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Production and membrane fractionation of bioactive peptides from a whey protein concentrate

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ABSTRACT

Whey proteins carry bioactive sequences that can be released by enzymatic hydrolysis. Often, the resulting hydrolysates are in the need of a fractionation process to improve or define their bioactivity. In this work, a whey protein concentrate was hydrolysed with trypsin and the obtained peptides were separated by means of membrane ultrafiltration/nanofiltration. Three pH values (2, 6 and 8) were assayed for two polyethersulfone membranes having different pore sizes (1 and 5 kDa). β-lactoglobulin peptides predominated in the hydrolysate as it was preferentially cleaved. Peptides net charge, charge distribution and size explained peptide transmissions. The highest transmissions were achieved at pH values near peptides isoelectric point. The best separation factors were obtained at basic pH values. A new membrane strategy was developed for obtaining permeates enriched in bioactive peptides from a complex hydrolysate.

1. Introduction

Traditionally, whey has been considered a troublesome by-product of the dairy industry, as it is produced in large amounts and entails high polluting power (Smithers, 2008). In order to find economical means to treat it, an intense scientific research was performed over whey components, especially proteins. Although whey proteins have been reported to possess relevant nutritional and biological properties (Power et al., 2014), their activities are mainly associated with the bioactive peptides encoded within the protein sequence (Da Costa et al., 2007). Biopeptides can be generated by *in vitro* hydrolysis of proteins (Madureira et al., 2010). They are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). These peptides can exert a large variety of activities (Hernández-Ledesma et al., 2008; Urista et al., 2011; Wada and Lönnerdal, 2014).

But even if hydrolysates are so rich in beneficial properties, they are still in the need of a fractionation or purification process, so as to turn them into saleable products with defined properties and characteristics. The main drawback for their fractionation is that most of the peptides share very similar physicochemical characteristics, therefore, only a separation technology able to distinguish between subtle differences in charge, size, solubility or hydropathicity results of utility (Fernández et al., 2014). Membrane technologies offer the possibility of a relatively easy scale up. Additionally, it is a low-cost technology, and mild operation conditions are used so substrate nutritional properties remain almost intact (Tavares et al., 2012). Among membrane processes, nanofiltration (NF) is considered as especially appropriate for peptide separation, due to the molecular weight cut-off (MWCO) used (within bioactive peptides range) and the importance of charge effects (as peptides are charged molecules) (Butylina et al., 2006).

Whey protein hydrolysates (WPH) have already been filtered with ultrafiltration (UF) and NF membranes, but with purposes such as obtaining permeate streams with low antigenicity (Guadix et al., 2006), collecting enriched fractions in a determined bioactivity (Demers-Mathieu et al., 2013; O'Keeffe and FitzGerald, 2014; Pan et al., 2012) or studying the influence of diverse parameters on the operation mode (Cheison et al., 2006). Even so, little effort has been done to attempt to elucidate the reasons of peptide transmission in those complex mixtures. Pouliot et al. (1999) fractionated a tryptic WPH in order to evaluate peptide separation under different conditions. However, they exclusively studied a selected group of previously characterized peptides, and the hydrolysate was previously fractionated with a 10 kDa MWCO membrane, so as to remove proteins and non-hydrolysed material.

The purposes of this research work were two: First, to develop a

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separation process by means of membrane filtration in order to obtain a permeate product with potential to become a functional ingredient. Second, to study the influence of feed pH, membrane pore size and peptides characteristics on the fractionation process. For this, a WPC was digested with trypsin in order to produce peptides with any reported bioactivity (antihypertensive, antioxidant, glucose regulatory, antimicrobial …); and the resulting hydrolysate was characterized in terms of peptide composition. The WPH was then filtered with two different UF/NF membranes at three different pH values, in order to compare separation performances. The mechanisms underlying peptide separation were assessed, and the enrichment in bioactive peptides under different conditions evaluated.

2. Materials and methods

2.1. Materials

WPC was kindly supplied by Armor Protéines (Saint Brice en Coglès, Brittany, France). The powder contained 82% protein by Kjeldahl [N*6,38 (FIL 20/ISO 8968)], comprising 60% of β-lactoglobulin (β-lg), 20% of α-lactalbumin (α-la), 10% of Immunoglobulin G (IgG), 8% of Bovine Serum Albumin (BSA), 1% of Lactoferrin (LF) and 1% of other proteins. Trypsin (T1426 from bovine pancreas TPCK treated) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Merck (Darmstadt, Germany). Acetonitrile (ACN), hydrochloric acid (HCl) and orthophosphoric acid (H_3PO_4) were obtained from VWR (Barcelona, Spain). Sodium hydroxide (NaOH) was from Panreac (Barcelona, Spain). Protein standards (bovine β-lg, α-la and BSA) were supplied by Sigma.

