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Proceeding Paper

Bovine Whey Hydrolysis with Pancreatin Produces a Functional Ingredient for Developing Antihypertensive Beverages †

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Abstract: Bovine whey remains an essential pollutant from food, and diverse strategies to mitigate it have been taken. Therefore, the work aimed to generate a functional ingredient from whey hydrolysis to be incorporated into beverage formulations. Hydrolysis kinetic at pH = 8 for 7 h was raised to determine the sample to be added to the beverage. It was elaborated according to local producer, and their ACE inhibition capacity was tested. Kinetics showed that only the times 0,4, and 7 h significantly differed in the hydrolysis degree. The exact times were submitted to the ACE inhibition test, but only 0 and 7 h showed higher activity (32.41 ± 0.63 and 29.63 ± 1.10%, respectively). As no statistical differences were found, both hydrolysates were incorporated into the beverage, finding an antihypertensive capacity of 74.84 ± 1.39 and 78.76 ± 1.39%. A better sensorial profile was determined in the beverage made with 7 h hydrolysate, where a lower salty taste was identified. Thus, pancreatin hydrolysis of whey generated peptides with antihypertensive activity and improved acceptance characteristics.

Keywords: ACE inhibition; functional beverage; whey biorefining

1. Introduction

The dairy industry grows yearly, with worldwide milk processing increasing by around 2.5% annually. Consequently, bovine whey generation has become a pollution concern due to the 160 million tons produced yearly [1]. To mitigate that problem, different strategies have been proposed as the main objective to achieve sustainable biorefining through whey valorization [1,2]. The recovery of high-added-value compounds such as protein and lactose, feeding farm animals, bioconversion to high-added-value compounds like nutrients, bioactive constituents, biofuels or prebiotics, as well as the

development of healthier food products by improving nutritional profile or food fortification are found in the proposal procedures [2–4].

In that context, the formulation of whey protein-based beverages has generated significant interest in recent years, especially that with healthy and functional functions [4]. Actual examples are the production of a naturally carbonated whey probiotic drink with antimicrobial activity and low alcohol tonics, both produced by fermentation processes. Indeed, the last technology has also been used for lactic acid production and bioactive peptides released from whey as valorizing approaches [1–4].

The Angiotensin Converting Enzyme (ACE) inhibition is found within the bioactivities mainly tested as a measure of antihypertensive capacity. Milk proteins have been widely used as antihypertensive peptide sources through enzymatic hydrolysis or fermentation, the last peptide-releasing method with the most extensive application [5]. It has been recognized that fermented milk maintains an antihypertensive power, even in commercial products such as Ameal S, Calpis, Danaten, and Evolus has been demonstrated that capacity [6].

Despite several reports for the generation of whey hydrolysates from exogenous enzyme applications that have been proposed, at this time, the use of an enzyme hydrolysate with antihypertensive properties for its incorporation in functional beverages has yet to be reported. Therefore, the objective of the work was to obtain an enzymatic hydrolysate with ACE inhibition capacity through bovine whey hydrolysis with pancreatin to propose a functional beverage.

2. Methods

Bovine whey hydrolysis: Dispersions of bovine whey powder were prepared at 10% (*w*/*v*) using Tris-HCl buffer (0.02 M, pH = 8) as solvent. Then, dispersions were thermally treated at 90 °C/10 min in an autoclave before beginning the enzymatic reaction. Hydrolysis of whey was performed by adding pancreatin in a mass ratio soluble protein: enzyme of 100:4, and the reaction proceeded for 7 h at 40 °C with oscillatory shaking at 130 rpm, according to the proposed by Abubakar et al. [7], with some modifications. Sampling was taken each hour, and supernatants used for the following determinations were obtained through enzyme inactivation in boiling water for 10 min and subsequent centrifugation at 13,000 rpm/4 °C for 15 min.

