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Effects of Two Pre-Treatment Methods on Functional Properties of Egg White Protein Hydrolysates Obtained by Pepsin

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Abstract: After Egg White Proteins (EWPs) were pre-treated by Conventional Heating (CH) Method and Microwave Irradiation (MWI), respectively, functional properties of protein hydrolysates from egg white hydrolyzed by pepsin was systemically investigated. The results indicated the maximum Degrees of Hydrolysis (DHs) of EWPs pre-treated by MWI could reach 19.81%, which was much more than that of EWPs pre-treated by CH. As the pH increased, the solubility of Egg White Protein Hydrolysates (EWPHs) gradually decreased, but the solubility of EWPHs originated from the proteins pre-treated by MWI was also significantly higher ($p<0.05$) than the EWPHs derived from the proteins treated by CH at pH 2. The Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI) of EWPHs exhibited the same trend with the solubility of the EWPHs. The water absorption ability of the EWPHs originated from the proteins pre-treated by MWI and CH, respectively, was higher than crude EWPs, but the former is far better than the latter. The experimental data also showed that MWI could improve the Oil Absorption Capacity (OAC) of EWPHs significantly ($p<0.05$) compared with CH treatment. The EWPHs had a greater ability to hold water than the FDEW and glycerol, but there were no difference in the WHC between the EWPHs originated from the proteins treated by CH and MWI ($p>0.05$). According to the experimental results, the EWPHs have potential for use as a natural ingredient for emulsifying and wetting agent.

Keywords: Egg white protein hydrolysates, emulsifying activity, hydrolysis, microwave irradiation, water absorption ability

INTRODUCTION

As raw materials in food industries, egg white is widely used as the important components because of its excellent emulsifier activity, good biocompatibility and good foaming properties. The functional properties of EWPs are mainly related to the type and number of peptides or amino acid residues on the surface of a protein molecular structure. It is well known that functional properties of protein can be improved by enzymatic hydrolysis under suitable conditions, DH is an important parameter for determination of functional properties of protein hydrolysates. Proteins can stabilize emulsions because of their amphiphilic nature and partially unfolding structures. Under certain conditions proteins will adsorb to a charged oil/water interface and form a solid viscoelastic layer, with the lipophilic portion in the nonpolar phase (oil) and the hydrophilic portion in the polar (water) phase, which has been well correlated with emulsion stability. In fact, all egg proteins tend to have similar, globular shapes, globular protein hydrolysates prepared by enzymatic hydrolysis may be able to unfold effectively proteins structures and they have more intermolecular bonds stabilizing their structure.

Enzymatic hydrolysis of proteins after MWI pre-treatment is a new method for accelerating the hydrolysis of proteins. Microwaves are electromagnetic waves and the heating of proteins by microwave energy is fast and uniform throughout the material, which facilitates more enzyme-cutting sites exposure to protease. Numerous studies have dealt with the application of microwave as a preference method to accelerate protein hydrolysis for preparing samples for amino acids analysis (Marconi *et al*., 1995; Chen *et al*., 1987; Chiou and Wang, 1989). Lin *et al*. (2010) also

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reported the efficacy of this technique for preparing ant oxidative peptides derived from enzyme hydrolysis of bone collagen after microwave assisted acid pre-treatment. According to these results, the denaturation of protein occurs in minutes using this technique, in contrast to the hours required by conventional heat treatments. However, the authors of the present study did not know whether MWI pre-treatment could improve the functional properties of protein hydrolysates.

In the present study, we have developed the method of preparation of protein hydrolysates by enzyme hydrolysis of EWPs after microwave irradiation pre-treatment, in the aim to accelerate the enzymatic reactions and also to find whether MWI pre-treatment can improve functional properties of protein hydrolysates.

MATERIALS AND METHODS

Materials and chemicals. Fresh eggs were obtained from the farmer's market of Center South University of Forestry and Technology (Changsha, Hunan, 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-dipheny l-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (Sigma Chemical Co., St. Louis, MO, USA). Other chemicals used were of analytical grade.

