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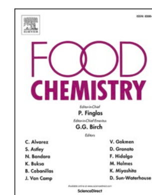

Development and sensory test of a dairy product with ACE inhibitory and antioxidant peptides produced at a pilot plant scale

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ABSTRACT

A scale-up process was carried out to obtain potent bioactive peptides from whey protein through a simple hydrolysis process. The scale-up was satisfactory, with results similar to those obtained at lab scale: a fraction of peptides < 1 kDa with ACE inhibitory activity of 18.44 ± 2.47 μg/mL, a DPPH value of 69.40 ± 0.44%, and an ORAC value of 3.37 ± 0.03 μmol TE/mg protein. The peptide sequences responsible for the ACE inhibitory activity were also similar to those identified at lab scale: PM, LL, LF, HFKG and PT. The hydrolysate was used as a functional ingredient in a low-fat yoghurt. The consumer sensory taste panel found no significant difference ($p >$ 0.05) between the bitterness of the control and the functional yoghurt, and about 50% of consumers would buy it. The hydrolysate maintained its bioactivities for 4 months at −20 °C (after thawing and pasteurisation), and for 1 week in yoghurt at 4 °C.

1. Introduction

Food industry wastes have been extensively used as a sustainable source for innovative products to manage efficiently the waste disposal and reduce production costs. In this regard, whey protein from cheese manufacturing has been broadly used as a source of bioactive peptides.

Bioactive peptides are short amino acid sequences that produce a positive effect on the consumer body functions by promoting health benefits or reducing disease risks (Jakubczyk, Karas, Rybczynska-Tkaczyk, Zielinska, & Zielinski, 2020). Among bioactive peptides from whey proteins, antihypertensive and antioxidant peptides have received important attention in the last decades (Brandelli, Daroit, & Corrêa, 2015). In fact, there are several products currently commercialised with antihypertensive claims, such as Lowpept®, Calpis®, Evolus® and Biozate®, mostly derived from caseins (Hsieh et al., 2015).

Bioactive peptides are commonly produced through enzymatic hydrolysis of whole whey proteins using proteolytic enzymes derived from several sources: animal (pepsin, trypsin and chymotrypsin), microbial (proteinase K or thermolysin), or vegetable (papain and bromelain) (Zhou, Sun, & Canning, 2012). Among them, microbial proteases have been pointed as interesting biocatalysts for the production of protein hydrolysates at a commercial scale as they can be easily produced under controlled and well-established methods (Brandelli et al., 2015). Moreover, enzymatic combinations including two or more proteases can be used simultaneously or sequentially, depending on the optimum hydrolysis conditions of each enzyme (Aluko, 2015).

The methods for obtaining bioactive peptides are soft and very specific (Adjonu, Doran, Torley, & Agboola, 2013), which facilitates the isolation and characterisation of the peptides generated. However, there are some disadvantages related to the products obtained, such as the appearance of bitter tastes due to the presence of hydrophobic amino acid residues in the peptides generated (Cheung, Aluko, Cliff, & Li-Chan, 2015).

The antihypertensive peptides generated from whey proteins are highly dependent on the hydrolysis conditions and the protease used. Thus, the most commonly used proteases for this purpose are digestive enzymes (López-Fandiño, Otte, & van Camp, 2006), such as trypsin (Naik, Mann, Bajaj, Sangwan, & Sharma, 2013), pepsin (Baba et al., 2021) and chymotrypsin (Lourenço da Costa, da Rocha, Gontijo, & Netto, 2007) using each alone, or in combination (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000).

Digestive enzymes are very useful as they mimic the physiological conditions of hydrolysis leading to bioactive peptides. But, for industrial applications, they are expensive compared to more readily available microbial enzymes. In this respect, we have previously demonstrated the suitability of two microbial proteases to hydrolyse and generate

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bioactive peptides to valorise cheese whey protein. These results are included in the patent WO 2012/172129 Al (*Optimised method for obtaining angiotensin converting enzyme inhibitory peptides, inhibitory peptides and food containing same*). Analytical grade proteases were optionally used for this purpose, which could comprise thermolysin, proteinase K, trypsin and chymotrypsin, or equivalent proteases with a technical grade of purity. Thus, depending on the desired application, different proteases were combined. Whey protein concentrate was hydrolysed with high purity technical proteases to obtain potent ACE inhibitory peptides. After six hours of hydrolysis and moderate temperatures, different inhibition percentages were obtained depending on the test conditions. Nevertheless, all the experiments showed peptides with>70% inhibition without exhaustive purification steps, which represent acceptable conditions for industrial purposes.

Therefore, the main objective of this work was to scale up the process described in the patent WO 2012/172129 to produce bioactive peptides from bovine whey protein. For that, whey protein hydrolysates were produced by enzymatic hydrolysis at the pilot plant scale. The final hydrolysate was analysed regarding its bioactivities (ACE inhibitory activity and antioxidant activity) and peptide composition. In addition, the potential application of the hydrolysate as a multifunctional food additive or supplement was determined by incorporating it into yoghurt. Consumer acceptance was also studied by sensory analysis, as well as the stability of the hydrolysate's bioactivity within the food matrix during extended storage periods.

2. Materials and methods

2.1. Materials

The substrate used in this study was bovine cheese whey protein obtained from QUEIZUAR S.L. (A Coruña, Spain). The microbial proteases used to hydrolyse the whey protein were from Sigma-Aldrich (St. Louis, MO, USA) and Novozymes (Copenhagen, Denmark). Bovine β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and glycomacropeptide (GMP) (>85% purity), azocasein, angiotensin I-converting enzyme (ACE) (EC 3.4.15.1, from rabbit lung), N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), acetonitrile (ACN) (HPLC grade), trifluoroacetic acid (TFA) (reagent plus grade, > 99%), trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein (FL), 2,2'-azobis-(2-methylpropionamidine)-dihydrochloride (AAPH), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade. All solutions were prepared with distilled or ultrapure water (Milli-Q Advantage A10, Millipore, CA, USA).

