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Effects of hydrolysis degree and type of protease on antioxidant activity and functionality of egg white protein hydrolysates

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Abstract

Enzymatic hydrolysis of egg white proteins has shown great potential to improve their functional properties such as increased solubility, stability, and digestibility, and to reduce protein allergenicity while still retaining their nutrition value. However, the enzymatic hydrolysis process is still poorly defined and difficult to control at the industrial scale resulting in peptide mixtures poorly characterized and with unpleasant bitter taste that make them unsuitable for human consumption. Thus, the hydrolysis reaction must be carefully controlled in order to produce new value-added egg white hydrolysates with improved properties and specialized functionality. In this paper egg white protein solution was hydrolysed with several enzymes using both, one-step and two-step hydrolysis. The hydrolysate was then tested on antioxidant activity, flavour, solubility, digestibility emulsifying activity, foaming capacity and stability. All protein hydrolysates showed higher solubility and digestibility than intact proteins, especially at pHs near isoelectric point of egg white proteins. Moreover, all hydrolysates had better functional properties, except emulsifying activity, than the native protein solution.

INTRODUCTION

Enzymatic hydrolysis of egg white proteins (EWPs) has shown great potential to improve their functional properties such as increased solubility, stability, and digestibility, and to reduce protein allergenicity while still retaining their nutrition value [1]. The high selectivity and mild reaction conditions associated with the enzymatic process have made this approach an attractive alternative in the production of egg white protein hydrolysate with improved functional properties, which are often difficult to obtain by conventional chemical route. Moreover, certain oligopeptides released during protein hydrolysis have been shown to possess distinctive physiological activities, such as anti-hypertensive activity, antioxidant activity, immunostimulating activity, and ACE inhibiting activity and as such may contribute to enhanced biological activities and health benefits of the hydrolysate [2].
Two major problems associated with this process have so far limited its general use. First, thermal treatment of fresh chicken egg white is often necessary prior to enzyme hydrolysis to obtain microbial safety product as well as for deactivation of Alcalase inhibitor. Furthermore, heat denatured ovalbumin and other EWPs, show an increased susceptibility to proteases [3]. Uncomplicated but relatively harsh, this treatment often decreases the nutritional and sensory properties of protein hydrolysate due to heat induced chemical reactions such as Maillard browning and destruction of cystine, arginine and lysine. Second problem is related to the

enzymatic hydrolytic step which could result in peptide mixtures poorly characterized and with unpleasant bitter taste that make them unsuitable for human consumption. Thus, the hydrolysis reaction must be carefully controlled in order to prevent bitter peptide formation and the effect of various process treatments prior to hydrolysis on the functional properties of proteins must be taken into account.

In recent years, the ultrasound treatment has been increasingly investigated as an alternative to heat pasteurization (conventional heating) for various food systems because it can be used to obtain stable products with minimal effects on flavour, colour and nutritional value or to create novel texture and taste [4-6]. To date, there seem to be limited number of commercial process based on ultrasonication, mainly because the process has not yet been optimized with respect to yield and process cost and the overall lack of knowledge of ultrasonication effects on food systems. Furthermore, the effect of ultrasound on the structural and functional properties of proteins, especially EWPs, has been less studied.

In this paper, we present the results of an investigation of combination of ultrasound pre-treatment of EWPs and their controlled enzymatic hydrolysis on the nutritional and functional properties of obtained hydrolysates. The aim of this work is to design a complete process for the preparation of egg white hydrolysate comprising the following steps: I) optimizing the process of EWPs denaturation prior to enzymatic hydrolysis, II) and determining optimal conditions for enzymatic hydrolysis in order to obtain hydrolysate with high yield of soluble protein and improved functional properties. For this purpose EWP soulution was hydrolysed with several proteases using both, one-step and two-step hydrolysis. The hydrolysate was then tested on antioxidant activity, flavour, solubility, emulsifying activity and stability, foaming capacity and stability. For comparison purposes, the pretreatment was also performed under conventional heating in a thermostatic water bath without ultrasound.

EXPERIMENTAL

Materials

Chicken egg white obtained from a local supermarket was separated from the yolk and gently stirred without foam formation to provide homogeneous mixture. Alcalase 2.4L (E.C. 3.4.21.62, proteinase from *Bacillus licheniformis* Subtilisin), Neutrase 0.8L (E.C. 3.4.24.28, protease from *Bacillus amyloliquefaciens*), papain from *Carica papaya* (E.C. 3.4.22.2) and Flavourzyme 500L (protease from *Aspergillus oryzae*) were obtained from Sigma Aldrich (St Louis, MO, USA). The enzyme activity was ≥0.8 U/g , ≥2.4 U/g and ≥500 U/g Anson Units, for Neutrase, Alcalase and Flavourzyme, respectively. One Anson unit is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25 °C at pH 7.50. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) used for radical scavenging test was also purchased from Sigma Aldrich (St Louis, MO, USA). Other chemicals were of analytical grade.