Following free amino groups by the trinitrobenzene sulfonic acid (TNBS) method: To assess the hydrolysis level during the enzymatic process, the TNBS method proposed by Adler-Nissen [8] was performed. In brief, sample supernatants (0.250 mL), 0.21 M phosphates buffer (2 mL) at pH = 8.2, and picryl sulfonic acid solution (2 mL) at 0.1% (v/v) were mixed and carried to react at 50 °C for 1 h in the absence of light. HCl addition at 0.1 N (4 mL) arrested the reaction, and the absorbance was recorded at 340 nm. A calibration curve of glycine (0–200 ppm) was used to determine the free amino groups concentration.

ACE in vitro *inhibition test:* The antihypertensive capacity was measured by the ACE inhibition test proposed by Hussein et al. [9] with some modifications. In brief, two systems were evaluated. The control system (A_{100}) comprised saline borates buffer (80 μL) at 0.05 M, pH = 8.2 with NaCl at 0.3 M, hippuryl-histidyl-leucine (200 μL) at 5 mM, and ACE from rabbit lung (20 μL) at 0.1 U/mL. In the sampling system (A_s), the saline borates buffer was substituted for the sample supernatants (80 μL). Both systems were carried out to react for 80 min at 37 °C to be later arrested by HCl 0.1 M (250 μL) addition. The last total content was mixed with ethyl acetate (1.7 mL), and organic extraction was performed, taking 800 μL of the organic layer, which was evaporated at 80 °C for 1 h. Then, the extracted content was reconstituted with deionized water (500 μL) and mixed with pyridine (300 μL) and benzene sulfonyl chloride (150 μL).

Finally, absorbance in each system was recorded at 410 nm, and the ACE inhibition percentage was obtained from the following equation.

$$\textbf{ACE inhibition } (\%) = \frac{\mathbf{A_{100} - A_s}}{\mathbf{A_{100}}} \mathbf{x\ 100}$$

Antihypertensive beverage formulation: The functional beverage was formulated according to a local ice cream bases factory with a percentual final composition of water (77%), fructose (13%), hydrolyzed whey protein (7.7%), butyric fat (1%), flavoring (0.32%), stabilizing salts (0.17%), and emulsifier (0.1%). The making process was the next: whey powder and stabilizing salts were blended and dissolved in purified water to reach a whey and salts concentration of 10 and 0.22% (*w*/*v*), respectively. Afterward, enzymatic hydrolysis was initialized with the same soluble protein: enzyme mass ratio described before, and it was performed for 0 and 7 h at the resulting pH (6.69) and 37 °C/130 rpm. Once the reaction time elapsed, pancreatin was inactivated with the previously mentioned method, and 77% of the obtained hydrolysate was used as a beverage base, blended with the corresponding percentages of fat and emulsifier melting at 60 °C. The last mix was homogenized with a turbo mixer at a low rate, then the fructose and flavoring were incorporated, the temperature increased to 70 °C, and the mechanical agitation was maintained. Beverage complete homogenization was carried out for 5 min with the turbo mixer at a high rate, and thermal treatment was made in an open system at 80 °C/15 min.

Antihypertensive test for the functional beverage: Once the drink was finalized, it let to rest, and the supernatant was obtained under the same centrifugation conditions described in the bovine whey hydrolysis section. The ACE inhibition capacity followed was the same that used for pancreatin hydrolysates but tested the supernatants from beverage centrifugation.

Statistical analysis: All analyses were duplicated, and one-way ANOVA was used to determine significant differences through Tukey's contrast at a 95% confidence level using the Minitab 18 package.

3. Results and Discussion

Following of free amino groups and ACE inhibition: As the first step, whey hydrolysis was raised to determine the hydrolysate to incorporate into the beverage. The following of free amino groups was done each hour, where statistical analysis showed that only the times 4 and 7 h were significantly different from the rest of the hydrolysis times (thorough hydrolysis study not shown). Results (Table 1) exhibited a slight but significant decrease in free amino groups from 0 to 4 h with a substantial rise at 7 h. This behavior has been previously found by Silvestre et al. [10], who did not observe changes in whey hydrolysis with pancreatin from 1–5 h by the OPA method and only at 3 and 5 h by Lowry's soluble protein method.