2.2. Enzymatic hydrolysis of whey protein

Before hydrolysis, the ultrafiltration (UF) process was applied. It was performed in the industrial plant at a scale of 4,000 L to remove the maximum amount of lactose and minerals from whey and concentrate the protein fraction. A retentate still rich in lactose (~3%, w/v) with 8.5% (w/v) protein concentration was obtained as starting material. As shown in Table 1S of the supplementary material, this was a whey protein concentrate rich in β-Lg and with a significant content of GMP.

Whey protein concentrate was hydrolysed using a combination of two microbial proteases as described in patent WO 2012/172129 Al. The process was scaled up to a pilot plant scale (200 L) using a 500 L vertical-stirred tank bioreactor located at QUEIZUAR S.L. (A Coruña, Spain). The reactor operated at 200 rpm and was equipped with a heating water jacket through which water circulated at different temperatures. The pH of the mixture was adjusted to be 8 at 60 °C with 0.02 M Tris-HCl buffer with 0.01 M $CaCl_2$. A temperature ramp was applied from 26 °C to 60 °C (0.2 °C/min) to pre-incubate the mixture, and the proteases were then added to start the reaction. At the end of the reaction (6 h), the enzymes were inactivated by a slight increase in temperature (up to 65 °C) and a decrease in pH (down to 5.0). After the inactivation of the enzymes, the hydrolysate at 6 h was stored directly at −20 °C for further analysis.

2.3. RP-HPLC

The samples were analysed with an Agilent 1200 series system (Agilent Technologies, Waldbronn, Germany), as described in Estévez et al. (2017). Samples were filtered through 0.45 μm filters and 20 μL of each sample were injected and eluted in an ACE 5 C18 column (250 × 4.6 mm, 5 μm, 300 Å, Advanced Chromatography Technologies) at 0.7 mL/min. Elution was performed by applying 100% of the solvent A (0.1% (v/v) TFA in Milli-Q water) for 5 min, then a linear gradient of 0–50% of the solvent B (0.1% (v/v) TFA in 100% (v/v) ACN) over the following 50 min. Throughout the next 2.5 min, a linear gradient from 50 to 100% of the solvent B was applied and then maintained at 100% for another 2.5 min. Finally, solvent A was increased to 100% during 5 min and the column was re-equilibrated for 5 min more. Absorbance was monitored at 220 nm.

The retention time of the main whey proteins (α-La, β-Lg, BSA and GMP) were determined using standard proteins. The concentration of each protein was determined using calibration curves of the standard proteins with concentrations ranging from 0.5 to 14 g/L.

2.4. Determination of protein content

Protein concentration was determined by the bicinchoninic acid

Table 1
Characterisation of the fractions obtained after whey protein hydrolysis at pilot plant-scale for 6 h, followed by ultrafiltration through 10 and 1 kDa MWCO membranes and desalting.

Fraction		Protein content (mg)	Recovery yield (%) *	I_{ACE} (%)	IC_{50} (μg/mL)	DPPH (%)	ORAC (μmol TE/mg protein)
Hydrolysate 6 h		1,233.01 ± 152.07[1]	n.d.	95.54 ± 1.74[1]	44.77 ± 1.91[1]	46.36 ± 1.27[1]	1.36 ± 0.02[1]
Ultrafiltration	Retentate 10 kDa	580.16 ± 21.12[2]	47.05[a]	90.40 ± 0.37[23]	n.d.	32.29 ± 1.02[2]	n.d.
	Permeate 10 kDa	644.33 ± 36.44[2]	52.26[a]	92.38 ± 1.69[12]	n.d.	31.89 ± 8.29[2]	n.d.
	Retentate 1 kDa	513.20 ± 62.35[2]	79.65[b]	90.09 ± 2.60[23]	n.d.	33.95 ± 0.83[12]	n.d.
	Permeate 1 kDa	91.23 ± 16.56[3]	14.16[b]	86.68 ± 0.82[3]	14.16 ± 0.65[2]	24.17 ± 9.21[2]	2.81 ± 0.05[2]
Permeate 1 kDa desalted		45.68 ± 7.27[3]	50.07[c]	93.80 ± 0.40[12]	18.44 ± 2.47[2]	69.40 ± 0.44[3]	3.37 ± 0.03[3]

*Recovery yield calculated respect: [a] the hydrolysate 6 h; [b] the permeate 10 kDa; [c] the permeate 1 kDa. [1,2,3]Different numbers in the same column indicate significant differences ($p < 0.05$). n.d.: not determined.

assay (Pierce, Rockford, IL, USA), following the manufacturer's instructions and using BSA as standard.

2.5. Purification and identification of bioactive peptides

2.5.1. Ultrafiltration

The selected protein hydrolysates were ultrafiltered in an Amicon stirred cell (Millipore, CA) under 40-psi pressure of nitrogen gas at room temperature. 10 and 1 kDa MWCO membranes were used separately in two consecutive steps. Firstly, the hydrolysate was ultrafiltrated using a 10 kDa MWCO membrane. Then, the 10 kDa permeate was fractionated using a 1 kDa MWCO membrane. Retentates and permeates were collected separately for further analyses.

2.5.2. Desalting

The 1 kDa permeate obtained in the previous step was subjected to a desalting process to avoid interfering salts. For this purpose, Sep-Pak Plus C18 cartridges (Waters, Milford, USA) were used. The cartridges were sequentially conditioned by rinsing them with 50% ACN (12 mL) and 0.1% TFA (12 mL), both prepared in Milli-Q water, using a syringe. TFA was added to the sample of 1 kDa permeate until it reached 0.1% (v/v) in the solution, and then was drop by drop loaded into the cartridge. The unbound compounds, i.e., buffer and salts, were washed with 0.1% TFA (12 mL). After that, the peptides were eluted using 100% ACN with 0.1% TFA (12 mL), and 2 mL of Milli-Q water were added to the collected fraction. To remove the ACN, the mixture was dried under a N_2 stream in a Turbo Vap LV evaporator (Caliper Life Sciences, Hopkinton, MA, USA) and concentrated to < 2 mL. Finally, the peptide fraction was lyophilised (LyoQuest −85 °C, Telstar, Spain), reconstituted to 45 g/L in Milli-Q water, and the pH was adjusted to about 7.