Methods: Preparation of EWPs 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-21M 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 (DH). The 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):

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

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, pK = 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 solubility properties of EWPHs. 0.1 g of protein hydrolysates with DH 17.89% were dispersed in 10 ml of distilled water. Then, the pH of the mixture was adjusted to pH 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0, respectively, The mixture was stirred for 30 min at room temperature and centrifuged at 7500 g for 15 min at 4°C. The protein content of supernatant was measured using the Biuret method (Gornall *et al*., 1949), the samples of protein hydrolysates were directly solubilized by 0.5 M NaOH for determination of total protein. The solubility of the EWPHs was calculated as follows:

$$Solubility\ (\%) = (A/B) \times 100\% \quad (3)$$

where,

A = The protein content in the supernatant
B = The total protein content in the sample

Determination of emulsifying properties of EWPHs. The Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI) were determined by the turbidimetric method of Pearce and Kinsella (1978). 3 mL of protein hydrolysates solution (2.0 mg/mL) was dispersed in 0.2 M phosphate buffer, the pH of the mixture was adjusted to pH 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0, respectively. Then, 1 ml of tea oil was emulsified by mixing the buffer solution containing protein hydrolysates with a Sorvall Omni-Mixer for 1 min. Fifty-microliter portions of the emulsion were pipetted from the bottom of the container at 0 and 10 min after

homogenization. Each portion was diluted with 5 mL of 0.1% Sodium Dodecyl Sulfate (SDS) solution. The absorbances of these diluted solutions were measured at 500 nm. The absorbances measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the EAI and ESI. EAI and ESI were calculated by the equation:

$$\text{EAI } (m^2/g) = 2\times T\times A_0 \times \textit{dilution factor}/(c\times \Phi\times 10000) \quad (4)$$

$$\text{ESI (min)} = 10\times A_0 /(A_0 - A_{10}) \quad (5)$$

where T = 2.303, *dilution factor* = 100, c is the weight of protein per unit volume (g/ml) and Φ is the oil volumetric fraction (0.25).

Determination of Water Absorption Properties (WAC) of EWPHs. The WAC of EWPHs was determined using the method of Li *et al*. (2009) with small modifications. The FDEW was used as a control. 1.0 g of the sample was spread evenly over the surface of a dried culture dish, with a diameter of 8 cm. The dishes containing the test samples were then placed in an incubator at 31°C and a relative humidity of 85%. The dishes were weighed every 6 h until its weight changed little. WAC was calculated using the following equation:

$$\text{WAC (g water/g sample)} = (W_1 - W_0)/W \quad (6)$$

where,

W_0 = The weight of the dried culture dish
W_1 = The total weight of the culture dish and the water absorbed by the sample
W = The initial weight of the sample

Determination of Oil Absorption Capacity (OAC) of EWPHs. The OAC of EWPHs was evaluated as described by Li *et al*. (2009) with a slight modification. 0.3 g of the sample and 2.0 mL of refined tea oil were taken into a centrifuge tube, then the centrifuge tube was stirred vigorously for 15 min at room temperature. After being allowed to stand at room temperature for 30 min, the mixture was centrifuged at 100×g for 25 min. Finally, the volume of free oil was determined after centrifugation. The OAC was calculated using the following equation:

$$\text{OAC (mL/g)} = (V_1 - V_0)/W \quad (7)$$

where,

V_0, V_1 = The volumes of initial oil and free oil
W = The weight of the sample

FDEW was used as a control.

Determination of water-holding capacity (WHC) of EWPHs. The WHC of EWPHs was determined according to the method of (Li *et al*., 2009) with small modifications. 10 mL of 10 mg/mL sample solution was poured into a dried culture dish with a diameter of 8 cm. The dishes containing the test samples were then placed in an incubator at 37°C and a relative humidity of 75%. The ratio of residual water to initial water was measured every 20 min for 80 min. WHC was calculated according to the following equation:

$$\text{WHC (\%)} = 100 \times(W_0 - \triangle W) / W_0 \quad (8)$$

where, $\triangle W$ and W_0 represent the amount of water loss during incubation and the initial amount of water, respectively. Distilled water was used as a control.

