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Impact of ultrasound pretreatment on whey protein hydrolysis by vegetable proteases

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ABSTRACT

The ultrasound treatment on whey protein before enzymatic hydrolysis using vegetable proteases was studied. Ultrasound density, temperature, and pretreatment time effects were evaluated on enzymatic degree of hydrolysis, release of Angiotensin I-Converting Enzyme (ACE) inhibitors, and antioxidant activity of the hydrolysates generated. In addition, thermal properties as measured by Differential Scanning Calorimetry (DSC) and changes in secondary structure by circular dichroism (CD) were measured. The results showed that ultrasound density was the only factor that exerted a significant effect on proteolysis increasing the ACE inhibition by 13% and a 95% reduction of hydrolysis time in bromelain hydrolysates, but did not improve the ACE inhibition on papain hydrolysates. Observed changes in denaturation enthalpy (ΔH), reduction of reactive thiol groups, and changes in secondary structure suggest protein rearrangements and aggregate formation. The effect of ultrasound on the whey protein structure produced changes increasing the bioactivity of hydrolysates that could enhance the enzymatic hydrolysis process.

Industrial relevance: Whey proteins are an important source of bioactive peptides. However, due to their globular structure, they are a complex substrate for enzymatic hydrolysis. Ultrasonic pretreatment could induce changes increasing susceptibility to enzymatic hydrolysis resulting in production of more bioactive peptides. Therefore, treatment of whey proteins with ultrasound waves before enzymatic hydrolysis, offers higher efficiency of enzymatic bioconversion of proteins and production of new biologically active peptide mixtures.

1. Introduction

The use of ultrasound technology is an innovative topic for the food industry, as it has been used as a tool to modify and to enhance different food processes (Knorr, Zenker, Heinz, & Lee, 2004; Tao & Sun, 2015). Ultrasound technology is based on the use of mechanical waves with frequencies above 20 kHz. When a liquid is subjected to high intensity ultrasound, a physical phenomenon called acoustic cavitation is generated. Cavitation involves the formation, growing, and collapse of small bubbles inside the liquid as a result of pressure changes. This process induces physical, mechanical, and chemical effects on liquid systems (Mason & Peters, 2002).

The application of ultrasound on proteins has shown effects on their hydration, molecular size, hydrophobicity, and conformation (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Jambrak, Mason, Lelas, Paniwnyk, & Herceg, 2014; O'Sullivan, Murray, Flynn, & Norton, 2016). Observed structural changes in proteins due to ultrasound treatment, also affects many functional properties such as solubility, viscosity, emulsification, gelation, among others (Arzeni et al., 2012; O'Sullivan, Arellano, Pichot, & Norton, 2014). The use of ultrasound for improving properties and performance of high-value protein based ingredients represents an emerging technology for the modification of their properties for their final application in food systems.

Proteins are used as a substrate in the production of bioactive peptides (BAPs). These BAPs are short amino acid sequences that are inactive in their precursor protein. However, once released by technological means, they may interact with selected receptors and regulate physiological functions (Dziuba & Dziuba, 2014). Potential health

Abbreviations: ABTS, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); ACE, Angiotensin-I-converting enzyme; BAPs, Bioactive peptides; CD, Circular dichroism; DH, Degree of hydrolysis; DSC, Differential scanning calorimeter; DTT, Dithiothreitol; HLL, Hippuril-His-Leu; MRW, Mean residue molecular weight; OPA, o-phtaldialdehyde; PBS, Phosphate buffer saline; ΔH, Denaturation enthalpy.

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benefits associated with BAPs consumption have caused them to be highly interesting to many researchers. (Li-Chan, 2015). Most studies on BAPs bioactive effects are focused on ACE inhibition. This enzyme plays a key role in controlling blood pressure in the rennin angiotensin system (Vermeirssen, Camp, & Verstraete, 2004).

Release of BAPs from their parent proteins by proteolysis is affected by several factors such as hydrolysis time, pH, temperature, and enzyme-substrate ratios (Udenigwe & Aluko, 2012). Currently, the release efficiency is not sufficient enough for use in the food industry. Therefore, an emerging strategy being researched in the BAPs production is the use of treatments prior to enzymatic hydrolysis (Adjonu, Doran, Torley, & Agboola, 2013; Hernández-Ledesma, del Mar Contreras, & Recio, 2011). Thermal treatment has been applied to modify the peptide profile of the hydrolysates (Leeb, Kulozik, & Cheison, 2011). However, emerging technologies such as high hydrostatic pressure (Garcia-Mora, Peñas, Frias, Gomez, & Martinez-Villaluenga, 2015; Quirós, Chichón, Recio, & López-Fandiño, 2007) and ultrasound (Huang, Liu, Ma, & Zhang, 2014; Uluko, Zhang, et al., 2014) are advantageous for being environmentally friendly and for having promising results, such as increased determinate bioactivity and reduced hydrolysis time. The effect of ultrasound on proteins before the enzymatic hydrolysis can improve the release of BAPs (Kadam, Tiwari, Álvarez, & O'Donnell, 2015; Ozuna, Paniagua-Martínez, Castaño-Tostado, Ozimek, & Amaya-Llano, 2015), due to the unfolding and increased accessibility of enzymes, however it is affected by protein structure and its purity.

