food Chem., Vol. 51, pp. 3661-3667.

Sakanaka, S. and Y. Tachibana, 2006. Active oxygen scavenging activity of egg-yolk protein Hydrolysates and their effects on lipid oxidation in beef and tuna homogenates. Food Chem., 95: 243-249.

Sakanaka, S., Y. Tachibana, N. Ishihara and L.R. Juneja, 2004. Antioxidant activity of egg-yolk protein hydrolysates in a linoleum acid oxidation system. Food Chem., 86: 99-103.

Shahidi, F., J. Synowiecki and J. Balejko, 1994. Proteolytic hydrolysis of muscle proteins of harp seal (*Ponca greenlandic*). J. Agr. Food Chem., 42: 2634-2638.

Shimada, K., K. Fujikawa, K. Yahara and T. Nakamura, 1992. Ant oxidative properties of xanthenes on the ant oxidation of soybean oil in cyclodextrin emulsion. J. Agr. Food Chem., 40: 945-948.

Sun, Q., H.X. Shen and Y.K. Luo, 2010. Antioxidant activity of hydrolysates and peptide fractions derived from porcine hemoglobin. J. Food Sci. Tech., 48: 53-60.

Thiansilakul, Y., S. Benjakul and F. Shahidi, 2007. Ant oxidative activity of protein hydrolysates from round scald muscle using Alkalize and Flavourzyme. J. Food Biochem., 31: 266-287.

Yildirim, A., A. Mavi, M. Oktay, A.A. Kara, F. Algur and V. Bilaloglu, 2000. Comparison of antioxidant and antimicrobial activities of tilia (Tilia argental Deaf Ex DC), sage (*Salvia trilobite* L.) and black tea (*Camellia saneness*) extracts. J. Agr. Food Chem., 48: 5030-5034.

Yildirim, A., A. Mavi and A.A. Kara, 2001. Determination of antioxidant and antimicrobial activities of Remix crisp us L. extracts. J. Agr. Food Chem., 49: 4083-4089.

You, L.J., M.M. Zhao, C. Cui, H.F. Zhao and B. Yang, 2009. Effect of degree of hydrolysis on the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates. Inn. Food Sci. Emerg. Tech., 10: 235-240.