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Enzyme inactivation and drying technologies influencing the vasorelaxant activity of a whey protein hydrolysate in semi-pilot scale

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ABSTRACT

A vasorelaxant whey hydrolysate at semi-pilot scale was generated, assessing the influence between two different enzyme inactivation conditions (pH and temperature) and drying technologies (freeze- and spray-drying). Hydrolysis was performed with 1.25% (w/v) whey protein concentrate solution and 1.91% (w/w) pepsin (pH 2, 3 h, 37 °C). Reactions were stopped either by increasing the pH to 7 (5 M NaOH) or by heating the solution at 80 °C for 5 min. Thermally inactivated samples induced a vascular relaxation ex vivo above 70%, while the chemically inactivated ones reached only 20%. Both drying technologies were effective in maintaining the bioactivity of the hydrolysate. These results brought the possibility of developing a whey-based product with high vasorelaxant potential in semi-pilot scale; thermal treatment followed by spray drying seemed to be the more affordable alternative for food and nutraceutical industries to generate this bioactive product.

1. Introduction

The use of whey proteins and whey peptides in sports nutrition, bakery and infant formulae is well consolidated (Allied Market Research, 2017). Despite its diversified use, large part of the whey produced remains under-utilised, representing a serious environmental concern, because of its high chemical and biochemical demands of oxygen (Dereli, van der Zee, Ozturk, & van Lier, 2019). This factor, together with the need to create new market niches and value old products, makes industries seek for new alternatives to their residues (Yadav et al., 2015).

Among other functionalities, whey proteins and whey peptides are associated with modulation of hypertension (Beltrán-Barrientos, Hernández-Mendoza, Torres-Llanez, González-Córdova, & Vallejo-Córdoba, 2016; O'Keeffe, Conesa, & FitzGerald, 2017), a chronic disease with high prevalence, which its complications account for 9.4 million deaths every year (WHO, 2015). Despite the lack of molecular studies, it is generally described that whey peptides may induce vascular relaxation and putatively reduce the blood pressure through different mechanisms, including inhibition of the angiotensin converting enzyme (ACE) and renin, stimulation of nitric oxide production, and blockage of calcium channels, among others (Udenigwe & Mohan, 2014). Besides all well-known health benefits, there are only a few commercial products available on the market based on the antihypertensive activity of whey peptides (Beltrán-Barrientos et al., 2016), highlighting the need of more studies in this field.

The main challenge of food and nutraceutical industries consists of high costs of production of the antihypertensive peptides since different advanced technologies are required. Therefore, the present study aimed at producing a whey protein hydrolysate with vasorelaxant effect by the use of commercial pepsin in a semi-pilot scale, assessing the influence of two different enzyme inactivation conditions and drying technologies.

2. Materials and methods

2.1. Materials

Bovine whey protein concentrate (WPC), containing 88% protein, 2% carbohydrate, 1.6% lipid and 8.4% cholesterol, vitamins and minerals, was provided by Alibra Ingredientes Ltda (Campinas, SP, Brazil). Porcine pepsin (enzyme activity: 0.28 μU mL^{-1}) was provided by Bela

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Vista Produtos Enzimáticos Ind. e Com. Ltda (Bela Vista, SC, Brazil). Phenylephrine hydrochloride, acetylcholine chloride, and all salt used to prepare the physiological saline solution (PSS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Whey hydrolysate preparation

Hydrolyses were performed according to Mellinger-Silva et al. (2015). Briefly, in a semi-pilot scale reactor of 20 L, WPC was suspended in distilled water at a concentration of 1.25% (w/v) and the pH was adjusted to 2 with 1 M HCl. Pepsin (4.2 g) was used to prepare 1.05 L of enzyme solution (0.4%, w/v), corresponding to 1.91 g of pepsin per gram of protein (1.91%, w/w). Pepsin solution was further added to the WPC solution and the reaction occurred during 3 h at 37 °C. Temperature and pH were monitored during all the process. Pepsin was either thermally (80 °C for 5 min) (TI) or chemically inactivated (CI) by increasing pH up to 7 with 5 M NaOH. Samples were either freeze-dried at −52 °C, 67.73 Pa (Liotop, São Paulo, Brazil) or spray-dried at 0.52 MPa (inlet temperature: 170 °C; outlet temperature: 85 °C) (Buchi Labortechnik, Flawil, Switzerland).

