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EFFECTS OF SONICATION AND HIGH-PRESSURE CARBON DIOXIDE PROCESSING ON ENZYMATIC HYDROLYSIS OF EGG WHITE PROTEINS

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The objectives of this study were to examine the effect of sonication and high-pressure carbon dioxide processing on proteolytic hydrolysis of egg white proteins and antioxidant activity of the obtained hydrolysates. It appeared that the ultrasound pretreatment resulted in an increase in the degree of hydrolysis of the enzymatic reaction while the high-pressure carbon dioxide processing showed an inhibition effect on the enzymatic hydrolysis of egg white proteins to some extent. The antioxidant activity of the obtained hydrolysates was improved by ultrasound pretreatment of egg white proteins at the pH 8.3. Thus, the combination of ultrasound pretreatment at the pH 8.3 and subsequent enzymatic hydrolysis with alcalase at 50°C and pH 8.0 could offer a new approach to the improvement of the functional properties of egg white proteins and their biological activity.

KEY WORDS: Egg white proteins, alcalase, antioxidant activity, ultrasound pretreatment, high-pressure carbon dioxide processing

INTRODUCTION

Egg producers are faced with the problems of excess of egg white because the mayonnaise and bakery industries use relatively large amounts of egg yolk, and egg white is the remainder. Although egg white proteins (EWPs) have unique functional characteristics such as excellent gelling and foaming and almost perfect amino acid composition, their high viscosity and allergenicity are limiting factors for their widespread use in food products, especially in the case of medical, dietary and infant foods (1).

Enzymatic hydrolysis of egg white proteins has been proven to be an effective approach to improve their properties such as increased solubility, emulsification, water holding capacity, stability, digestibility, and to reduce protein allergenicity while still retaining their nutrition value (2). 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 as such may contribute to enhanced biological activities and health benefits of the hydrolysate (3). However, the production of these functional ingredients requires new and innovative technologies

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because they are sensitive to a variety of environmental processing factors that may cause the loss of nutritional quality and chemical degradation.

Major proteins in chicken egg white including ovalbumin and ovotransferin, in their native forms have a low susceptibility to digestion by alcalase, trypsin or α-chymotrypsin. Heat denatured ovalbumin however, shows an increased susceptibility to these proteases (4). However, thermal treatments are obviously the most critical steps able to impair protein functionalities, inducing various chemical reactions such as Maillard browning which may lead to nutritional, sensory and safety deterioration in egg white hydrolysates. Several papers reported a decrease in the nutritional properties of different natural protein sources due to thermal processing. Thus, a lot of research works have been performed to investigate the effectiveness of replacing conventional thermal treatment with several non-thermal alternative approaches for improvement of the overall process performances of EWP hydrolysis and produce protein solutions with new functional properties (4,5).

High-pressure processing (HPP) is popular as an alternative to heat pasteurisation 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 (6,7). To date, there seem to be a limited number of commercial processes based on HPP, mainly because the process has not yet been optimized with respect to yield and process cost and the overall lack of knowledge of HPP effects on food systems, especially egg white proteins.

The purpose of this research was to study the effect of ultrasound pretreatment and high-pressure carbon dioxide processing on the *in vitro* digestibility of egg white proteins by alcalase. The influence of pretreatment parameters including pH, pressure, and holding time on enzymatic hydrolysis of pretreated EWPs was studied. The antioxidant activity of the obtained hydrolysates has also been determined and compared.

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 (**proteinase from *Bacillus licheniformis* Subtilisin)** was obtained from Sigma Aldrich (*St Louis, MO,* USA). The enzyme activity was ≥2.4 U/g Anson Units, where 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 colour with the Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25°C at the pH 7.50. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) used for radical scavenging test was also purchased from Sigma Aldrich (St Louis, MO, USA). Commercial carbon dioxide (99% purity) was supplied by Messer-Tehnogas (Serbia). Other chemicals were of analytical grade.