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 MWI could be used to enhance the enzymatic proteolysis. Besides, the rate and degree of hydrolysis depend also on the protein substrate used, proteolysis conditions, the specificity of the enzyme used for the proteolysis and the DH. After the EWPs were pre-treated by CH and MWI, respectively, protein hydrolysates with different DH were obtained using pepsin. The hydrolysis curves of EWPs after 1 to 7 h of incubation were shown in Fig. 1, the results showed that the DH of the egg white proteins increased rapidly within the first 2 h, the higher levels of proteolysis were obtained in the MWI digestions after 7 min of digestion in comparison with CH digestion. After 1, 3, 5 and 7 h of hydrolysis, the DHs of EWPs denatured by CH were 6.47, 13.37, 16.93 and 18.22%, respectively. However, after 1, 2, 4 and 7 h of hydrolysis, the DHs of EWPs pre-treated by MWI could reach 7.25, 11.79, 16.98 and 19.81%, respectively. Increase of enzymatic hydrolysis by MWI has also been reported on the case of preparing samples for amino acids analysis (Chiou and Wang, 1989; Lin *et al*., 2010).

Solubility properties of EWPHs. Production of high solubility protein hydrolysate in a pH range is very interesting for food applications, because solubility greatly influences the other functional properties such as emulsifying, foaming and gelling abilities. The solubility's of the EWPHs in the pH range of 2-12 were

Table 1: The solubility, emulsifying ability and emulsion stability of the EWPHs

	Solubility (%)		EAI (m^2/g)		ESA (min)	
pH	CH	MWI	CH	MWI	CH	MWI
2	86.70±2.17	91.23±2.10*	145.35±4.67	152.63±4.77*	92.17±3.04	95.17±3.14*
4	70.94±1.63	73.77±1.65*	116.60±3.31	122.74±2.82*	88.10±2.91	91.12±2.73
6	68.12±1.43	70.25±1.44	116.38±3.31	118.97±2.26	65.36±2.09	70.52±1.83
8	61.37±1.22	65.31±1.27	110.92±2.89	114.43±2.06	49.62±1.54	56.88±1.36
10	61.15±1.10	62.28±1.15	100.28±2.41	105.88±1.86	33.93±0.95	39.51±0.79
12	58.55±0.94	59.15±1.06	96.15±2.08	99.25±1.49	31.30±0.78	37.25±0.67

*: $p<0.05$; Each value is expressd as mean±SD of five independent sample with five measurements per (n = 5)

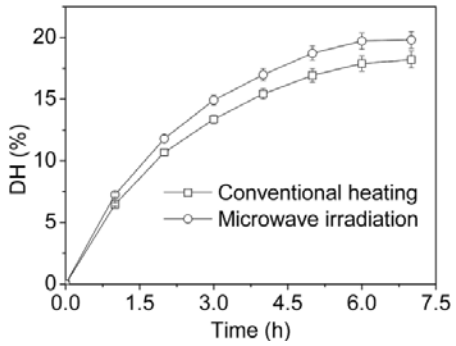

Fig. 1: The hydrolysis curve of EWPs hydrolyzed by using pepsin

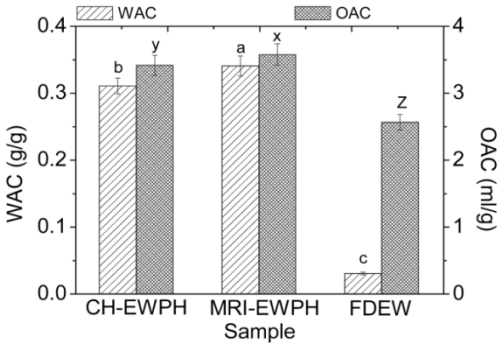

Fig. 2: Water and oil absorption capacities of the EWPHs derived from the proteins treated by CH and MWI, respectively; (CH-EWPH: EWPHs derived from the proteins treated by conventional heating; MWI-EWPHs: EWPHs derived from the proteins pre-treated by MWI; FDEW: the freeze-dried egg white); Each result is the average calculated in 95% condense interval of five parallel studies

shown in Table 1. All hydrolysates were soluble over a wide pH range with more than 58% solubility. After the EWPs were treated by CH, as the pH increased from 2 to 12, the solubility of EWPHs decreased from 86.70±1.95% to 58.55±0.98%. However, the solubility of EWPHs originated from the proteins pre-treated by MWI was significantly higher than the EWPHs derived from the proteins treated by CH at pH 2, the differences between the former and the latter gradually reduced with the decrease of EWPHs solubility. The differences in solubility of EWPHs might be attributed to the production of more alkaline amino acid residues in the EWPHs originated from the proteins treated by MWI, which made it more easily soluble in acidic conditions.