2.5.3. Liquid chromatography-mass spectrometry (LC-MS)

Samples were analysed by liquid chromatography coupled to a mass spectrometer by the BioCentre Facility of the University of Reading (Reading, United Kingdom) using the method described by Welderufael, Gibson, and Jauregi (2012). Briefly, peptides were separated on a reverse-phase column (Nova-Pak C18 column 150 × 2.1 mm i.d.) coupled to a Bruker MicroTof Qii high-resolution TOFMS with an electrospray ionization (ESI) source. Peaks were identified using the Bruker Data Analysis software Version 4.

The peptide identification based on the experimental masses, for intensities above 12,000 units, was carried out using the FindPeptide tool (ExPASy) for the four main proteins in *Bos taurus* milk: β-Lg, α-La, BSA and GMP, with a mass tolerance of 0.003 Da and with specific enzymatic cleavage. After that, peptide sequences with mass error [(theoretical mass-experimental mass)/theoretical mass) × 10^6] > 5 ppm were discarded. BioPep database was used to contrast the peptides identified with those previously described as ACE inhibitors (https://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

2.6. Determination of ACE inhibitory activity

The ACE inhibitory activity was determined in 96 wells plates following the FAPGG-based spectrophotometric method described by Shalaby, Zakora, and Otte (2006) with some modifications, as is described in Fuciños, Estévez, Pastrana, Tovar, and Rúa (2021). Briefly, 10 μL of each tested sample were placed in a 96-wells microtiter plate together with another 10 μL of ACE solution (0.5 U/mL distilled water). Immediately, the microplate was transferred to a thermostatic microplate scanning spectrophotometer FLUOstar Omega (BMG Labtech, Offenburg, Germany) at 37 °C. Then, 150 μL of 0.88 mM FAPGG in 50 mM Tris–HCl, pH 7.5 containing 0.3 M NaCl and preheated at 37 ∘C, was automatically pumped into each well to start the reaction. The absorbance at 340 nm was recorded for 30 min. Control samples were prepared in the same way with 10 μL of buffer (50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl) instead of the protein sample. The analysis was performed in triplicate samples.

The mean slope of the linear fit of the absorbance data versus time was used to calculate the ACE inhibition (I_{ACE}, %) was as follows:

$$I_{ACE} = \left(1 - \frac{\rho A_{inhibitor}}{\rho A_{control}}\right) \times 100 \quad (1)$$

where $\rho A_{inhibitor}$ and $\rho A_{control}$ are the slopes determined with the hydrolysate and control samples, respectively.

Concentration-response curves were obtained by plotting the ACE inhibition percentage (I_{ACE}) as a function of the protein concentration of six sample dilutions. The experimental data were fitted with the mechanistic model developed in Estévez et al. (2012) to obtain the IC_{50} value defined as the concentration of ACE inhibitors to inhibit 50% of ACE activity.

2.7. Determination of antioxidant activity

2.7.1. DPPH free radical scavenging assay

The antioxidant activity was determined using DPPH as a free radical according to the method described by Amado, Vázquez, González, and Murado (2013) with some modifications. A volume of 10 μL of each tested sample was placed in a 96-well microplate, and 200 μL of a 0.3 mM solution of DPPH in 50% (v/v) ethanol (freshly prepared under dark in 100% (v/v) ethanol) were added. The microplate was covered with an adhesive transparent sealer (A5596-100EA, Sigma-Aldrich) to prevent evaporation, incubated at 37 °C in a microplate reader (FLUOstar Omega, BMG Labtech, Germany), and the absorbance was recorded at 515 nm every 5 min until the reaction reached a plateau (120 min). Two blanks were carried out: colour blank (B_C, 10 μL of tested sample and 200 μL of 50% (v/v) ethanol) and reactive blank (B_R, 10 μL of 50% (v/v) ethanol and 200 μL of DPPH). Assays were carried out in triplicate. The radical scavenging activity (RSA) was calculated as follows:

$$RSA(\%) = \left(1 - \frac{AS - AB_C}{AB_R}\right) \times 100 \quad (2)$$

where AS, AB_C and AB_R are the absorbances of the sample, colour blank and reactive blank obtained at 120 min, respectively.

2.7.2. ORAC assay

The antioxidant activity was determined according to the method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004). The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture (200 μL) contained: FL (120 μL; 70 nM), AAPH (60 μL; 12 mM) and the antioxidant (20 μL) [Trolox (0.5–8 μM) or sample (1–10 mg/L; final concentrations)]. AAPH and trolox were prepared daily and FL was diluted from a stock solution (117 μM) in 75 mM phosphate buffer (pH 7.4). The mixture of the antioxidant and FL was dispensed in a black 96-well microplate and preincubated for 15 min at 37 °C in a microplate reader (FLUOstar Omega, BMG Labtech, Germany). After that, the AAPH was automatically pumped into each well to start the reaction. The fluorescence was recorded every 5 min for 120 min, shaking the plate automatically before each reading. 485-P excitation and 520-P emission filters were used. Blank samples (FL + AAPH) were prepared using phosphate buffer instead of the antioxidant solution. Assays were performed in triplicate.

To determine the antioxidant activity, first, it was necessary to calculate the area under the curve (AUC) as:

$$AUC = 1 + \sum_{i=1}^{i=120} f_i/f_0 \quad (3)$$

where f_0 is the initial fluorescence reading at 0 min after the addition of AAPH and f_i is the fluorescence reading at the time i. Then, the net AUC was calculated as follows:

$$NetAUC = AUC_{Sample} - AUC_{Blank} \quad (4)$$

Finally, the ORAC value (μmol Trolox Equivalent (TE)/mg protein of each tested sample) was determined from the linear regression fit of the net AUC versus the antioxidant concentration for each sample and Trolox according to:

$$Antioxidant\ activity = \frac{Slope\ for\ each\ sample\ (mgL^{-1})}{Slope\ for\ trolox(\mu molL^{-1})} \quad (5)$$

2.8. Sensory evaluation and stability assays

2.8.1. Incorporation of the whey hydrolysate as a functional ingredient into yoghurt

The whey hydrolysate produced in the pilot plant was stored frozen for four months. It was then thawed, pasteurised and incorporated into natural skimmed yoghurts by gently homogenisation under aseptic conditions. Control yoghurt samples were prepared by adding water instead of hydrolysate.