Sonication pretreatment

Ultrasonic denaturation was investigated in the pH range of 6.0 to 10.0 at 27°C in an ultrasonic water bath. Prior to sonication, the pH of the 10% (w/w) egg white solution was adjusted to 6.0-10.0 using 0.1 M HCl or 0.1 M NaOH. The ultrasound treatments were performed for 15, 30, 60, and 180 min under a power setting of 30 kHz. The samples were half-immersed in an ice-water bath to avoid temperature increase during sonication. Each treatment was conducted in duplicate.

High-pressure carbon dioxide processing

The experiments of denaturation of EWPs were conducted in the Autoclave Engineers Screening System with the configuration previously described (8), shown in Figure 1. The extractor vessel (150 ml) was filled with 10% (w/w) egg white solution, and temperature was risen to 35°C. Heaters were supplied on the extractor vessel for temperature elevation. A thermocouple, connected to a temperature controller, was used to control and maintain a constant temperature.

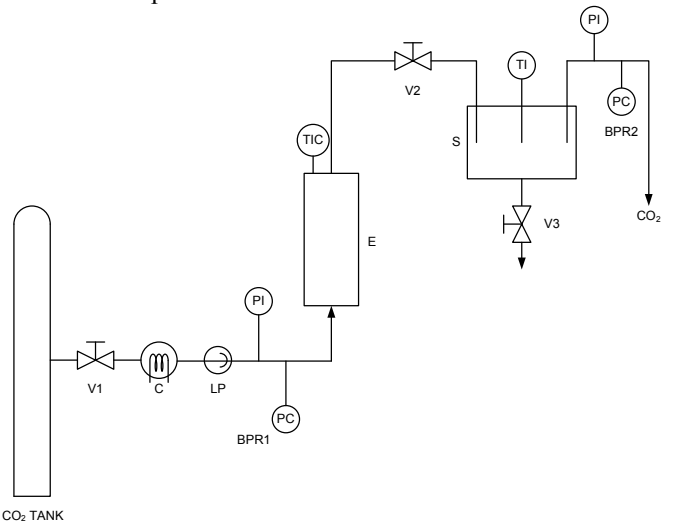

Figure 1. Scheme of the autoclave engineers screening system - C: cryostat; LP: high pressure liquid pump; BPR: back pressure regulators; E: extractor vessel; S: separator vessel (8).

Liquid CO_2 was supplied from a CO_2 cylinder by a siphon tube. The liquid CO_2 was cooled in a cryostat between the cylinder outlet and the pump. The pump operated at a maximum output pressure of 41.3 MPa and an adjustable flow rate from 38 to 380 mL/h. The CO_2 was pumped into the system until the required pressure was obtained. Back pressure regulators were used to set the system pressure (in the extractor and separator).

The sample was exposed to SC CO_2 at 35°C and 10 MPa, 20 MPa and 30 MPa for 20 min in a batch vessel. After 20 min of exposure, the pressure was decreased very fast to atmosferic conditions. During the decompression, the SC CO_2 flew through the extractor

and entered the separator vessel (500 ml) with treated egg white solution. The CO_2 continued to flow out of the separator through the flowmeter/totalizer and out to atmosphere. The pressure and temperature were controlled to an accuracy of ±0.4 MPa and ±0.5°C, respectively.

Samples of the treated egg white solution were taken by opening the ball valve located at the bottom of the separator vessel.

Enzymatic hydrolysis of EWPs

The pretreated aqueous egg white solutions (360 mL, 10 mg of protein/mL, pH 8.0) were hydrolysed at 50 °C by adding 2.3 Units of alcalase. The pH was kept at 8.0 by adding 0.01 M NaOH, using a pH-stat (Metrohm, Basel, Switzerland) with automatic dosage of the base. The degree of hydrolysis (DH) was used as a parameter to measure the effect of ultrasound and high-pressure pretreatment on the susceptibility of egg white proteins to enzymatic hydrolysis. After 4 h of incubation, the reaction was terminated by heating during 10 min on a boiling water bath. The DH was calculated by the pH stat method according to Adler-Nissen (9), using the following equation:

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} = N_{\mathrm{b}} \times B \times \frac{1}{\alpha} \times \frac{1}{m_{\mathrm{p}}} \times \frac{1}{h_{\mathrm{tot}}} \times 100 \qquad [1]$$

where h is the number of equivalents of peptide bonds hydrolysed at the time t; h_{tot} is the total number of peptide bonds in protein substrate in mmol/g_{protein}; B is the base consumption in mL; N_{b} is the base normality; α is the average degree of dissociation of the α-NH groups, and m_{p} is the mass of protein in g.

