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High hydrostatic pressure assisted enzymatic hydrolysis of whey proteins

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ABSTRACT

Whey proteins, due to their high nutritional value, are generally hydrolyzed to reduce the allergenicity and used as ingredients in many special products, such as infant formulae, geriatric products, highly energetic supplements or dietetic foods or in foods produced to prevent nutrition related diseases, like food intolerances and allergies. The aim of this work was to assess the applicability of innovative technologies, such as high hydrostatic pressure (HHP) processes, to assist the enzymatic hydrolysis of target proteins, namely whey protein concentrate (WPC-80), in order to modify their antigenicity. Experiments were carried out to verify the effectiveness of HHP technology to accelerate whey protein hydrolysis reaction with selected enzymes (α-chymotrypsin, bromelain), and to affect the protein allergenic power. To this purpose, different HHP treatments were carried out at several pressure levels (100, 200, 300 and 400 MPa) and the untreated whey protein samples were used as control. A defined enzyme/substrate ratio of 1/10 w/w was used in the experiments, while the treatment time was changed from 0 to 30 min (0, 5, 15, or 30 min).

The experimental data demonstrated that High Hydrostatic Pressure (HHP) induced WPC-80 unfolding at the highest value of the pressure applied (400 MPa) as indicated by the higher exposure of free sulfhydryl groups. When HHP was used in combination with enzymatic hydrolysis, the degree of hydrolysis increased not only with the pressure level applied but also with the processing time. These results suggested that, even if the exposure of hidden epitopes upon protein unfolding increased the antigenicity of whey proteins, further peptide bonds cleavage also took place after hydrolysis. This effect could modify whey proteins antigenic sequences, and thus their antigenic power.

1. Introduction

Whey, a by-product of the dairy industry, represents an excellent source of functional and nutritional compounds (proteins and peptides, lipids, vitamins, minerals and lactose). Sweet whey (pH > 6.4) derives from cheese manufacturing and casein production by the rennet coagulation of milk, while acidic whey results from processes based on destabilization of milk casein colloids by acidification at pH level below 5.0 (Carvalho & Maubois, 2010; Tamime, 2009). Whey proteins recovered from whey are generally characterized by a high biological value mainly due to the high concentration of essential amino acids (isoleucine, leucine, threonine, tryptophan and valine), which play an important role as metabolic regulators in protein and glucose homeostasis and lipid metabolism (De Wit, 1998).

Whey proteins consist mainly of β-lactoglobulin (55–60%) and α-lactalbumin (15–20%), but also other minor proteins are present, such as bovine serum albumin (5–10%), immunoglobulins, lactoferrin, phospholipoproteins, as well as bioactives and enzymes (Heine, Klein, & Reeds, 1991; Peñas, Prestamo, Luisa Baeza, Martinez-Molero & Gomez, 2006; Peñas, Restani, et al., 2006; Peñas, Snel, Floris, Prestamo & Gomez, 2006). β-lactoglobulin (β-lg), which represents approximately 50% of total whey proteins, is a globular protein extremely stable in acidic environment and produced in the mammary gland and secreted in milk. The primary structure of β-lactoglobulin contains 162 amino acids, one free thiol group and two disulfide bridges and has a molecular weight of 18.3 kDa. α-Lactalbumin is a globular protein found in the milk of all mammals. Its primary structure contains 123 amino acids, has a molecular weight of 14.2 kDa and plays an important role in lactose biosynthesis. The primary structure of Bovine Serum Albumin (BSA), a residual protein found in both blood serum and in milk of all mammals, contains 582 amino acids and has a molecular weight of 66 kDa. BSA is the only whey protein that is not produced by the mammary gland but enters in the milk by passive diffusion from blood streams (Heine et al., 1991; Peñas, Prestamo, et al., 2006; Peñas, Restani, et al., 2006; Peñas, Snel, et al., 2006).

Whey protein concentrate (WPC) and whey protein isolates (WPI) are used in the manufacturing of yogurt, processed cheese, infant

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formulae, products for athletes and weight management products and in various bakery applications, since it is combining the effects of protein, lactose and minerals, as well as ingredients in the cosmetic and pharmaceutical sectors.

However, notwithstanding the extensive utilization of whey protein in many industrial applications, there are many concerns in their uncontrolled diffusion mainly related to their allergenicity.

β-lactoglobulin, α-lactalbumin and caseins are the main allergens in cow's milk and whey proteins, while other proteins, namely BSA and even lactoferrin (present in traces) are also potential allergens (Bu, Luo, Chen, Liu, & Zhu, 2013; Castro, Peryronel, & Cantera, 1996; Fritsche, 2003; Sharma, Kumar, Betzel, & Singh, 2001). Some processing technologies (glycation, enzymatic hydrolysis and lactic acid fermentation) have been investigated with the aim of reducing the allergenicity of milk proteins by controlling and optimizing the processing conditions (Bu et al., 2013).

Among these processes, protein hydrolysis represents a well-known method to reduce the allergenicity, to improve functional properties (foaming, solubility, etc.) and to preserve the nutritional value of whey proteins (Castro et al., 1996). Different processes were developed to carry out protein hydrolysis, including enzymatic hydrolysis, which is widely used to produce high quality protein hydrolysates at the industrial scale (Clemente, 2000). Proteolytic enzymes, extracted from animal sources (pancreatin, trypsin, pepsin) and plant sources (bromelain, papain), allow to carry out the hydrolysis reactions in milder conditions and to control the hydrolysis degree and the fragmentations in peptides to higher extent (Clemente, 2000; Clemente et al., 1999). Protein hydrolysates have enhanced nutritional, functional and biological properties with respect to the original proteins due to their smaller size and structural rearrangements, which cause the exposure of some hydrophobic regions, originally buried within the protein molecule, to the contact with the aqueous phase.