For the triangle tests, the hydrolysate was incorporated into yoghurt the same day that the tests were performed and stored at 4 °C until testing. For the consumer test, the hydrolysate was incorporated into the yoghurt and stored at 4 °C for 7 days until the test was performed. Yoghurt samples prepared for the consumer tests were also used in the stability assays. For this purpose, aliquots of each yoghurt were centrifuged at 12,000 × *g* for 30 min (Beckman Coulter, Model Avanti J-26 XP) to obtain water-soluble extracts which were kept at −20 °C until use.

2.8.2. Triangle test

The bitterness effect of whey hydrolysate products was evaluated by sensory analysis using a triangle test according to (ISO 4120, 2004). The hydrolysate was incorporated into commercial yoghurt at three different concentrations (low, medium and high) ranging from 0.1 to 1% (w/w). An untrained sensory panel (N = 29, ages 18 to 60) consisting of people recruited from students and staff from the University of Vigo (Campus of Ourense, Spain) was used to find differences in bitterness of each protein hydrolysate. In each triangle test, two samples of yoghurt containing hydrolysate and a control (or vice versa) were labelled with a randomly selected three-digit number. Samples (10 mL) were placed into 50 mL plastic glasses and presented to the panel in a random order of 6 portions (AAB, ABA, BAA, ABB, BAB, and BBA). The panellists were instructed to taste each sample from left to right and were asked to select the different samples among the three samples and to describe the difference(s) perceived. Due to the strong aftertastes associated with the samples, the panellists were provided with room temperature water and unsalted crackers for palate cleansing between samples.

2.8.3. Consumer test

The whey hydrolysate was incorporated into yoghurt elaborated by the company QUEIZUAR S.L. at the concentration optimised in the above sensory test. A minimum of fifty ratings per product is considered desirable for the precision of the statistical analysis for consumer acceptance sensory tests (Moskowitz, Beckley, & Resurreccion, 2012). Thus 123 participants from the University of Vigo (Campus of Ourense, Spain) and QUEIZUAR S.L. (A Coruña, Spain) were recruited through poster advertisement to take part in a consumer taste panel. Poorly answered tests and those corresponding to non-regular yoghurt consumers were rejected, resulting in a total of 103 participants (62 females and 41 males) for the consumer acceptability study.

Approximately 60 g of yoghurt samples were placed in uniform plastic glasses and presented to the panel labelled with random three-digit codes. Participants were initially asked to fill out a brief questionnaire about demographic information (gender, age, education level, occupation) and consumption habits of dairy products and functional foods. The socio-demographic characteristics and consumption habits of the participants in the consumer test are shown in Tables 2S and 3S, respectively, in the supplementary material.

Then, each panellist evaluated the two samples of yoghurts for odour, appearance (colour, brightness, uniformity), flavour (sweet, sour, bitter and astringent) and mouthfeel (creaminess) and overall acceptability on a 5-point hedonic scale: 1) dislike it very much, 2) dislike it moderately, 3) neither like nor dislike it, 4) like it moderately and 5) like it very much. Room temperature water and unsalted crackers were provided to panellists to cleanse their palates between samples.

Table 2
Major peptides identified by mass spectrometry in the desalted fraction below 1 kDa from whey protein hydrolysed at a pilot plant scale for 6 h. Peptides are presented in decreasing order based on their intensity.

Experimental mass (Da)	Theoretical mass (Da)	Protein source	Amino acid sequence[a]
247.1111	247.1116	β-Lg or BSA	PM or MP
245.1852	245.1865	α-La, BSA or β-Lg	LL, IL, LI or II
279.1694	279.1709	α-La, BSA or β-Lg	LF, FL or FI
488.2639	488.2621	BSA	HFKG
217.1183	217.1188	GMP, β-Lg or BSA	PT or TP
368.1556	368.1570	BSA	DPH
431.2429	431.2407	BSA	HFK
535.2707	535.2728	GMP or BSA	QVTST or VSTQT
479.2232	479.2254	BSA	FQNA
631.2962	631.2939	GMP or β-Lg	EASPEV or EPTSTP or TPEGDL
611.3772	611.3768	BSA	LPPLTA
448.2400	448.2407	GMP or β-Lg	TIASG or TINT or AEKT or VTQT or KATE
467.2151	467.2142	BSA	TEFA
844.4065	844.4052	β-Lg	AEPEQSLA or PTQLEEQ

[a] Amino acids are designated using the one-letter code. Hydrophobic amino acids are underlined.

2.9. Statistical analysis

The triangle test data (total number of responses and number of responses identifying the different sample) were analysed using the binomial probability model with a probability of 1/3 (Meilgaard, Civille, & Carr, 2006). The difference between the yoghurt with and without hydrolysate addition was considered statistically significant when the error was less than or equal to 5% (α = 0.05), which corresponded to a level of confidence greater than or equal to 95%.

For all other analysis, statistical calculations were performed at α = 0.05 using GraphPad Prism 6 software (GraphPad Software Inc.). The students *t*-test was used to analyse differences between two samples for a single variable, and one-way analysis of variance (ANOVA) was performed for multiple comparisons.

3. Results and discussion

3.1. Quantification of the biological activity of the hydrolysate generated at pilot plant scale

The ACE inhibitory activity at the end of the hydrolysis was determined. Moreover, since many peptides derived from whey proteins are known to be multifunctional peptides, i.e., they can exert more than one health-promoting activity (Udenigwe & Aluko, 2012), it was interesting to study also the antioxidant activity of the peptides obtained in this process.

The results showed that the scaling of the process described in the patent WO 2012/172129 Al resulted in ACE inhibitory peptides with an IC_{50} value of 44.77 ± 1.91 μg/mL (Table 1), similar to those obtained at the laboratory scale (unpublished results of our lab). Moreover, this value is comparable and even lower than those found by other authors for the large-scale production of whey or casein hydrolysates. For instance, Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, and

Recio (2011) reported IC_{50} values of 53.93 and 39.47 μg/mL for casein hydrolysates (150 L) obtained with pepsin and dehydrated by freeze and spray drying, respectively. While, O'Loughlin, Murray, FitzGerald, Brodkorb, and Kelly (2014) reported IC_{50} values ranging from 2.25 to 5.80 mg/mL for WPI hydrolysed with Corolase PP (400 L), i.e. between 50 and 130-fold less potent than our whey protein hydrolysate.