Antioxidant activity measured by DPPH assay

The antioxidant activity of EWP hydrolysates was measured by their ability to scavenge DPPH radical, which was monitored via the decrease of the absorbance at 517 nm, as described elsewhere (10). A volume of 200 μL of samples was mixed, in spectrophotometric cuvette, with 1800 μL of methanolic DPPH solution (0.1 mM), vortexed and left in dark and after 30 min absorbance was measured on 517 nm. The calculation was done as follows:

$$RSA(\%) = \left[1 - \frac{(A_s - A_0)}{A_b} \right] \qquad [2]$$

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.

RESULTS AND DISCUSSION

Sonication pretreatment

As sonication-induced protein denaturation strongly depends on the temperature and pH at which pretreatment occurs, the effect of ultrasound pretreatment was studied at

constant temperature and at three pH values: 6.0, 8.3 and 10.0. The aim of optimization was to find the conditions that would lead to irreversible denaturation of the alcalase inhibitor, but would not result in the formation of dense and compact precipitates, which are poor substrate for proteolysis.

As shown in Figure 2, it appeared that for all cases studied, the ultrasonic pretreatment changed the proteolytic pattern of EWPs, resulting in an increase in their digestibility by alcalase. This ultrasound-dependent increase in the susceptibility to enzymatic hydrolysis is in accordance with the results of Su et al. (11), who observed an increase of susceptibility to alcalase hydrolysis of egg white after 120 min treatment at 40 kHz. It is considered that the sonication produces hemolytic water molecule cleavage, generating high-energy intermediates such as hydroxyl and hydrogen free radicals, and therefore, causing structural changes of proteins.

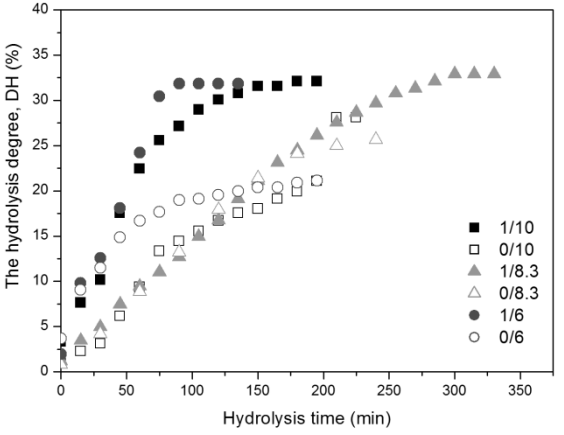

Figure 2. Comparison of the DH profiles of EWPs hydrolysis by alcalase at the pH 8.0 and 50°C. The labels indicate samples with different sonication pretreatment (0/10 means alkali pretreatment at the pH 10 without ultrasound treatment; 1/10 means 1 h sonication at the pH 10.0; 0/8.3 means alkali pretreatment at pH 8.3 without ultrasound treatment; 1/8.3 means 1 h sonication at pH 8.3; 0/6 means acid pretreatment at the pH 6.0 without sonication; and 1/6 means 1 h sonication at the pH 6.0).

In contrast, an increase in the sensitivity of ovotransferrin to proteolysis by using thermolysin after sonication was not observed by Lei et al. (4). However, these authors studied the effect of ultrasound pretreatments on model egg white protein – ovotransferrin. In this case, the reaction mixture seemed to be free from proteases' inhibitors such as ovomucoid. It is one of the major egg white protein accounting for 11% in egg white, well known as a strong inhibitor of alcalase. The increase in the susceptibility of egg white solutions to enzymatic hydrolysis after sonication could also be due to the ovomucoid denaturation.

The strongest increase in the susceptibility of egg white solution to enzymatic hydrolysis was observed after ultrasound treatment at an acidic or alkaline pH. The partially unfolded conformation of the major EWPs formed by sonication may be attributed to the

increase of the susceptibility but this also could be the result of disulfide exchange and formation of non-native disulfide bonds in ovomucoid, resulting in its irreversible denaturation. It appeared that its effective denaturation was attained by combined alkali/acid and ultrasonic treatment.