2.3. Reversed-phase high performance liquid chromatography

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed according to Mellinger-Silva et al. (2015). A HPLC (Jasco, São Paulo, Brazil) coupled with a BDS Hypersil C18 (100 × 4.6 mm, particle size 2.4 μm, Thermo Scientific, USA) was used. Trifluoracetic acid (TFA) in ultrapure water (0.1%, v/v) and 0.1% TFA in acetonitrile (v/v) were used as solvents A and B, respectively. Hydrolysates were eluted as follows: 0–2 min, 5% B; 2–15 min, 5–20% B; 15–20 min, 20–30% B; 20–25 min, 30–40% B; 25–28 min, 40–50% B; 28–32 min, 50–60% B; 32–34 min, 60–70% B; 34–36 min, 70–80% B; 36–38 min, 80–5% B; 38–40 min, 5% B. Runs occurred at a flow rate of 1.0 mL min^{-1} for 40 min at ambient temperature (approximately 20 °C). The detection wavelength used was 216 nm. Bovine α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) from bovine milk (Sigma-Aldrich) were used as standards with retention times of 29.84 and 30.70 min, respectively.

2.4. Aortic ring preparation and analyses of vascular relaxation

Six male Wistar rats (250–300 g) provided by Universidade Federal de Santa Catarina (Florianópolis, Brazil) were used in the experiments, which were approved by our local Institutional Animal Care and Use Committee (authorisation number 5371190815). Animals were maintained under standard laboratory conditions at controlled temperature (22 ± 2 °C) with free access to water and diet ad libitum. For these experiments, animals were killed by anaesthetic overdose (ketamine and xylazine, 140 and 40 mg kg^{-1}, i.p.) and the preparation of endothelium-intact rat aortic rings was performed as previously described (Mellinger-Silva et al., 2015). Briefly, the aortic rings were mounted in organ baths and kept in 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, at 37 °C and continuously bubbled with 95% O_2/5% CO_2. To evaluate the presence of a functional endothelium, acetylcholine (1 μmol L^{-1}) was used to induce relaxation in phenylephrine (1 μmol L^1)-contracted preparations. Only the rings that achieved 80% relaxation to acetylcholine were considered with functional endothelium.

The changes in vascular tone were recorded by isometric force transducers, coupled to a recording system PowerLab® and its application software LabChart v7.4 (both from ADInstruments, Castle Hill, Australia). After stabilisation, the vessels were contracted by 1 μmol L^{-1} phenylephrine and cumulative concentrations of the hydrolysed fractions (1, 3, 5 and 10 mg mL^{-1}) were added.

The results were expressed as the mean ± SEM of 6 aortic rings from different animals per group. Each preparation was tested with only one of the hydrolysed fractions. The results were analysed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. A value of $p < 0.05$ was considered statistically significant. Graphs and statistical analyses were performed using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

Process scale up is essential when aiming to develop an ingredient for food industries; processes changes must be well controlled to avoid alterations in the final product. The integrative process for the development of whey-based products with high

