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Improvement of functional and antimicrobial properties of brewery byproduct hydrolysed enzymatically

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ABSTRACT

The aim of this study is to investigate the characteristics of brewer's spent grain (BSG) hydrolysates. Hydrolysis was performed using a *Bacillus cereus* sp. extracellular peptidase. The proteins were hydrolysed for 2, 8 and 24 h, achieving degree of hydrolysis ranging from 2.65 to 21.80%. During enzymatic hydrolysis, average peptide chain length decreased rapidly and the soluble forms increased. Solubility of the hydrolysates showed a good correlation with turbidity. The water/oil holding capacity, the emulsifying properties and the foaming expansion were analysed, and improved functional properties were found respect to the control. For the studied hydrolysates concentrations no gel formation were obtained. However, BSG hydrolysates exhibited also desirable rheological properties making their good candidate for many food formulations. Thermal characterization revealed the energy recovery by the enzymatic hydrolysis process. The understanding of hydrolysates antimicrobial properties may lead to utilize their as potent natural antimicrobial against *Escherichia coli* O157:H7. In the light of the results, hydrolysates made from BSG can be converted into a high value protein food ingredient.

1. Introduction

Protein-rich byproducts from agro industrial industry have limited uses due to their dark colour, susceptibility to oxidation and off-flavour. As a sequence, they are discarded or processed into low marked-value products. The application of enzyme technology to recover food protein may produce a broad spectrum of food ingredients or industrial products. Recent advances in biotechnology have also demonstrated the capacity of enzymes to produce novel food products, modified foodstuffs and improved waste management.

Technological advancement in the past two decades in the commercial protein production has focused on the use of plant protein sources instead of animal sources (Henry & Kettlewell, 1996). Barley (*Hordeum vulgare* L.) is an extensive grown cereal and is used mainly for the brewing industry. In recent years, increased incorporation of barley into the human diet is recommended (Newman, Lewis, Newman, Boik, & Ramage, 1989) due to nutraceutical properties. Brewer's spent grain (BSG), high-volume by-product from the brewing industry, primarily contains proteins, barley cell wall carbohydrates, and lignin (Treimo et al., 2009). However, to date its use has mainly been limited to animal feeding. To increase the potential application of such insoluble proteins, they can be hydrolysed.

With regard to BSG utilization, our research group has already optimized the conditions for fermentation using *Bacillus cereus* spp., a bacteria isolated from fermented cabbage (Pérez Borla, Davidovich, & Roura, 2010). The fermentation is an eco-friendly method for the recovery of biomolecules from BSG.

In recent years, a significant growth of interest in functional protein hydrolysates can be observed. Protein modifications by enzymatic or chemical means can usually improve certain functional properties. Particular attention has been paid to enzymatic hydrolysis of protein. Enzymatic modification using specific proteases has several advantages over acid or alkali hydrolysis; it is mild and does not destroy amino acids (Desslie & Cheryen, 1988). Protein hydrolysis has also been applied to improve the functional, organoleptic and nutritional value of foodstuff (Navarrete del Toro & García-Carreño, 2002). Functional protein hydrolysates can be

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used in food systems as additives, as food texture enhancers or as pharmaceutical ingredients. Some of these properties are water/oil binding, emulsification, foam formation, viscosity and gelation. Many studies have demonstrated that the enzymatic hydrolysis of various substrates improved its functional properties, including solubility, emulsifying and foaming characteristics (Puski, 1975; Were, Hettiarachchy, & Kalapathly, 1997). Functional properties of proteins are those physicochemical properties of proteins which affect behaviour of proteins in food systems during preparation, processing, storage, and consumption, and contribute to the quality and sensory attributes of food systems (Kinsella, 1976).

These properties are affected by intrinsic protein factors such as molecular structure and size, and other many environmental factors. The importance of these properties varies with the type of food products in which the hydrolysate is used (Yu, Ahmedna, & Goktepe, 2007). Is scare the information about the antimicrobial activity of hydrolysates from processing by-products against food pathogens. Protein hydrolysates are widely used as nutritional supplements, functional ingredients and flavours enhancers in food, coffee whiteners, cosmetics, personal care products and confectionery and in the fortification of food products. The latter is critically needed in developing countries where protein deficiencies remain a mayor health problem, especially among children (Yu et al., 2007). The hydrolysis process generates smaller peptides with improved nutritional characteristics compared to the original protein (Kalili, Mohamed, Taha, & Karisson, 2003).

The hydrolysed protein rich liquor is expected to show beneficial bio-functionalities like antibacterial properties. Protein hydrolysates from different sources, such as soy (Joo, Yi, Lee, Lee, & Oh, 2004), whey (Shinam, Radha, Prakash, & Kaul, 2007), meat and fish proteins (Jang, Jo, Kang, & Lee, 2008) and defatted peanut kernels (Huang, Shyu, Wang, & Hsu, 2010), have been found to possess antimicrobial activity. The operational conditions employed in the processing of protein isolates, the type of protease (Celus, Brijs, & Delcour, 2007) and the degree of hydrolysis (DH) also affect the antimicrobial activity.

In the light of the above facts, a characterization of the hydrolysates resulting from enzymatic hydrolysis of BSG protein was done. Firstly, protein hydrolysates, with variable DH, were prepared from pre-treated BSG by a *B. cereus* spp. extracellular protease. After that, the hydrolysates were characterized by their functional properties (such as solubility, clarity, foaming, emulsification and gelation) to determine the potential application in various food products.

Additionally, other physicochemical properties as the thermal characterization, reological behaviour and colour of the hydrolysis product were investigated.