Emulsifying properties of EWPHs. Emulsifying properties of food proteins or proteins hydrolysates are usually described as emulsion capacity or emulsion activity, which reflect the ability of the protein to aid formation and stabilization of newly created emulsions and the ability of the proteins to impart strength to emulsion for resistance to stress (Liu *et al*., 2008). The EAI and ESI of the EWPHs in the pH range of 2-12 were shown in Table 1, the results indicated that the EAI and ESI of the EWPHs depended on the pH of the solution and whether the EWPHs were derived from the EWPs treated by CH or MWI. The highest EAI and ESI were found at pH 2, respectively. As the pH increased, the EAI and ESI of EWPHs gradually decreased, which exhibited the same trend with the solubility of the EWPHs. The higher EAI of hydrolysates accompanied their higher solubility (Mutilangi *et al*., 1996). Hydrolysates with high solubility could rapidly diffuse and adsorb at the interface, therefore they possessed good emulsifying property. This also indicated that MWI pre-treatment could improve emulsifying properties of the EWPHs compared with CH treatment.

Water and oil absorption capacity of EWPHs. WAC is an important index, which gives valuable information on the behavior of weaning food products during reconstitution in hot or cold water. It expresses the result as millilitre of water absorbed per gram of protein in the sample. As shown in Fig. 2, the water absorption ability of the EWPHs originated from the proteins pre-treated by MWI was significantly higher ($p<0.05$) than the EWPHs derived from the proteins treated by CH and FDEW. The difference might be attributed to the presence of more hydrophilic groups in the EWPHs after the EWPs derived from the proteins pre-treated by MWI was hydrolyzed by pepsin compared with CH treatment (Kinsella, 1976). OAC is an important functional characteristic of protein ingredients used in meat and confectionary industries (Souissi *et al*., 2007). OAC of protein hydrolysates can be affected by many different factors, such as the type of protein, the type of enzyme used, the DH, the hydrolysis conditions and the oil used (Kristinsson and Rasco, 2000; Hou and Zhao, 2011). The data shown in Fig. 2 indicated that the OAC of EWPHs derived from the proteins treated by CH and MWI were 3.42±0.11 and 3.58±0.17, respectively, which were significantly

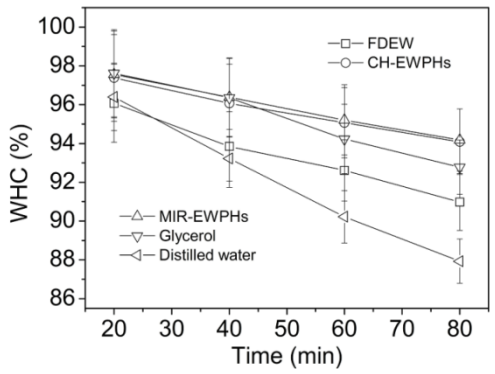

Fig. 3: A comparison of water holding capacity of the EWPHs, the FDEW, and glycerol; Each result is the average calculated in 95% condense interval of five parallel studies

higher ($p<0.05$) than that of the FDEW. This indicated that MWI could improve the OAC of EWPHs significantly ($p<0.05$) compared with CH treatment and the hydrolysis of EWPs could be applied to enhance its OAC.

Water-holding capacity of EWPHs. The functional properties of food proteins are important in food processing and food product formulation and some of these properties are related to hydration, such as WHC, WAC and solubility. As shown in Fig. 3, the EWPHs originated from the proteins pre-treated by CH and MWI had a greater ability to hold water than the FDEW and glycerol, but there were no difference in the WHC between the EWPHs originated from the proteins treated by CH and MWI ($p>0.05$). After the EWPs were hydrolyzed by pepsin, the increase in the WHC might be attributed to the presence of more hydrophilic groups in the EWPHs. Therefore, the EWPHs had potential applications in the food and cosmetic industries.

CONCULSION

The EWPHs originated from the EWPs pre-treated by MWI have a better functional properties compared with CH treatment, including high solubility, excellent emulsifying properties, water and oil absorption capacity and water-holding capacity, although there was no significant difference in water-holding capacity between the EWPHs originated from the proteins treated by CH and MWI ($p>0.05$). Therefore, the EWPHs can be used as a natural ingredient for emulsifying and wetting agent in the cosmetic industries.

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