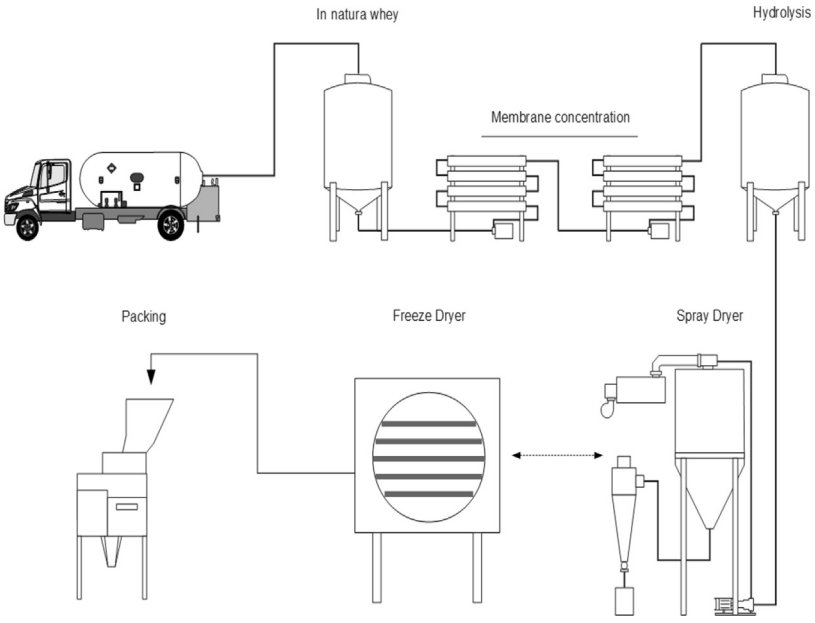

Fig. 1. Flow diagram of a proposed industrial plant for whey protein hydrolysate production.

economic value includes several steps, such as reception of the in natura whey, membrane concentration, hydrolysis, drying and packing (Fig. 1) (O'Keeffe et al., 2017; Tavares et al., 2012).

Enzymes are extensively used to generate biofunctional products (Le Maux, Nongonierma, Barre, & Fitzgerald, 2016). Pepsin presents high affinity for cleaving peptide bonds between hydrophobic and preferably aromatic amino acids (Bannigan et al., 2018). The presence of these amino acid residues at each of the C-terminal tripeptide positions seems to be related to the angiotensin I-converting enzyme (ACE) inhibition, which may be considered a good physiological target for the clinical treatment of hypertension, as it is involved in the control of blood pressure through two different systems, the renin-angiotensin and the kinin-nitric oxide systems (Ko et al., 2015; Norris & Fitzgerald, 2013). Pepsin is also found in