Whey proteins are recovered from cheese whey, an industrial byproduct with high environmental impact. However, it also can be transformed into high value-added products, such as BAPs, which is a successful handling strategy for its management (Smithers, 2015; Yadav et al., 2015). Whey hydrolysates from different enzymes have shown antihypertensive, antioxidant, immunomodulatory, and antimicrobial activities (Tavares et al., 2012). However, whey proteins are not easily broken down by proteases, due to their globular structure, and their hydrophobic core (Tavares & Malcata, 2013). Some studies have included a heating pretreatment to increase the hydrolysis rate using gastrointestinal enzymes (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010; Leeb, Götz, Letzel, Cheison, & Kulozik, 2015). Many enzymes have been used in BAPs production, mainly gastric and microbial, whose biotechnological production has a lowered their commercial cost. On the contrary, proteases from vegetables sources have been poorly studied and they present an opportunity as certain population sectors prefer natural alternatives for their production (Corrons, Bertucci, Liggieri, López, & Bruno, 2012). Vegetable proteases have shown highly proteolytic action when used in cheese making, which makes them an interesting alternative for their use in enzymatic hydrolysis for BAP production.

The objective of this study was to apply ultrasound pretreatment on whey proteins in order to evaluate the ultrasonic pretreatment, and to identify the main factors involved in enzymatic hydrolysis of whey proteins. This was done by using a central composite design and evaluating two vegetable proteases (bromelain and papain). The bioactivity (ACE inhibition and antioxidant activity) of the hydrolysates generated was also assessed, focusing on the correlation of the structural modification of whey proteins due to ultrasound pretreatment.

2. Materials and methods

2.1. Whey preparation

Sweet whey was prepared according to Fuda and Jauregi (2006), where fresh whole pasteurized milk was heated to 37 °C, then 0.03% v/v commercial calf rennet solution was added (Caglificio Clerici S.p.A, Italy) and stirred gently for 5 min. Casein coagulation was performed for 1 h. The curd was then cut and left to settle for another 30 min then whey was filtered using cheesecloth. Remaining protein particles and fat were eliminated by centrifugation at 10,000 $\times g$ at 4 °C for 30 min. The whey was then separated by filtration through a Whatman no. 4 filter paper. The protein content on whey (8.13 ± 0.13 g/L) was determined by the Bradford method (Bradford, 1976) and the sample was stored at −18 °C. The analyses were carried out by thawing the stored whey samples at 4 °C for 12 h before its use.

2.2. Experimental design of ultrasound pretreatment

The experimental design for the study of factors involved in ultrasonic pretreatment included three input variables (k = 3), ultrasound energy density (W/mL), ultrasound pretreatment time (min), and temperature under ultrasound treatment (°C), each of which were set at two levels. Table 1 shows the complete central composite design with three central points. The degree of hydrolysis (%), ACE inhibitory activity (%), and antioxidant activity (%) were the dependent variables used to evaluate the effects on whey hydrolysates.

Thawed whey samples were pretreated using an ultrasound homogenizer (750 W nominal power at 20 kHz) with a 13 mm titanium diameter probe (Cole Palmer, Instruments Company, USA) at 30, 45 and 60% of amplitude. In order to avoid overheating the solution due to sonication, it was jacketed with chilled water while undergoing sonication and the temperature was set at different levels according to the conditions defined by the experimental design. The temperature under sonication was measured using a thermocouple (HI 9063, Hanna instruments Ltd. Texas, USA).

The acoustic energy densities of the three levels used in the experimental design were determined by calorimetry, recording the temperature as a function of time using a thermocouple (HI 9063, Hanna instruments Ltd. Texas, USA). The following equation was used (Margulis & Margulis, 2003), where the absolute power P is given as:

$$P = m \times Cp \times \left(\frac{dT}{dt}\right)$$

where: m is the mass of the sonicated liquid (g), Cp is the specific heat of the medium at a constant pressure (4.081 J/gK^{-1} data from Walstra, Walstra, Wouters, and Geurts (2005)) and dT/dt is the slope at the origin of the curve. The Acoustic Energy Density (AED) was calculated by dividing absolute ultrasound power (P) by the volume (V) of the medium (200 mL).

$$\mathrm{AED} = \frac{\mathrm{P}}{\mathrm{V}}$$

The three levels of ultrasonic density applied to the whey pretreatments with a 20 kHz probe, were 0.092, 0.151 and 0.220 W/mL.

2.3. Enzymatic hydrolysis

After applying ultrasound pretreatment and during the hydrolysis, the pH of the sweet whey was corrected to 7.0 using 0.1 M NaOH. The hydrolysis temperature was set at 50 °C for bromelain and 60 °C for papain (Enzyme Development Co. NY, USA). Enzymes were added in a 1:20 Enzyme/Substrate ratio and immediately stirred at 180 rpm in a magnetic stirrer for 180 min. Protein hydrolysis was stopped after 180 min by heating the mixture in a water bath at 90 °C for 15 min. Kinetics for the hydrolysis of the control sample were carried out using the thawed native whey without applying pretreatment.

2.4. Quantification of degree of hydrolysis using OPA method

The degree of hydrolysis (DH) was evaluated based on the number of peptide bonds cleaved. The o-phtaldialdehyde (OPA) method as described by Nielsen, Petersen, and Dambmann (2001), which measures the reaction between free amino group, OPA and dithiothreitol (DTT)