Ultrasound and Thermal Pretreatments

Egg white was dissolved in distilled water at a concentration of 10 mg mL^{-1}. Ultrasonic denaturation was investigated at pH 8.6 and at 27 °C in an ultrasonic water bath. The ultrasound treatments were performed for 30 min under a power setting of 35 kHz. The samples were half-immersed in an ice-water bath to avoid temperature increase during sonication. Each treatment was conducted in duplicate.

Thermal treatment of the egg white solutions (10 mg mL^{-1}) was performed in 500 mL glass in a temperature-controlled water bath set at a constant temperature of 75 ^{o}C for 30 minutes. After that, samples were taken out of the water bath and immediately transferred to an ice bath to stop further denaturation.

One-step Enzymatic Hydrolysis of Egg White Proteins

The one-step enzymatic hydrolysis was performed for 4 h in a batch reactor with continuous stirring by using the three enzymes separately, namely Alcalase, Neutrase and papain. These enzymes were selected on the basis of their differing activity towards EWPs. The temperature and pH during the hydrolysis was 50 ^{o}C and 8 when the EWPs solution was used for the hydrolysis by Alcalase, 50 ^{o}C and 7.0 by Nautrase and 55 ^{o}C and 7 for the hydrolysis with papain. The pH was kept at constant value by adding 0. 1 M NaOH, using a pH-stat with automatic dosage of the base. The DH was used as a parameter to measure the effect of ultrasound and thermal pretreatment on the susceptibility of EWPs to enzymatic hydrolysis. After 4 h of incubation, the reaction was terminated by heating during 10 min at a boiling water bath. The extent of hydrolysis was determined as a degree of hydrolysis (DH), by quantification of hydrolysed peptide bonds using the pH-stat technique, as previously described [7].

Two-step Enzymatic Hydrolysis of Egg White Proteins

In a two step process: step 1 consisting of hydrolysis using only one proteolytic enzyme, Alcalase, followed by step 2 which involved treatment with Flavourzyme. First EWP solution was subjected to a partial hydrolysis with one endoprotease (Alcalase) as previously described. Then, the pH value was adjusted to 7.0 by addition of 0.1 M aqueous solution of HCl and 750 µL of Flavourzyme was added. After addition of the Flavourzyme, the hydrolysis was carried out for 60 to 80 minutes and stoped by heating in a boiling water bath for 10 min.

Antioxidant Activity of Egg White Hydrolysates measured by DPPH assay

Antioxidant activity of EWP hydrolysates was measured by their ability to scavenge DPPH radical, which was monitored by decrease of absorbance on 517 nm. A volume of 200 µL of samples was mixed, in spectrophotometric cuvet, with 1800 µL of metanolic DPPH solution (0.1 mM), vortexed and left in dark and after 30 min absorbance was measured on 517 nm. Calculations were done as follow:

$$\boldsymbol{RSA}(\%) = \left[1 - \frac{(\boldsymbol{A}_s - \boldsymbol{A}_0)}{\boldsymbol{A}_b}\right] \quad (1)$$

where A_s is the absorbance of the tested sample, A_0 is the absorbance of the sample in methanol, and A_b is the absorbance of the DPPH solution without the sample.

Reducing power

The sample solution (0.5 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ^{o}C for 20 min. An aliquot (2.5 mL) of 10% trichoroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 0.1% ferric chloride and the absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicates increasing reducing power.

Functional Properties of EWP Hydrolysates

Solubility of EWP hydrolysates was determined at different pH and compared. Samples were suspended in water (5% w/v) and pH was kept at different values between 2 and 10 using 1 M NaOH or 1 M HCl while stirring at room temperature for 1 h. The samples were then centrifuged at 10,000g for 15 min and protein content was determined in the supernatants by using Lowry method [8]. Solubility was expressed as the percentage of protein remaining in the supernatant as

compared to the untreated sample. Foaming capacity and stability were determined as previously described [9].

RESULTS AND DISCUSIONS

One-Step Enzymatic Process

The Effect of Pretreatment of EWPs on Their Susceptibility to Proteases

To study the effect of ultrasound treatment on susceptibility of EWPs to proteases, different endoproteases were separately added to the thermal or ultrasound treated egg white solution and the reaction was carried out under optimum conditions for each protease. Hydrolytic curves of pretreated EWPs by Alcalase, Neutrase and Papain were compared (Fig. 1) for both pretreatments.