Regarding the antioxidant activity, the obtained hydrolysate showed moderate activity with DPPH and ORAC values of 46.36 ± 1.27% and 1.36 ± 0.02 μmol TE/mg protein, respectively. Compared with other studies, this hydrolysate exhibited comparable antioxidant activity as other whey protein hydrolysates. For example, de Castro and Sato (2014) reported DPPH values ranging from 36.46% to 73.62% for whey hydrolysates obtained with three different microbial proteases. Moreover, Contreras et al. (2011) reported ORAC values that ranged from 0.704 to 1.122 μmol TE/mg protein and from 0.832 to 2.321 μmol TE/mg protein for hydrolysates obtained with Corolase PP and thermolysin after 24 h, respectively; which agrees with the results found by Hernández-Ledesma, Dávalos, Bartolomé, and Amigo (2005) who obtained activities between 0.667 and 2.954 μmol TE/mg protein for α-La and β-Lg hydrolysates produced with different proteases.

3.2. Characterisation of bioactive peptides generated at pilot plant scale

As shown in Fig. 1, analysis of the hydrolysate by RP-HPLC revealed that the main whey proteins were almost completely hydrolysed (no peaks were detected between 50 and 60 min), and no secondary products were detected (peaks between 40 and 50 min). The formation of the peptides can be seen after 6 h of hydrolysis. When superimposing the chromatograms at 0 h and 6 h, it can be seen that the most hydrophilic compounds (peptides) were found after 6 h of hydrolysis, whereas the 0 h treatment contained more hydrophobic compounds (intact whey proteins) (Fig. 1). These results were similar to those obtained at the lab scale (unpublished results of our lab). An aliquot of the hydrolysate was sequentially subjected to UF and desalting, as explained in Section 2.5, to characterise and identify the main peptides responsible for the bioactivity.

The ACE inhibitory and antioxidant activities of each fraction were determined and the results are shown in Table 1. The fraction containing the smallest peptides (permeate with MW < 1 kDa) caused an increase of the inhibitory potency, resulting in a product enriched in powerful peptides with an IC_{50} value of 14.16 ± 0.65 μg/mL, significantly lower ($p < 0.05$) than the IC_{50} value of the initial hydrolysate (Table 1).

The recovery yield in the fraction below 1 kDa with respect to 10 kDa-Permeate was 14.16%, i.e., only 7.4% of the total protein content of the hydrolysate at 6 h (Table 1). These results therefore confirm that these powerful peptides were obtained after filtering out the larger, less active peptides (>1 kDa).

In addition, after the desalting step, the ACE inhibitory activity was maintained at the same level (18.44 ± 2.47 μg/mL) without significant differences ($p > 0.05$) regarding the same permeate without desalting (Table 1). Besides, these values were similar to that previously obtained in the lab scale assays (unpublished results of our lab).

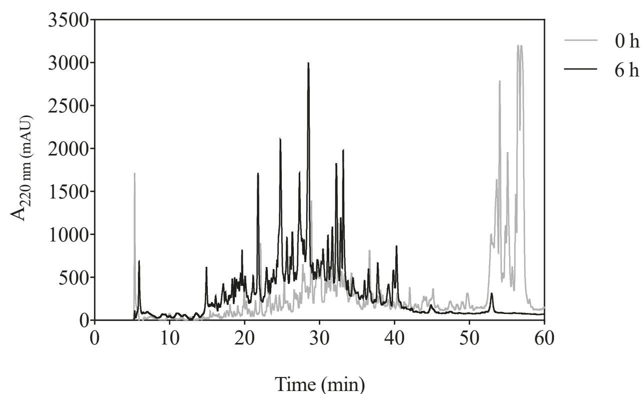

Fig. 1. RP-HPLC profile of the hydrolysate obtained at 0 and 6 h from whey protein hydrolysis at the pilot plant scale.

Concerning the antioxidant activity, during the UF a different pattern was observed depending on the method used. The activity decreased significantly ($p < 0.05$) to 24.17 ± 9.21% when it was determined by DPPH method and increased significantly ($p < 0.05$) to 2.81 ± 0.05 μmol TE/mg protein with ORAC method (Table 1). Nonetheless, after the desalting step, the activity increased significantly ($p < 0.05$) with both methods, obtaining a DPPH value of 69.40 ± 0.44% and an ORAC value of 3.37 ± 0.03 μmol TE/mg protein, which were 1.5 and 2.5-fold more potent, respectively, than the hydrolysate of 6 h. This antioxidant capacity was greater than that reported by other authors. For example, a fraction with MW < 3 kDa from α-La Corolase PP hydrolysate presented an ORAC value of 2.315 μmol TE/mg protein (Hernández-Ledesma et al., 2005), and the synthetic peptides PYVRYL (α_{s2}-casein f(203–208)) (López-Expósito, Quirós, Amigo, & Recio, 2007) and WYSLAMAASDI (β-Lg f(19–29)) (Hernández-Ledesma et al., 2005) showed values of 1.82 and 2.621 μmol TE/mg protein, respectively.

Improvement of the antioxidant activity of whey protein hydrolysates after a desalting step was recently described by Zhang, Wu, Ling, and Lu (2013) who obtained a decrease of 6.89% in the IC_{50} values of DPPH-scavenging activity after treatment of the hydrolysate with a macroporous adsorption resin. These authors demonstrated that the improvement was due to an enrichment of peptides containing hydrophobic amino acid residues, at their three C-terminal positions.

