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Dual function peptides from pepsin hydrolysates of whey protein isolate

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ABSTRACT

The aim of this study was to investigate the effect of pepsin hydrolysates of whey protein isolate (WPI) on vascular relaxation and emulsifying capacity. WPI was subjected to pepsin hydrolysis for 5 h. The chromatographic profiles of the samples showed the formation of a wide variety of peptides. Addition of WPI hydrolysates in phenylephrine-contracted rat aortic rings induced a similar concentration-dependent relaxation in both endothelium-intact and endothelium-denuded preparations. In endothelium-denuded vessels the maximum relaxation induced by WPI fractions increased along the time, reaching over 70% after 3 h-hydrolysis on. In addition, the vascular relaxation was not associated with an inhibition of the angiotensin-converting enzyme or activation of K^+ channels. Hydrolysed fractions were further evaluated for the emulsifying capacity (EC) and all tested fractions were able to keep an EC over 60%. These results reinforce the potential of WPI pepsin-hydrolysates as an option in the search for dual function peptides from whey proteins.

1. Introduction

Whey proteins are being extensively studied for the last decades, especially after the consolidation of technologies for processing whey into concentrated or isolated protein-enriched ingredients, giving rise to valorized products for food, nutraceutical and pharmaceutical industries (Tavares et al., 2012; Tavares & Malcata, 2013).

Recent studies on whey proteins generally concern issues related to their structures and functionalities, and how processes, in varied conditions, could interfere on such a relation (Hernández-Ledesma, Contreras, & Recio, 2011; Lam & Nickerson, 2013; O'Loughlin, Murray, FitzGerald, Brodkorb, & Kelly, 2014a). In this respect, enzymatic hydrolysis of whey proteins has also been extensively explored, and is considered the most promising process for producing bioactive peptides, which, in many instances enhance biological functionalities when compared to intact proteins (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010; O'Loughlin, Murray, FitzGerald, Brodkorb, & Kelly, 2014b). Among the biological functionalities described for peptides from whey proteins, emphasis has been given to actions on the cardiovascular, nervous, gastrointestinal, and immune systems (Madureira et al., 2010).

Cardiovascular diseases, mainly involving hypertension, are the major cause of morbidity and mortality around the world (Celermajer, Chow, Marijon, Anstey, & Woo, 2012). In spite of the drug therapy currently available, the development of alternative strategies to prevent or reduce high blood pressure, including the intake of functional food, is highly desired (Norris & FitzGerald, 2013).

Several reports have suggested the ability of whey proteins and their peptides in modulating the cardiovascular function. However, the majority of the developed studies, including those involving hydrolysates obtained from the well-known gastrointestinal enzymes, such as pepsin and trypsin, associate the putative vascular effects of whey protein hydolysates with the inhibition of angiotensin-converting enzyme (ACE) activity (Mullally, Meisel, & FitzGerald, 1997; Otte, Shalaby, Zakora, Pripp, & El-Shabrawy,

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2007). Importantly, the mediators responsible for such effects remain under investigation (O'Loughlin et al., 2014b). Moreover, functional evidence of the inhibitory effects on ACE in vessels, or the existence of additional structures involved in the vascular effects of whey protein hydrolysates, remain to be investigated (Norris & FitzGerald, 2013).

In addition to the bio-functionalities, whey peptides may also present technological properties on food formulations, such as water binding, solubility, gelation, and emulsification (Adjonu, Doran, Torley, & Agboola, 2014; Solak & Akin, 2012), and the possibility of joining biological and technological functionalities into a dual functional ingredient is of great interest for the food industry sector. In this way, the main goal of this study was to investigate the effect of WPI pepsin-hydrolysates on vascular relaxation and evaluate the influence of this enzymatic hydrolyses on its emulsifying capacity.

2. Materials and methods

2.1. Materials

A spray-dried whey protein isolate (WPI) from bovine milk, composed of 98% protein (*w/w*), was gently donated by Alibra Ingredientes Ltda (Campinas, Brazil), and was used as substrate. Pepsin from porcine gastric mucosa (activity $\geq$ 2500 BAEE units mg^{-1} of protein), phenylephrine hydrochloride, acetylcholine, captopril, tetraethylammonium and glibenclamide, as well as the salts used in nutritive solutions, were purchased from Sigma–Aldrich (St. Louis, MO, USA), or Merck (Darmstadt, Germany).

2.2. Enzymatic hydrolysis

WPI hydrolysates were prepared by suspending the powder in an 0.1 M HCl at a concentration of 1.25% (*w/v*). The suspension was then mechanically stirred (350 rpm) and the temperature was kept at 37 °C. Prior the reaction, the system was allowed to rehydrate and solubilize for 30 min. The suspension was then adjusted to pH 2.0 using 4 M NaOH. At this point, an aliquot (control – PC) was collected. The enzyme was then added (0.1%, *w/v*) as recommended by the manufacturer. Temperature, pH, and speed of stirring were monitored throughout the reaction, and aliquots were collected after 1 min (P0), 1 h (P1), 2 h (P2), 3 h (P3), 4 h (P4), and 5 h (P5) of hydrolysis. All hydrolysates were heated to 80 °C for 5 min to inactivate the enzyme, and then cooled down under tap water temperature (~20 °C). Samples were freeze-dried or kept at −20 °C for further analyses. Hydrolysis curves were built by measuring the release of aromatic amino acids at 280 nm. The amount of released amino acid was calculated from a tyrosine standard curve (Goodwin & Morton, 1946). Protein content was measured according to Bradford (1976).