2.2. Preparation of WPC solutions

WPC solutions were prepared by dissolving 50 g of WPC in 1 L of ultrapure water. The powder was allowed to dissolve for 15 min at 37 °C under constant agitation in a magnetic stirrer hotplate (MR Hei-standard, Heidolph, Schwabach, Germany). Complete solubilization of WPC in water was verified by 10 min centrifugation at 12.000*g* at ambient temperature.

2.3. Enzymatic hydrolysis

For all experiments, hydrolysis conditions were pH 8, temperature 37 °C (trypsin optimum conditions) and constant agitation at 375 rpm. The pH was maintained throughout reactions by titration with 2 M NaOH, using a pH burette 24 2S unit (Crison, Barcelona, Spain). The enzyme:substrate (E/S) ratio was 1:200 (w/w). Reactions were stopped by the addition of 0.1 M HCl after 6 h. Aliquots of the hydrolysate were collected before enzyme addition and every 15 min, and stored at −40 °C for further analyses.

2.4. Calculation of the degree of hydrolysis

The degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved during the enzymatic reaction. Thus, a DH of 100% means the total degradation of a protein to free amino acids. However, when enzymes with specificity to individual peptide bonds are used, DH values are always lower than 100%. In case of the system WPC-trypsin, the maximum theoretical DH ($DH_{max\ the}$) was calculated as 10.70%.

The DH was calculated according to the amount of base consumed to maintain constant the pH value (pH-stat), using Eq. (1) (Adler-Nissen, 1986):

$$DH(\%) = \frac{B \cdot Nb}{\alpha \cdot MP \cdot htot} \times 100 \quad (1)$$

Where B is the base consumption (mL), N_b the normality of the base (meq/mL), MP is the mass of protein (g), h_{tot} corresponds to the total number of peptide bonds in the substrate protein (meq/g) and α is the average degree of dissociation of the α-NH groups. The parameters α and h_{tot} for whey proteins were previously calculated and take the values of 1 and 8.8 respectively (Nielsen et al., 2001).

2.5. Membrane fractionation

2.5.1. Membrane rig

The filtration system consisted of a Pellicon 2 mini cassette holder (88 cm^2 & 0.11 m^2, Millipore, Billerica, MA, USA) and a GJ series 120 pump (I-Drive, Micropump Inc., Vancouver, WA, USA), connected to a 1 L jacketed glass tank reactor coupled to a thermostatic water bath for temperature control (Ultraterm, P Selecta, Barcelona, Spain).

2.5.2. Characterization of the UF/NF membranes

Two different polyethersulfone (PES) membranes, each with a filtration area of 0.1 m^2, were used for the fractionation experiments. They had a nominal molecular weight cut-off (NMWCO) of 5 (PES5) (Millipore) and 1 kDa (PES1) (Sartorius, Goettingen, Germany) respectively.

The membranes were characterized with distilled water at two temperatures, room temperature (25 °C) and filtration temperature (37 °C). Permeate flux (J_p) (L/m^2h) was measured vs. transmembrane pressure (TMP) (MPa), temperature (°C) and time (min).

The membranes cleaning procedure was performed before first use and after every filtration run, according to the membrane supplier recommendations, rinsing thoroughly with distilled water before and after every cleaning process. Cleaning agents used were 1 M NaOH and 2% H_3PO_4; and cleaning protocol was a 10 min rinse followed by a 60 or 30 min recirculation at 40 °C respectively. The constant TMP values used were 0.12 MPa for PES1 membrane and 0.20 MPa for PES5 membrane, and the respective recirculation rates were 4 and 55 L/hm^2. The pure water flux (J_w) was measured before use and after each cleaning procedure, so as to check cleaning efficiency. J_w was always 100% recovered. The membranes were stored in 20% ethanol (PES1) or 0.1 M NaOH (PES5) under refrigeration (2−8 °C).

2.5.3. Fractionation of the WPH

The fresh hydrolysate was diluted at a ratio of 1:15 with ultrapure water in order to minimize concentration polarization effects and allow acceptable permeate flow rates.

For each experiment run, 1.5 L hydrolysate dilutions were adjusted to pH 2, 6 or 8 with 0.1 M HCl or NaOH, and temperature and pressure set to the corresponding values. Then, the filtration was started, letting the system equilibrate for 15 min. Retentate was returned to the feed tank and permeate was collected into a beaker. All filtration experiments were performed at fixed conditions of temperature (37 °C) and TMP (0.15 MPa).

The observed transmissions (Tr_{obs}) of individual peptides through the membranes were calculated using Eq. (2):

$$Tr(\%) = \frac{A_{Pi}}{A_{Ri}} \times 100 \quad (2)$$

where A_{Pi} and A_{Ri} are the i peptide peak areas obtained from the HPLC chromatograms of each membrane permeates and retentates, respectively.

The theoretical transmissions (Tr_{theo}) were also calculated