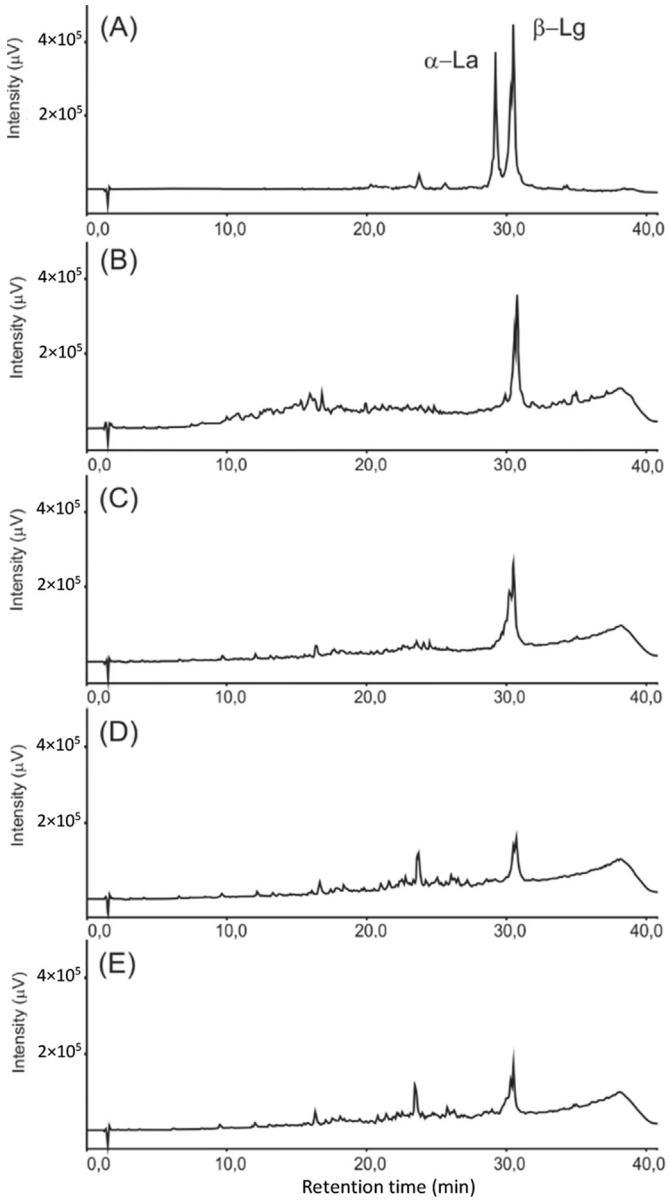

Fig. 2. Peptide profiles of whey hydrolysates assessing different enzyme inactivation conditions and drying technologies: (A) control sample, whey protein concentrate 88%; α-La: α-lactalbumin; β-Lg: β-lactoglobulin; CI (adjusted to pH 7) lyophilised (B) and spray-dried (C) samples; TI (80 °C × 5 min) lyophilised (D) and spray-dried (E) samples.