3.3. Identification of major bioactive peptides in fractions with low MW (<1 kDa)

To identify the main bioactive peptides obtained at the pilot plant scale, the desalted fraction below 1 kDa was directly subjected to RP-HPLC coupled on-line to a mass spectrometer. As shown in the total ion current (TIC) chromatogram (Fig. 1S in the supplementary material), the mass spectrum analysis of the 1 kDa-permeate was composed of an extremely complex mixture of peptides. This result was expected because the 1 kDa-permeate was not subjected to a semi-preparative RP-HPLC fractionation. For this reason, the peptide identification was based on the experimental masses for intensities higher than 12,000 units.

As summarised in Table 2, a total of 14 peptide sequences, predominantly derived from β-Lg and BSA, were identified. These were short peptides with 2–8 amino acid residues.

Concerning the peptides responsible for the antioxidant activity of the hydrolysate produced at the pilot plant scale, none of the peptides identified here has been previously reported as antioxidant peptides. However, due to their small size and structural characteristics, some of them may be the main responsible for the antioxidant activity detected. For example, high content of hydrophobic amino acid residues, such as Pro (P), Met (M), Tyr (Y), Trp (W) and Phe (F), and their location at the C-terminal can enhance the activity of the antioxidant peptides (Li & Li, 2013). This is the case with of some the most abundant peptides detected (Table 2). Hydrophobic amino acids in purified peptides may contribute to lipid peroxidation inhibitory activity by increasing the solubility of peptides in lipids and thereby facilitating a better interaction with radical species (Power, Jakeman, & Fitzgerald, 2013). Some amino acids, such as Trp (W), Tyr (Y), Met (M), Cys (C), His (H), and Phe (F) (in this order) have been described as the main ones responsible for the antioxidant activity of peptides in the ORAC-Fluorescein model (Hernández-Ledesma et al., 2005). In addition, peptides containing His (H) exhibit a strong radical scavenging activity due to the decomposition of its imidazole ring (Zhang et al., 2013). Table 2 showed that most of the peptides identified in this work contain one or more of the amino acid residues described, which explains the antioxidant activity detected. Besides, ORAC method has been usually used to assay the hydrophilic antioxidant activity, in contrast with DPPH method which works

better in hydrophobic systems as it uses organic media. So, the higher content on peptides with hydrophobic amino acids (Table 2), could explain the higher values obtained with the DPPH method compared to those obtained by ORAC.

Regarding the ACE inhibitory activity, the dipeptide PM/MP showed the highest peak intensity in the mass spectrometry analysis, which had the same C-terminal as the ACE inhibitory tripeptide GPM (Gly-Pro-Met, IC_{50} = 17.13 μM) that was previously identified from an enzymatic hydrolysate of fish protein (Byun & Kim, 2001). Furthermore, the results revealed that the next most abundant peptides were LL/IL/LI/II, LF/FL/FI, HFKG and PT/TP (Table 2), which had already been identified in the whey hydrolysis at lab scale. Moreover, the peptides LL/IL/LI/II, LF/FL/FI and PT/TP also were three of the main ones responsible for the ACE inhibitory activity of the hydrolysate at the lab scale (unpublished results from our lab). Therefore, the whey hydrolysate obtained at the pilot plant scale resulted in practically the same peptide composition as obtained at the lab scale.

3.4. Sensory analysis

The main considerations for the industrial application of protein hydrolysates containing bioactive peptides are the organoleptic properties of these ingredients and their resistance to processing conditions (Hernández-Ledesma, del Mar Contreras, & Recio, 2011).

An important limitation in the practical use of whey protein hydrolysates is the formation of off-flavour bitterness during the hydrolysis, which can be attributed mainly to the release of peptides containing hydrophobic amino acid residues. Moreover, hydrophobic amino acid residues, especially at the C-terminal end, have been associated with a strong ACE inhibitory effect of different peptides (Wu, Aluko, & Nakai, 2006). Therefore, there is a direct correlation between ACE inhibitory activity and bitterness, which means that whey protein hydrolysates may only be incorporated into foods at very low concentrations to prevent consumer rejection (Cheung et al., 2015). Thus, the next objective of this study was to evaluate the effect of the bitterness of the hydrolysate produced at the pilot plant scale on the organoleptic characteristics of the foods into which it was incorporated by sensory analysis.

3.4.1. Triangle test

Initially, a sensory discrimination test was performed to select the most suitable concentration of the hydrolysate that did not affect the sensory attributes of the final product. For this purpose, the whey protein hydrolysate obtained at 6 h (H) was incorporated into yoghurt at three different concentrations (low, medium and high) between 0.1 and 1% (w/w). Yoghurt with added hydrolysate was compared to a control yoghurt sample (with water instead of hydrolysate).

Results from each triangle test are shown in Table 3. The number of correct answers increased with increasing concentrations of the hydrolysate. For a total of 29 evaluations and a significance level of 5% (α = 0.05), at least 15 correct answers are required for significance differences (ISO 4120, 2004). Thus, the panellists detected significant differences ($p < 0.05$) between the two highest concentrations (H_{Medium} and H_{High}) and the control. Moreover, based on the comments given by the panellist, the samples H_{High} and H_{Medium} were described as less sweet and more bitter and astringent compared with the control sample. The bitterness of these yoghurt samples could be related to the fact that the most abundant peptides previously identified in the whey hydrolysate were: PM/MP, LL/IL/LI/II, LF/FL/FI, HFKG and PT/TP, which are short peptides (<3 kDa) containing hydrophobic amino acids (phenylalanine (F), proline (P), isoleucine (I) or histidine (H)) (Welderufael, Gibson, Methven, & Jauregi, 2012).

Table 3
Results of the triangle tests performed to evaluate the influence of the incorporation into yoghurt of three different concentrations (low, medium and high) of a whey protein hydrolysate.

Triangle test	Sample	N° of panellists	N° of responses identifying the different sample
1	H_{Low}	29	11
2	H_{Medium}	29	20*
3	H_{High}	29	27*

*Statistically significant differences between the sample and the control ($p < 0.05$).

Nonetheless, there was no significant difference ($p > 0.05$) between the sample with the lowest hydrolysate concentration (H_{Low}) and the control. Therefore, the addition of the hydrolysate at this concentration did not affect the taste of the yoghurt. For this reason, the lowest hydrolysate concentration was selected for the next sensory analysis.