The extent of the enzymatic hydrolysis mainly depends on the accessibility of the peptide bonds, which stabilize the protein structure and control the processing time and the composition of the mixture of peptides produced. Protein unfolding, which increases the number of the binding sites exposed to the enzymatic attack, may be used as a strategy to fasten the hydrolysis reactions. To this purpose, in industrial practice enzymatic treatments are carried out at temperatures able to induce the modification of the protein structure, in particular the unfolding.

Among novel methods to induce protein unfolding, high hydrostatic pressure (HHP) has been specially focused, since this technology brings about structural changes in milk proteins able to modify epitopes, such as denaturation and formation of aggregates (Iametti et al., 1996). Pressure denaturation is a complex phenomenon that depends on protein structure, pressure range, temperature, pH, and solvent composition, and where electrostatic and hydrophobic interactions in protein molecules can be modified (Palou, Lopez-Mato, Barbosa-Canovas, & Swanson, 1999). High pressure causes deprotonation of charged groups and disruption of salt bridges and hydrophobic interactions, thereby resulting in conformational and structural changes of proteins (Martin, Barbosa-Canovas, & Swanson, 2002), as well as aggregation and gelation. Most of the whey proteins have a globular conformation and are susceptible to denaturation and aggregation induced by heat as well as by HHP. Among the whey proteins, β-lactoglobulin is the most sensitive to high pressure. Exposure to pressure levels higher than 300 MPa causes irreversible changes to the tertiary and quaternary structure of β-lactoglobulin, resulting in the formation of homopolymeric aggregates, in contrast to heat induced unfolding where heteropolymers may be detected.

α-lactalbumin is resistant to denaturation at pressures up to 500 MPa while it undergoes thermal unfolding at a lower temperature with respect to β-lactoglobulin. Differences in pressure stability of these whey proteins is due to the more rigid structure of the former, caused partially by the number of intramolecular disulfide bonds present in both proteins and the lack of free sulfhydryl groups in α-lactalbumin (Hinrichs, Rademacher, & Kessler, 1996; Hinrichs and Rademacher 2004; Huppertz, Fox, & Kelly, 2004; Huppertz, Smiddy, Upadhyay, & Kelly, 2006). According to these observations, HHP treatments induce irreversible structural and functional changes in α-lactalbumin. Reversible unfolding begins at 200 MPa and the loss of native conformation becomes irreversible beyond 400 MPa. The extent of HP-induced denaturation of α-lactalbumin and β- lactoglobulin increases with treatment time and temperature (Huppertz et al., 2004, 2006). BSA is relatively stable to high pressures (800 MPa) despite the presence of a free thiol group (De Maria, Maresca, & Ferrari, 2015, 2016). BSA undergoes substantial secondary structure changes but, differently from β-lactoglobulin, the changes are reversible, apparently due to the protection of the hydrophobic core of the protein by the large number of disulfide bonds (Huppertz et al., 2004, 2006).

When applied to whey proteins, HHP treatments were found to enhance their antigenicity, which was associated to the exposure of epitopes buried in the native protein becoming accessible for the antibodies (Kleber, Maier, & Hinrichs, 2007). Although HHP-induced unfolding may have a negative effect on the allergenicity of the proteins, the conformational changes may improve the efficiency of the enzymatic digestion, by allowing the access of the enzymes to previously hidden sites (Chicón, Belloque, Recio, & López-Fandiño, 2006). Thus, HHP technology can be proposed, alternatively to thermal treatments, to assist the reduction of food allergenicity.

The aim of the present work was to assess the applicability of HHP treatments to assist the enzymatic hydrolysis of whey protein concentrate solutions (WPC-80), in order to decrease their antigenicity. Therefore, experiments were carried out to verify the effectiveness of HHP technology to accelerate the hydrolysis reaction of whey protein concentrates with α-chymotrypsin and bromelain, and to affect the proteins allergenic power.

2. Materials and methods

2.1. Preparation of the samples

Whey protein concentrate solutions (WPC-80, Lacprodan 80, Arla foods®) were prepared by adding the proteins in Sodium phosphate buffer (100 mM, pH = 7.5) at a concentration of 1% (w/v) under gentle mixing until complete solubilization at 25 °C. The pH of the protein solutions was measured with a pH-meter (S400 SevenExcellence, Mettler Toledo International Inc.) and adjusted to the final value of 7.5. The protein concentration in the prepared solution, determined by Kjeldahl method, was 8 mg/mL.

Two different enzymes, α-chymotrypsin and bromelain (Sigma-Aldrich, Italy), were used in the experimental design. The enzymatic solutions were prepared by dissolving the enzymes (50 mg/mL) at a temperature of 25 °C in Sodium Phosphate Buffer (100 mM and pH = 7.5 for α-chymotrypsin; 100 mM and pH = 6.5 for bromelain) and stored in refrigerated conditions at 4 °C before utilization.

2.2. Experimental apparatus

The HHP system U22 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland), which is a laboratory scale unit provided with a vessel with a maximum processing volume of 50 mL, was used during the experiments. The system can be operated in a wide pressure range (0–1400 MPa) under controlled thermal conditions (25–120 °C). Operating pressure, ramp rate and processing time are set up on a control panel, which, in turn, allows the opening and closure of the HHP vessel. A portable Temperature Power and Control Unit (TCU), connected to the main unit with electrical cables and thermocouples (K-type), permits the set up and control of the operating temperature in the HHP vessel. The vessel can be heated with electrical heaters and cooled with compressed air. The pressurizing