Table 1. Whey hydrolysis with pancreatin and ACE inhibition properties from each beverage formulated.

Following Whey Hydrolysis with Pancreatin			**Antihypertensive Capacity in Beverage**	
Hydrolysis time (h)	Hydrolysis degree (ppm)	ACE inhibition (%)	Hydrolysate was used as a beverage base	ACE inhibition (%)
0	748.04 ± 67.64 ^{bc}	32.41 ± 0.63 ^{a}	Time 0	74.84 ± 1.39 ^{a}
4	727.39 ± 7.69 ^{c}	16.67 ± 1.57 ^{b}	Time 7	78.76 ± 1.39 ^{a}
7	1206.74 ± 138.35 ^{a}	29.63 ± 1.10 ^{a}		

Different lowercase letters indicate significant differences ($p < 0.05$).

Due to only the sampling times 4 and 7 h showing differences during hydrolysis degree analysis, it was submitted to the ACE inhibition test. At the same time, the hydrolysis beginning was also tested to contrast initial ACE inhibition with the later times. Results showed that from 0 h, antihypertensive activity was present, probably for the bovine whey

processing obtention; once that has been identified, the presence of soluble peptides at 2.27% in whey powders, which contains proline, tyrosine, and lysine [11], amino acids structurally associated with antihypertensive properties [12].

In the case of the hydrolysis at 4 h, it showed lower ACE inhibition than the other evaluated times. The result can be explained by the lower free amino groups concentration found, which affects the capacity to inhibit the ACE because other studies have demonstrated that antihypertensive activity is lost by excessive hydrolysis [13,14]. However, in this specific case, no over-hydrolysis was determined. In the same context, the last time (7 h) showed a higher hydrolysis degree, the ACE inhibition was slightly lower than the initial time (0 h) but statistically the same. It confirms that free amino groups concentration strongly influences the bioactivity assessed.

ACE inhibition power from beverage formulations: According to the previous results, it was decided to formulate the functional beverages with the 0 and 7 h hydrolysates. However, changes in enzymatic hydrolysis must be made to engage the producer formulation with pancreatin hydrolysis following. Thus, stabilizing salts and whey powder were mixed before hydrolysis. The pH was measured as these salts differed from those used in previous enzymatic hydrolysis assays, originating with a pH of 6.69. This change was associated with increased ACE inhibition activity found for beverage formulations (Table 1), where 74.84% and 78.76% with 0 and 7 h hydrolysates were reached. However, no statistical differences were found between them as in previous experiments. Indeed, Guo et al. [13] found similar results, where at pH near 7, a higher ACE inhibition was achieved compared to that found at pH = 8.

Both beverage formulations can be considered as potential functional food from a similar product but based on low-fat skim milk has been proposed by Ahtesh et al. [15], where ACE inhibition of around 80% was obtained after 8 h of hydrolysis with the combination of flavourzyme and *Lactobacillus helveticus*. Nevertheless, it is essential to highlight that the formulation proposed in this work with the highest probability of acceptance will be the 7 h hydrolysate because a preliminary sensory analysis showed a diminishing salty taste for that sample. Its diminishing could be associated with the degradation of some peptides with salty characteristics in combination with the fructose addition [16,17].

4. Conclusions

Whey hydrolysis with pancreatin propitiated a functional ingredient, which can be incorporated into functional formulations to produce antihypertensive beverages. Despite the ACE inhibition being the same at the beginning and the final hydrolysis, pancreatin allowed to obtain a hydrolysate with better sensorial characteristics, mainly orientated in diminishing the salty taste. Therefore, this work shows that enzymatic hydrolysis can be oriented in the obtention of higher bioactivity and the improvement of sensorial characteristics provided by peptides.

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