3.4.2. Consumer test

Based on the results of the discriminatory sensory test, a consumer acceptance test was conducted to evaluate the acceptability of yoghurt formulated with whey protein hydrolysate as a functional ingredient. The comparisons of the qualitative attributes for control and functional yoghurt are shown in Fig. 2.

Both, the control and functional yoghurt, showed scores between 2.0 (dislike it moderately) and 4.0 (like it moderately) on a 5-point hedonic scale for all attributes studied. Significant differences ($p < 0.05$) were observed between control and functional yoghurt for only two attributes: odour and creaminess (Fig. 2), indicating that the incorporation of the hydrolysate into the yoghurt brought some negative notes to the functional product. The lowest score received for the attribute creaminess was expected since the yoghurt matrix (without the addition of water or hydrolysate) was already quite fluid and showed little consistency. These changes in the individual sensory attributes of the functional yoghurt concerning the control were also reflected in the overall acceptability causing significant differences ($p < 0.05$) between both samples (Fig. 2).

Nonetheless, significant differences were not detected ($p > 0.05$) for the bitterness (Fig. 2), which is the sensory attribute more negatively affected due to the use of protein hydrolysates as a functional food ingredient (Cheung et al., 2015; Welderufael et al., 2012).

In addition, at the end of the test, consumers answered the question: *Would you buy this yoghurt if you knew that it has functional properties?*. The answers for the functional yoghurt showed that 46.60% of consumers would buy it. While in the case of the control yoghurt, 65.05% of consumers would buy it. Many of the consumers who participated in the consumer test commented that they preferred yoghurts containing sugar and fruit pulp in the product formulation.

3.5. Stability of the bioactive properties of hydrolysates during storage and processing

The conditions of processing and storage commonly used in the industry may be detrimental to bioactive peptides, resulting in a loss of activity after their incorporation into the food matrices (Hernández-Ledesma et al., 2011). Therefore, the final objective of this work was to evaluate the stability of the bioactivities of the hydrolysate produced at pilot plant scale, and incorporated into natural skimmed yoghurt, under processing and storage conditions that could be used in the industry. For this purpose, samples of the same yoghurts (control and functional) used in the consumer test were stored at 4 °C for 1 week and then centrifuged to obtain water-soluble extracts, on which the ACE inhibitory and antioxidant activities were determined. Additionally, the activities of the hydrolysate mixed with yoghurt were also analysed after 1 h storage at 4 °C.

As described earlier, the whey hydrolysate produced at pilot plant scale had an ACE inhibitory activity of 95.54 ± 1.74% (IC_{50} = 44.77 ± 1.91 μg/mL) and antioxidant activity of 46.36 ± 1.27% and 1.36 ± 0.02 μmol TE/mg protein determined by DPPH and ORAC method

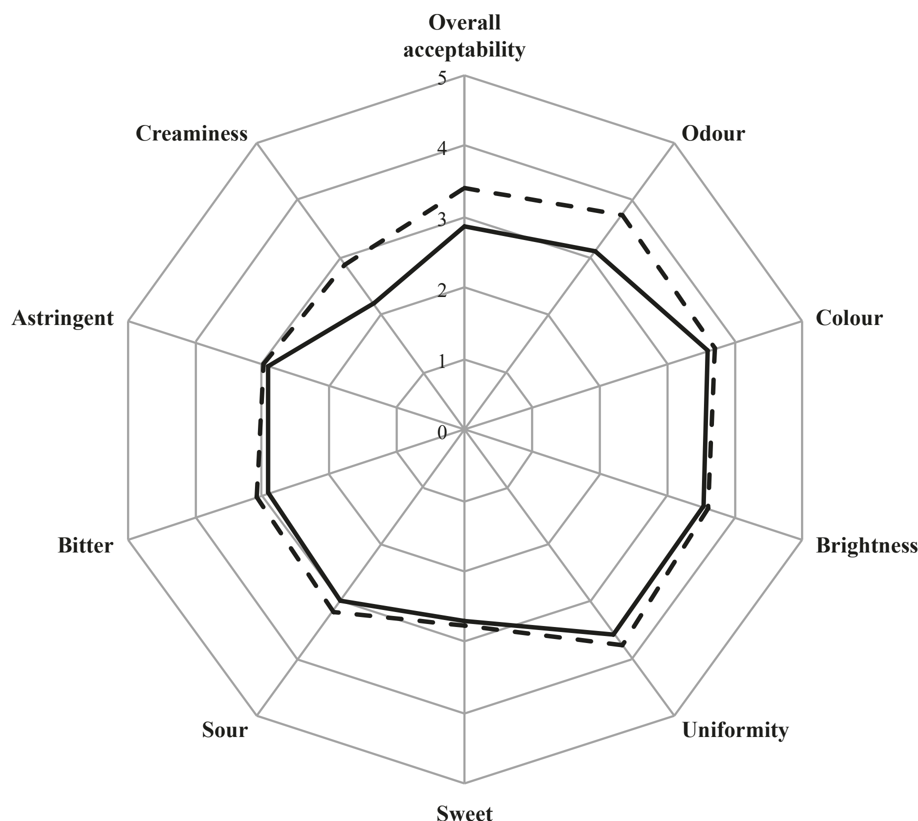

Fig. 2. Spider plot showing the mean acceptance ratings for the control (– –) and functional (—) yoghurt samples. 5 points scale: 1 = Dislike it very much and 5 = Like it very much. Statistically significant differences ($p < 0.05$) were found between the two yoghurt samples in terms of odour, creaminess and overall acceptability.

respectively (Table 4). The same hydrolysate after undergoing various processing conditions, i.e. storage for 4 months at −20 °C, thawing and pasteurisation, exhibited no significant ($p > 0.05$) decrease in I_{ACE}, DPPH and ORAC values up to 93.46 ± 11.83%, 50.01 ± 0.68% and 1.12 ± 0.15 μmol TE/mg protein respectively (Table 4). Therefore, the whey hydrolysates retained their bioactivity, indicating that they were stable against the different processing conditions that could be used in the industry and long storage times.