A longer treatment time (up to 60 min) resulted in a higher subsequent susceptibility to enzymatic hydrolysis (Figure 3).

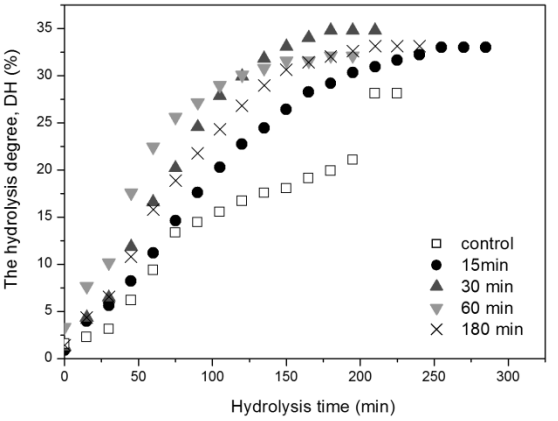

Figure 3. Time-dependent changes in the susceptibility of egg white solutions due to ultrasound pretreatment at the pH 10 lasting 0 min (□); 15 min (●), 30 min (▲), 60 min (▼), and 180 min (×). Hydrolysis condition: 2.3 Units of alcalase, 10% w/w egg white, pH 8.0, 50 ^{o}C.

The prolonged exposure to ultrasound of 60 min at the pH 10.0 seemed to have a negative effect on the EWPs hydrolysis. Under this condition, a turbid suspension of protein aggregates could be observed, which might lower the accessibility of the unfolded proteins to the alcalase.

High-pressure carbon dioxide processing

As shown in Figure 4, the high-pressure carbon dioxide processing showed an inhibitory effect on the enzymatic hydrolysis of EWP. The highest degree of hydrolysis was observed under the pretreatment at 30 MPa, but it was still lower than that achieved without pretreatment. Hayashi et al. (12) reported an extensively improved digestibility by subtilisin of homogenized pressure-induced egg white gels as compared to raw egg white.

Although some other researchers showed that carbon dioxide treatment could inactivate pectin esterase, lipoxygenase, polyphenol oxidase and peroxidase either in pure enzymatic solutions or in real food systems, the understanding of the synergetic effect between ultrasounds and enzymes is still far from being completely understood.

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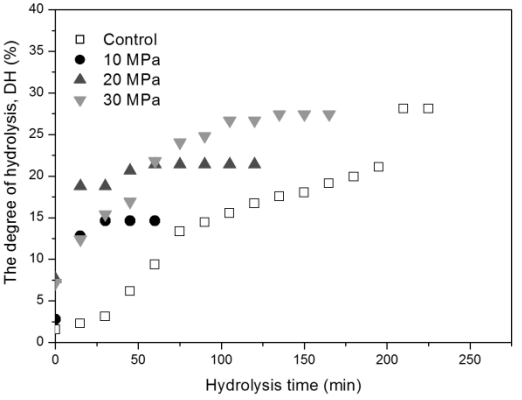

Figure 4. Comparison of the DH profiles of EWPs hydrolysis by alcalase at the pH 8.0 and 50 ^{o}C. The samples were pretreated under different high pressure carbon dioxide conditions: (●) 10 MPa; (▲) 20 MPa; (▼) 30 MPa.

Antioxidant activities of sonication-pretreated alcalase hydrolysates

The release of bioactive peptides from their parent proteins is affected by various factors such as temperature, pH, enzyme, pressure, sonication, and others. The production of bioactive peptides was monitored after the hydrolysis. The antioxidant activities of samples treated under different ultrasound conditions followed by alcalase hydrolysis are compared in Figure 5.