2.3. Chromatographic analysis of peptides and proteins

RP-HPLC was used to analyse the peptidic profiles generated from hydrolysis. An analytical HPLC unit from Waters (São Paulo, Brazil) (Alliance, separation module 2695) with a C18 column, held at ambient temperature (~20 °C), was used. Gradient elution was carried out by the use of two solvents. Solvent A: 0.1% trifluoroacetic acid (TFA) in ultrapure water (*v/v*), and Solvent B: 0.1% TFA in acetonitrile (ACN) (*v/v*). Hydrolysates were eluted as follows: 0–2 min, 95% A; 2–15 min, 95–80% A; 15–20 min, 80-70% A; 20–25 min, 70–60% A; 25–28 min, 60–50% A; 28–32 min, 50–40% A; 32–34 min, 40–30 % A; 34–36 min, 30–20% A; 36–38 min, 20–95% A; 38–40 min, 95%A. The flow rate was 1.0 mL min^{-1}, and the detection was at 216 nm (Waters PDA Detector, 2996). Total running time was 40 min. For the chromatographic analysis, freeze-dried samples were ressuspended into ultrapure water (2 mg mL^{-1}), reaching a pH of ~5.5, similar to the mobile phase. Injections were of 20 μL. Bovine α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) were used as standards, and the retention times were 28.12 and 29.32 min, respectively.

2.4. Protein electrophoresis

Samples (100 μL) were collected from the hydrolysis flasks at different reaction times and added to sample buffer (200 μL), following pH adjustment to ~7.0 by using 1 M NaOH. All samples were kept frozen (−20 °C) until use. Proteins were analysed by SDS-PAGE in a Bio-Rad vertical PROTEAN II xi cell (Hercules, CA, USA). Stacking and running gels were prepared by using 8% and 12% (*w/v*) acrylamide solutions, respectively. A constant voltage of 100 V was used throughout the entire running time (8 h). Gels were stained with Coomassie Brillant Blue. High (48–204 kDa) and low (20–103 kDa) molecular weight standards from Bio Rad were run in parallel (Laemmli, 1970).

2.5. Emulsifying capacity

Emulsifying capacity (EC) of hydrolysates was evaluated by the emulsifying index of the sample in hexadecane (Fontes, Amaral, Nele, & Coelho, 2010), immediately after the hydrolysis process and as soon the hydrolysates cooled down from the enzyme inactivation. For this, 1 mL of each hydrolysate was added of 1 mL of hexadecane in a tube test, followed by vigorous and non-interrupted stirring for 3 min. Then, the tubes rested for 24 h at room temperature. The EC was calculated by expressing the height of the emulsified phase (H_e) as a percentage of total solution height (H_t).

Analyses were carried out on five sequencing replications. Means were compared by the Tukey test at a 5% significance level.

2.6. Vascular relaxation

2.6.1. Animals

Male Wistar rats (250–350 g) provided by the Central Vivarium facilities of the Federal University of Santa Catarina (Florianópolis, Brazil) were used in this study. The animals were housed in controlled temperature (22 ± 2 °C) and light/dark cycle (12/12 h) with free access to water and food. All procedures were approved by the Institutional Animal Care and Use Committee of the Federal University of Santa Catarina (Florianópolis, Brazil).

2.6.2. Isolated aortic rings

The animals were killed by anaesthesia overdose (ketamine and xylazine, 150 and 40 mg kg^{-1}, i.p.) and had the descending thoracic aorta removed and immediately accommodated in a Petri dish containing cold (~4 °C) physiological saline solution (PSS, composition in mM: NaCl 131.3, KCl 4.7, KH_2PO_4 1.18, $MgSO_47H_2O$ 1.17, $NaHCO_3$ 14.9, D-glucose 5.5, $CaCl_22H_2O$ 1.6, EDTA 0.08, pH 7.4). The perivascular tissues were removed and the aorta was cut into rings 3–4 mm length. For those experiments performed with endothelium-denuded aortic rings, the endothelium was damage by rubbing a small needle around the lumen. The aortic rings were transferred to organ baths (2 mL) filled with PSS (37 °C, constantly bubbled with 95% O_2/5% CO_2), and subjected to a resting load of 3 g. The generation of tension in response to vasoconstrictor and vasodilator stimulus was recorded by isometric force transducers, coupled to a recording system PowerLab® and its application software LabChart v. 7.2, (both from AD Instruments, São Paulo,