These results are consistent with those reported by other authors. Thus, Hwang (2010) reported that ACE inhibitory peptides derived from tuna cooking juice were stable after incubating at different temperatures (20–100 °C, for 2 h), levels of pressure (50–300 MPa, for 30 min) and pHs (2–10, for 2 h at 40 °C). In addition, Zhao, Huang, Chen, and Jiang (2011) reported that a hydrolysate obtained from shrimp processing by-products retained>80% of its antioxidant activity after heating it to 100 °C and maintained nearly 70% of the original activity at low pH (2.0). Kurosaki, Maeno, Mennear, and Bernard (2005) and Mizuno, Mennear, Matsuura, and Bernard (2005) also have shown the stability of the potent ACE inhibitory tripeptides IPP and VPP in refrigerated conditions and in powered preparations stored at room temperature, respectively.

The control yoghurt showed a very low ACE inhibitory activity (<5%) by itself (Table 4). Conversely, the yoghurt with hydrolysate incorporated one week before presented a significant ($p < 0.05$) higher inhibitory activity of 52.70 ± 2.71% and an IC_{50} value of 315.90 ± 34.32 μg/mL, which was, therefore, due to the presence of the active peptides. This activity was expected considering the dilution factor of the active peptides in the yoghurt and the increase in protein concentration due to the yoghurt matrix. Furthermore, the yoghurt with hydrolysate incorporated one hour before showed a very similar activity (58.24 ± 1.15% and IC_{50} = 266.85 ± 34.71 μg/mL), with no significant ($p > 0.05$) differences against the values obtained after 1 week (Table 4). This means that the ACE inhibitory peptides were stable against incorporation into yoghurt and subsequent storage at 4 °C for one week.

On the other hand, the control yoghurt presented a slight antioxidant activity with a DPPH value of 17.74 ± 0.97% and an ORAC value of 0.35 ± 0.03 μmol TE/mg protein (Table 4). After 1 h or 1 week storage from the incorporation of the hydrolysate into the yoghurt, the activity increased slightly with the DPPH method, but without significant ($p > 0.05$) differences, obtaining values 1.04 and 1.23 times, respectively, higher than the yoghurt alone. In the case of the ORAC method, the differences were higher, where the antioxidant activities of yoghurt with peptides incorporated 1 week or 1 h early were 1.67 and 1.76 significant ($p < 0.05$) higher with than the control yoghurt. Besides, there were also

Table 4
Effects of storage and processing conditions on the bioactive activities of a whey protein hydrolysate obtained at a pilot plant scale for 6 h, and their stability after incorporation into yoghurts as a functional ingredient.

Sample	Storage and processing conditions	I_{ACE} (%)	IC_{50} (μg/mL)	DPPH (%)	ORAC (μmol TE/mg protein)
Hydrolysate 6 h	< 48 h/−20 °C	95.54 ± 1.74[1]	44.77 ± 1.91[1]	46.36 ± 1.27[1]	1.36 ± 0.02[1]
	4 months/−20 °C	93.46 ± 11.83[1]	n.d.	50.01 ± 0.68[1]	1.12 ± 0.15[2]
Yoghurt	1 week/4 °C	4.53 ± 1.17[2]	n.d.	17.74 ± 0.97[2]	0.35 ± 0.03[3]
Yoghurt + hydrolysate	1 h/4 °C	58.24 ± 1.15[3]	266.85 ± 34.71[2]	21.78 ± 1.17[2]	0.62 ± 0.04[4]
	1 week/4 °C	52.70 ± 2.71[3]	315.90 ± 34.32[2]	18.52 ± 3.07[2]	0.59 ± 0.04[4]

[1,2,3,4]Different numbers in the same column indicate significant differences ($p < 0.05$). n.d.: not determined.

no significant ($p > 0.05$) differences against the values obtained after 1-hour and 1-week storage (Table 4), which reinforces the stability of the bioactive peptides present in the hydrolysate against incorporation into yoghurt and subsequent storage at 4 °C.

4. Conclusions

The results demonstrated that the pilot plant scale (200 L) process developed was effective in reproducing the whey protein hydrolysis process using a combination of two microbial proteases as described in patent WO 2012/172129 Al, resulting in peptides with a potent ACE inhibitory (IC_{50} = 44.77 ± 1.91 μg/mL) and antioxidant activity (DPPH and ORAC values of 46.36 ± 1.27% and 1.36 ± 0.02 μmol TE/mg protein, respectively). Besides, potent bioactive peptides (PM/MP, LL/IL/LI/II, LF/FL/FI, HFKG and PT/TP), already produced at the lab scale, have been identified in the pilot plant scale whey hydrolysed product.

The amount of hydrolysate incorporated into a natural skimmed yoghurt was also optimised in such a way that no significant differences ($p > 0.05$) were detected with respect to a control yoghurt without hydrolysate with regards to the bitterness, which is the sensory attribute more negatively affected due to the use of protein hydrolysates as a functional food ingredient. Although significant differences ($p < 0.05$) were detected odour and creaminess. On the other hand, the bioactive peptides present in the hydrolysate obtained at pilot plant scale have shown high stability, maintaining ACE inhibitory and antioxidant activities for at least 4 months of storage at −20 °C and after undergoing various processing conditions (IC_{50} = 44.77 ± 1.91 μg/mL and antioxidant activity of 50.01 ± 0.68% and 1.12 ± 0.15 μmol TE/mg protein, quantified by DPPH and ORAC methods respectively). Furthermore, bioactivities were maintained for at least 1 week at 4 °C, after incorporation of the hydrolysate into natural skimmed yoghurt (IC_{50} = 315.90 ± 34.32 μg/mL and antioxidant activity of 18.52 ± 3.07% and 0.59 ± 0.04 μmol TE/mg protein, quantified by DPPH and ORAC methods respectively).

Therefore, the results obtained in this study represent a new opportunity for the development of functional ingredients that could be used as food additives at an industrial scale, as it contributes to a higher depuration of food industry waste, leading to a decrease in its contamination effect.

CRediT authorship contribution statement

Natalia Estévez: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Clara Fuciños:** Validation, Formal analysis, Writing – review & editing. **Andrea Rodríguez-Sanz:** Formal analysis, Writing – review & editing. **María Luisa Rúa:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133459.

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