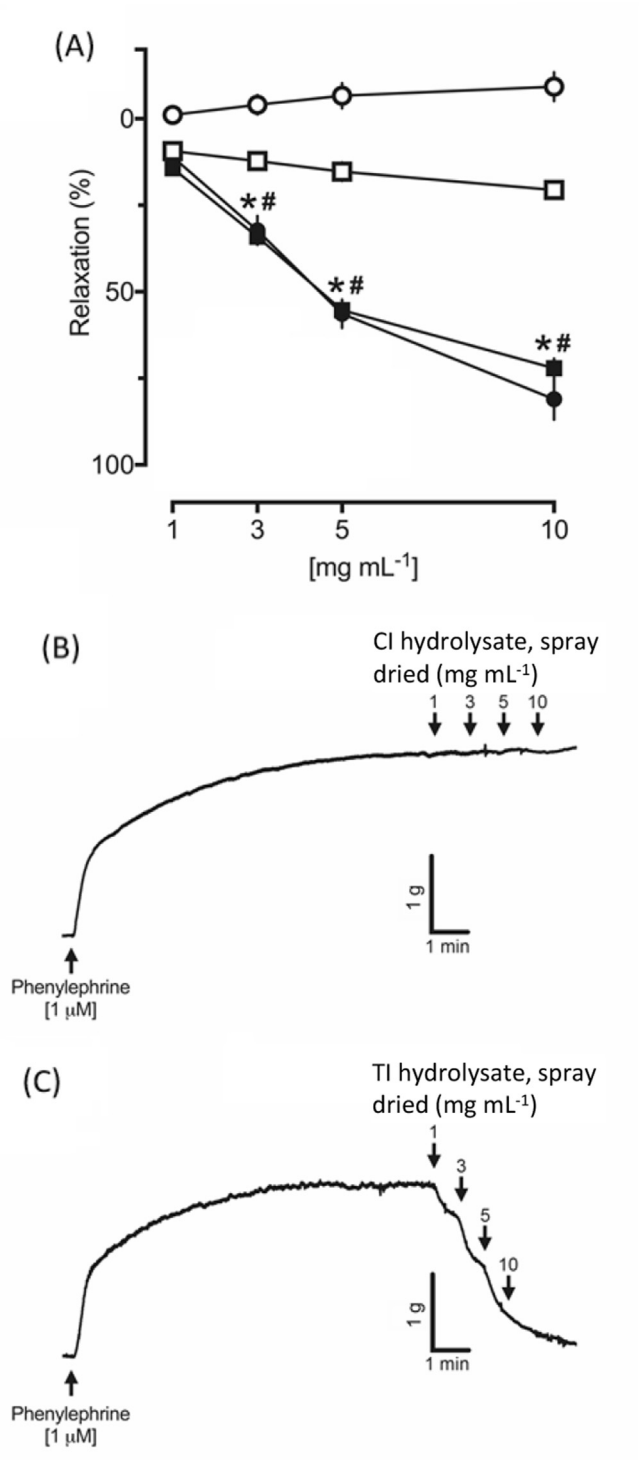

Fig. 3. Vascular relaxation induced by whey hydrolysates in rat aortic rings pre-contracted by phenylephrine. Panel A: percentage relaxation as a function of cumulative concentrations of 1, 3, 5, 10 mg mL^{-1} of the whey hydrolysates CI (adjusted pH to 7; ○, □) and TI (80 °C × 5 min; ●, ■) lyophilised (circles) or spray-dried (squares) were added; * indicates significant vascular relaxation ($p < 0.005$) for both TI samples, # indicates $p < 0.005$ compared with the previous concentration of used in TI samples (two-way ANOVA followed by Tukey's post hoc test. The results were expressed as the mean ± SEM of 6 aortic rings from different animals per group. Panels B and C, patterns of change in vascular tone contracted by phenylephrine followed by addition of (B) CI spray-dried hydrolysate and (C) TI spray-dried hydrolysate.

the human gastrointestinal tract, in this manner, after a previous peptic hydrolysis of the WPC, any in vivo gastric digestion is expected further, possibly allowing a controlled formation of the vasorelaxant peptides. More studies are being carried out to evaluate the influence of the gastrointestinal digestion in the release of the bioactive peptides.

Besides the choice of the enzyme, its inactivation and the method used to dry the solution are also parameters that may influence the composition of the hydrolysate and its bioactivity. Among the drying methods, lyophilisation is well-known for preserving quality and the integrity of bioactive compounds in food matrices; however, it requires high energy and presents operational costs about six-times higher than spray-drying (Haque & Adhikari, 2015; Schulze, Hubbermann, & Schwarz, 2014). The spray-dryer, more commonly used in the food industries, applies high working temperatures, possibly causing heat degradation and affecting the stability of thermally labile compounds (Drosou, Krokida, & Biliaderis, 2016).

Fig. 2B and C reveals that the drying method may influence the peptide formation of the CI samples, as the freeze-dried hydrolysate presented a more varied peptide composition than the spray-dried one. This may be related to the heat and pressure applied during spray-drying, causing sample degradation with generation of free amino acids (Drosou et al., 2016). The same was not observed for the TI samples, as both lyophilized (Fig. 2D) and spray-dried (Fig. 2E) samples presented similar peptide profiles, including hydrophobic and hydrophilic molecules. Comparing CI and TI samples, it is possible to observe higher β-Lg hydrolysis in the TI ones than in the CI samples, which may be a result of the thermal denaturation of this protein during the thermal inactivation process (Fig. 2).

As shown in Fig. 3, the enzyme inactivation method directly influenced the biofunctionality of the hydrolysates, as TI samples were able to induce greater vasorelaxant effect when compared with CI samples (Fig. 3A). Notably, CI spray-dried hydrolysate completely lacked capability to induce vascular relaxation, compared with TI sample (Fig. 3B and C). Furthermore, the TI hydrolysate induced significant levels of vascular relaxation in pre-contracted aortic rings, achieving 72.1 ± 11.4% and 81.0 ± 14.3%, for the freeze- and spray-dried samples, respectively.

4. Conclusions

The present study revealed that enzyme inactivation was an important step in the process of generation of a whey hydrolysate with vasorelaxant activity. The heat inactivated hydrolysates presented higher vascular relaxant potential than those that were chemically inactivated. Despite applying high temperatures and pressure, the spray-drying technology presented a tendency to be more efficient in maintaining vascular relaxation capacity of the hydrolysate, besides presenting lower working costs and reduced process time compared with freeze-drying, being a more feasible technology to food industries. In this manner, thermal inactivation of pepsin followed by spray-drying the hydrolysate might be recommended for the generation of an antihypertensive whey hydrolysate in semi-pilot scale.

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References

Allied Market Research. (2017). *Whey protein ingredients market - global opportunity analysis and industry forecast, 2014–2022*. Retrieved from https://www.alliedmarketresearch.com/whey-protein-ingredients-market.