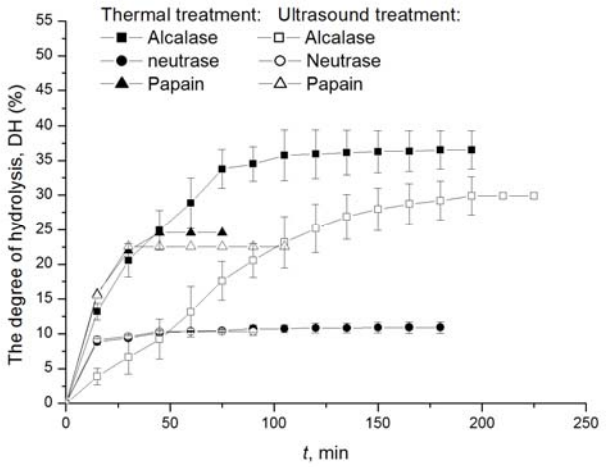

Figure 1. The comparison of the DH profiles of EWPs hydrolysis by several endoproteases after thermal treatment (full symbols) and ultrasound treatment (open symbols). The reaction was run for 4 h at pH 8.0, 50 °C, at pH 7.0 and 50 °C and at pH 7.0 and 55 °C for Alcalase, Neutrase, and papain, respectively.

The highest hydrolysis degree was achieved for Alcalase followed by papain and Neutrase, irrespective of the pretreatment used. However, for thermal treated sample, the initial reaction rates were slightly higher with Papain than with Alcalase and, for ultrasound treated EWPs, with both Papain and Neutrase. In the case of hydrolysis by papain, a rapid reaction rate was obtained in the first 15 min, then the rate of hydrolysis subsequently decreased. A constant rate was also observed with Neutrase after 15 min of reaction time, suggesting that the enzymatic reaction reached the steady-state phase.

Antioxidant Activity of obtained EWP Hydrolysates

The ultimate aim of this study was to achieve a high degree of hydrolysis, but also an end product (hydrolysate) with improved biological and functional properties. Thus, the radical scavenging activity of hydrolysates during the one-step hydrolysis by the three proteases was tested and compared. The results are presented in Fig. 2.

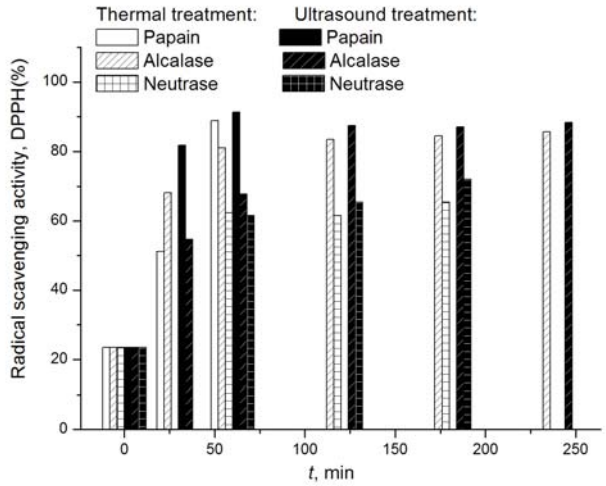

Figure 2. Antioxidant activities of EWP hydrolysates (DPPH activity) obtained with different commercial proteases for thermal (white patterns) and ultrasound pretreated EWPs (black patterns). The experiments were performed at 50 ^{o}C and pH 8.0 for Alcalase, at 50 ^{o}C and pH 7.0 for Neutrase, and at 55 ^{o}C and 7.0 for papain, after thermal heating (75 ^{o}C) and sonication at 35 kHz for 30 min.

It appeared that hydrolysates obtained by Alcalase, Neutrase and papain exhibited different radical scavenging activities depending on both pretreatment and type of protease, which can be directly attributed to differences in the specificity of the proteases used. The papain hydrolysate obtained after ultrasound pretreatment demonstrated the highest radical scavenging activity at 30 and 60 min. Also, ultrasound pretreated samples had better DPPH properties compared to thermal pretreated for both, Alcalase and Neutrase at higher degrees of hydrolysis, suggesting that the pretreatment could have effect on proteases` specificity. It appeared that mechanical effect of ultrasonication resulted in structural changes in EWPs that altered the secondary and tertiary structure of the macromolecule which may be attributed to the formation of an ultrasonically induced state that differs from a thermally induced state. However, only slight differences were found in terms of antioxidant activity between ultrasound and thermal pretreated samples and this should be further investigated in the two-step enzymatic process.