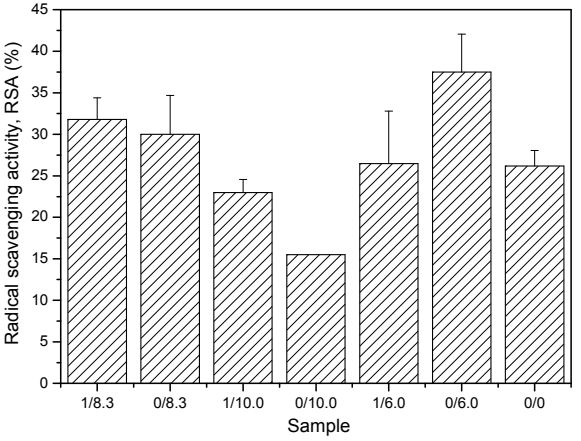

Figure 5. Effects of sonication on the radical scavenging activity of egg white hydrolysates obtained with or without sonication treatments. Data were the results of three individual determinations. Labels of 0/0 means raw egg white, 0/6.0 means acid egg white treatment without sonication treatment, 0/10 means alkali but no sonication treatment, 1.6.0, 1/8.3, and 1/10.0 means 1 hours sonication at the pH 6.0, 8.3, and 10.0, respectively. Error bars represent the standard deviations of triplicate measurement.

The results indicate that 1-hour sonication treatment at the pH 8.3 improved the antioxidant activity of raw egg white, while the sonication at the pH 6.0 or at 10.0 did not have a substantial effect on radical scavenging activity.

CONCLUSION

The study addressed the effect of the ultrasound pretreatment at 27^{o}C and at atmospheric pressure and of high-pressure carbon dioxide processing in the range of 10-30 MPa on the enzymatic hydrolysis of EWPs with alcalase. As the pH seemed to strongly affect the ultrasound induced protein denaturation, the effect of pH during sonication in the range 6.0-10.0 on the egg white susceptibility to enzymatic hydrolysis was investigated. The antioxidant activity of obtained hydrolysates was also tested. The ultrasound pretreatment of egg white proteins resulted in an increase in the degree of hydrolysis of the enzymatic reaction, while the high-pressure carbon dioxide processing showed an inhibition effect on the enzymatic hydrolysis of EWPs to some extent. The antioxidant activity of the obtained hydrolysates was improved by ultrasound pretreatment of EGPs at the pH 8.3. Thus, the combination of ultrasound pretreatment at the pH 8.3 and subsequent enzymatic hydrolysis with alcalase at 50^{o}C and pH 8.0 could offer a new approach to improve the functional properties of EWPs and their biological activity. The sonication procedure is simple, rapid and efficient, and could be useful for the industrial production of functional products from egg white.

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УТИЦАЈ СОНИКАЦИЈЕ И ПРЕТРЕТМАНА ВИСОКИМ ПРИТИСКОМ НА ЕНЗИМСКУ ХИДРОЛИЗУ ПРОТЕИНА БЕЛАНЦЕТА

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Протеини беланцета спадају у веома квалитетне протеине због свог јединственог аминокиселинског састава. Међутим, већа комерцијална примена хидролизата протеина беланцета је ограничена услед неадекватног процесног третмана при обради и стерилизацији беланцета термичким третманом као и хемијској хидролизи протеина, који доводе до значајне промене боје, укуса, функционалности и нутритивних својстава производа. Циљ овог рада је био да се испита могућност примене нетермичких третманима, као што су соникација и третман високим притиском, да би се унапредила ензимска хидролиза протеина беланцета и омогућило добијање хидролизата са антиоксидативном активношћу. Показано је да третирање протеина беланцета ултразвуком под одређеним условима доводи до њихове касније побољшане хидролизе алкалазом, док процесирање беланцета високим притиском има негативан утицај на активност алкалазе. Антиоксидативна активност добијених хидролизата је повећана након претретмана ултразвуком на pH 8,3. Тако, комбинација претретмана протеина беланцета ултразвуком на pH 8,3 и њихова сукцесивна хидролиза алкалазом на 50^{o}C и при pH 8,0 се показала као ефикасна алтернативна метода за добијање хидролизата протеина беланцета са унапређеним функционалним својствима и биолошком активношћу.

Кључне речи: протеини беланцета, алкалаза, антиоксидативна активност, претретман ултразвуком, процес под високим притиском угљен-диоксида

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