Bannigan, P., Durack, E., Mathur, H., Rea, M. C., Ross, R. P., & Hudson, S. P. (2018). Delivery of a hydrophobic drug into the lower gastrointestinal system via an endogenous enzyme-mediated carrier mechanism: An in vitro study. *European Journal of Pharmaceutics and Biopharmaceutics, 133*, 12–19.

Beltrán-Barrientos, L. M., Hernández-Mendoza, A., Torres-Llanez, M. J., González-Córdova, A. F., & Vallejo-Córdoba, B. (2016). Invited review: Fermented milk as antihypertensive functional food. *Journal of Dairy Science, 99*, 4099–4110.

Dereli, R. K., van der Zee, F. P., Ozturk, I., & van Lier, J. B. (2019). Treatment of cheese whey by a cross-flow anaerobic membrane bioreactor: Biological and filtration performance. *Environmental Research, 168*, 109–117.

Drosou, C. G., Krokida, M. K., & Biliaderis, C. G. (2016). Encapsulation of bioactive compounds through electrospinning/electrospraying and spray drying: A comparative assessment of food-related applications. *Drying Technology, 35*, 139–162.

Haque, M. A., & Adhikari, B. (2015). Drying and denaturation of proteins in spray drying process. In A. S. Mujumdar (Ed.), *Handbook of industrial drying* (4th ed., pp. 971–983). Boca Raton, FL, USA: Taylor & Francis.

Ko, S.-C., Jung, W.-K., Kang, S.-M., Lee, S.-H., Kang, M. C., Heo, S.-J., et al. (2015). Angiotensin I-converting enzyme (ACE) inhibition and nitric oxide (NO)-mediated antihypertensive effect of octaphlorethol A isolated from Ishige sinicola: In vitro molecular mechanism and in vivo SHR model. *Journal of Functional Foods, 18*, 289–299.

Le Maux, S., Nongonierma, A. B., Barre, C., & Fitzgerald, R. J. (2016). Enzymatic generation of whey protein hydrolysates under pH-controlled and non pH-controlled conditions: Impact on physicochemical and bioactive properties. *Food Chemistry, 199*, 246–251.

Mellinger-Silva, C., Rosa, L. O. L., Stephan, M. P., Brígida, A. I. S., Cabral, L. M. C., Da Silva, G. O., et al. (2015). Dual function peptides from pepsin hydrolysates of whey protein isolate. *International Dairy Journal, 48*, 73–79.

Norris, R., & Fitzgerald, R. J. (2013). Antihypertensive peptides from food proteins. In B. Hernandez-Ledesma, & C.-C. Hsieh (Eds.), *Bioactive food peptides in health and disease (Chapt. 3)*. London, UK: InTechOpen.

O'Keeffe, M. B., Conesa, C., & FitzGerald, R. J. (2017). Identification of angiotensin converting enzyme inhibitory and antioxidant peptides in a whey protein concentrate hydrolysate produced at semi-pilot scale. *International Journal of Food Science and Technology, 52*, 1751–1759.

Schulze, B., Hubbermann, E. M., & Schwarz, K. (2014). Stability of quercetin derivatives in vacuum impregnated apple slices after drying (microwave vacuum drying, air drying, freeze drying) and storage. *LWT Food Science and Technology, 57*, 426–433.

Tavares, T. G., Amorim, M., Gomes, D., Pintado, M. E., Pereira, C. D., & Malcata, F. X. (2012). Manufacture of bioactive peptide-rich concentrates from whey: Characterization of pilot process. *Journal of Food Engineering, 110*, 547–552.

Udenigwe, C. C., & Mohan, A. (2014). Mechanisms of food protein-derived antihypertensive peptides other than ACE inhibition. *Journal of Functional Foods, 8*, 45–52.

WHO. (2015). *Q&As on hypertension*. Geneva, Switzerland: World Health organisation. Retrieved from https://www.who.int/features/qa/82/en/.

Yadav, J. S. S., Yan, S., Pilli, S., Kumar, L., Tyagi, R. D., & Surampalli, R. Y. (2015). Cheese whey: A potential resource to transform into bioprotein, functional/nutritional proteins and bioactive peptides. *Biotechnology Advances, 33*, 756–774.