Two-Step Enzymatic Process

Two-step enzymatic process combined random activity of one endoprotease such as Alcalase with a specific activity of a commercial exopeptidase-rich preparation (Flavourzyme). For this purposis, EWPs were subjected to a partial hydrolysis (DH≈0.15) with Alcalase. Then, the resulting peptide mixture can be degraded completly or partly to the amino acids and di-, tri- and oligopeptides by the use of Flavourzyme. A graphical comparison of results obtained for the two step enzymatic EWPs hydrolysis for thermal and ultrasound pretreated EWPs under the same operating conditions is shown in Fig. 3.

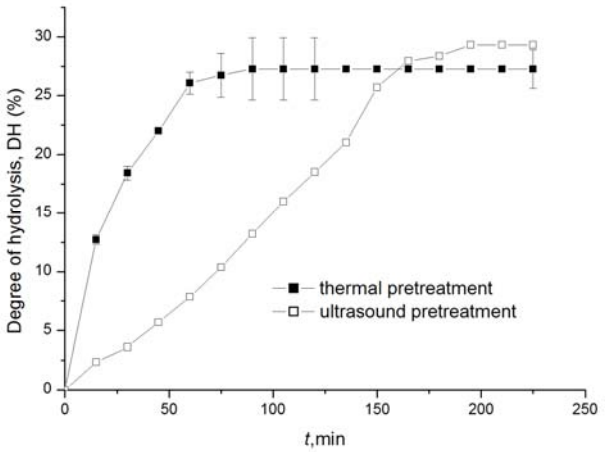

Figure 3. Hydrolysis curves of EWP solution (heated for 30 min at 75 °C or sonicated for 30 min at 35 kHz) during hydrolysis by Alcalase and Flavourzyme in the two-step enzymatic process

Although the initial rate seemed too be higher with thermal pretreated EWPs compared to that ultrasound pretreated, even higher equilibrium degree of hydrolysis was achieved with ultrasonicated protein sample. The aim is to obtain a high degree of hydrolysis but also an end hydrolysate with high nutritional value and improved functionality. Thus, antioxidant activity as measured by DPPH method and several functional properties were detected in all EWP hydrolysates during the course of hydrolysis. The results are summarized in Table 1. It appeared that the antioxidant properties were enhanced by hydrolysis in both of cases.

Table 1. DPPH radical-scavenging activity, reducing power, flavour, nutritional and functional properties of EWP hydrolysates produced using different combination of proteases for ultrasound (UP) and thermal pretreatment (TP)

Parameter	Intact EWPs, pH 8.4	Alcalase		Alcalase, Flavourzyme		Papain	
		UP	TP	UP	TP	UP	TP
Degree of hydrolysis, DH(%)	0	30.90	36.52	29.30	25.40	22.58	24.58
Foaming capacity, %	260	404	276	488	184	360	257
Foam stability, %	12	264	140	76	28	192	85
Emulsifying capacity, mL/g	30	20	10	38	18	32	30
Digestibility, %	9.21	26.87	58.90	10.62	50.65	28.40	41.59
Solubility, % at pH 6	51.18	96.82	100	83.11	72.83	91.81	88.15
DPPH activity, %	23.45	88.90	86.67	70.24	59.94	91.33	88.86
Reducing power,	0.019	0.017	0.014	0.016	0.036	0.021	0.020
Flavour	–	bitter	bitter	Pleasant taste	Pleasant taste	bitter	bitter

Enzymatic hydrolysis seemed to increase solubility, digestibility and antioxidant activity of EWPs in all cases. Hydrolysis also caused enhanced foaming properties of EWPs, but this effect was strongly dependent on the pretreatment before hydrolysis. Namely, the most voluminous foams were obtained in the two-step enzymatic process with Alcalase and Flavourzyme for

ultrasound pretreatment, while the most stable, dense foams were obtained in the one-step process with Alcalase, for both heat and ultrasound pretreatment. Overall, the relationship between the pretreatment-induced changes in the physicochemical properties and the functional properties of EWPs seemed to be clear. It was reported that application of ultrasound to wheat germ protein causes changes in protein structure, like increase in protein surface hydrophobicity, activity and charge and decrease in sulfhydryl group content, that may alter its bulk functionality [10]. However, for both pretreatments, a specificity of protease could be found to predict accurately the functional properties of EWP hydrolysates. For example, hydrolysate with high antioxidant activity determined by two methods was obtained by papain, while the hydrolysate with pleasant taste was obtained with combined activity of Alcalase and Flavourzyme.

Conclusion

Thus, in general, ultrasound treatment enhances EWP hydrolysis, and depending upon the choice of proteases, increases the antioxidant activity of the hydrolysates. Emulsifying and foaming properties of the protein hydrolysates were also dictated by both factors. The combined ultrasound pre-treatment and enzymatic hydrolysis, not only represents a rapid, efficient and reliable alternative to improve the quality of EWPs, but it also has the potential to develop new products with a unique functionality.

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