Finally, this study determined the antimicrobial activity of the protein hydrolysate against *Escherichia coli* and *Listeria monocytogenes*. The understanding of antimicrobial properties may lead to utilize it as a potent natural antimicrobial. In future works, we will emphasize the search of antioxidant capacity of these hydrolysates.

2. Materials and methods

2.1. Raw materials

Brewer's spent grain (BSG) [~32.5 g protein/100 g on a dry basis (db)] was purchased from Antares S.A. (Mar del Plata, Argentina). The BSG was pre-treated according to the procedure describing in Kotlar, Belagardi, and Roura (2011) in order to standardize the hydrolysis substrate (HS). Enzymatic hydrolysis was produced in our lab following the procedure described in Section 2.1.

2.2. Preparation of protein hydrolysate

2.2.1. Enzymatic hydrolysis

The enzyme source was obtained from a *B. cereus* strain previously isolated by Pérez Borla et al. (2010).

Thirty six gram of HS was added to 100 mL Minimal Salt Medium (MSM) which contained: K_2HPO_4 (0.1 g/100 mL), $MgSO_4 \cdot H_2O$ (0.02 g/100 mL), $CaCl_2$ (0.01 g/100 mL) and Na_2CO_3 (0.1 g/100 mL), and then was autoclaved at 121 °C for 15 min (Kotlar, Ponce, Sansevero, & Roura, 2010), to inactivate microorganisms initially present in the HS. Sodium carbonate was sterilized separately and added to the rest of the medium after cooling to room temperature. 5 mL of the enzyme source (crude enzyme) were added to 100 mL of the above mentioned medium in 150 mL Erlenmeyer, and incubated at 32 °C on an orbital shaker (TS-1000, Zhejiang, China) for 24 h. To evaluate the effect of the incubation medium alone over this timeframe, a control sample was prepared in a similar manner, but without enzyme (control).

Hydrolysate samples were collected at 0, 2, 8 and 24 h of incubation period (HP_0, HP_1, HP_2 and HP_3, respectively), and enzymatic activity stopped by heating in a water bath at 95 °C for 5 min.

Then, each sample was centrifuged at 2000 × *g* at 4 °C for 10 min using a Labnet 7M centrifuge (Labnet International, Inc., New York, USA) and the supernatant was collected.

Hydrolysates were freeze-dried using a freeze dryer (Karaltay Scientific Instruments Co., Ltd., Beijing, China) to yield a powered hydrolysate. Lyophilizes were stored at 4 °C until analysed.

2.2.2. Determination of degree of hydrolysis (DH)

The DH of BSG hydrolysates was measured by the o-phthaldialdehyde (OPA) method (Nielsen, Petersen, & Dambmann, 2001). The OPA reagent was prepared by combining 7.620 g disodium tetraborate, 200 mg sodium dodecyl sulphate (SDS), 160 mg OPA 97% (in 4.0 mL 95% ethanol), and 176 mg dithiothreitol (DTT) (Sigma, cod. D0632) and adding deionized water to a final volume of 200 mL. A L-serine (Merck, cod. Art. 7769) concentration range (0–0.2 mg/mL) was used as the standard curve. Total hydrolysate was diluted (1:80) in SDS (1 g/100 mL). OPA reagent (3.0 mL) was added to all samples (400 μL), and the absorbance (340 nm) was measured after 20 min. The increase in amino groups between control sample and hydrolysates was attributed to proteolysis, and the degree of hydrolysis (DH) was calculated by the following equation:

$$DH\_(\%) = \frac{h}{h_{tot}} * 100 \tag{1}$$

Where *h* represents hydrolysis equivalents [mequivalents (mequi)/g protein]; and h_{tot} is the total theoretical number of peptide bonds per unit weight present in BSG protein, for hordeins h_{tot} = 7.52 mequi/g protein (Bamdad, Wu, & Chen, 2011). The degree of hydrolysis expressed for each sample was the mean of three determinations.

The expression for *h* in the OPA method is:

$$\text{serine\_}NH_2 = \frac{\left(OD_{sample\_} - OD_{blank}\right)}{\left(OD_{standard} - OD_{blank}\right)} * 0.9516 \text{ mequi} / L * 0.1 * 100 / X * P \tag{2}$$

Where serine_NH_2 is the mequi serine NH_2/g protein; *X* are the grams of sample; *P* is the protein concentration in the sample; 0.1 is the sample volume in litre (L), *h* is then:

$$h = \frac{(\text{serine\_NH}_2 - \beta)}{\alpha\text{\_mequi/g\_protein}} \quad (3)$$

Where α and β for no examined material are estimated to be 1.00 and 0.40, respectively (Nielsen et al., 2001).

2.2.2.1. Determining of average peptide chain length. The average peptide chain length (PCL) of hydrolysates can be estimated using the degree of hydrolysis (Eq. (4)), assuming that the entire hydrolysate is soluble, as only the supernatants were analysed:

$$\text{PLC} = \frac{100}{\text{DH\%}} \quad (4)$$

2.3. Hydrolysate functional properties

Functional properties evaluated were protein solubility, emulsifying capacity, foaming capacity, water holding capacity, oil holding capacity and gelation capacity. Each assay was performed by duplicate in 3 independent experimental runs.

2.3.1. Clarity

This was evaluated according to the method reported by Desslie and Cheryen (1988). An aqueous solution of hydrolysates was blended (1 g/100 mL). The pH was adjusted with either HCl (0.1 mol equi/L) or NaOH (0.1 mol equi/L). Optical clarity was assessed quantitatively by turbidity measurements, as the optical density at 660 nm. Distilled water was used as the blank.

2.3.2. Determination of protein solubility

To determine the solubility of the hydrolysed proteins, 20 mg of lyophilized protein sample was redissolved in 20 mL of deionized water, adjusted to pH 3, 5, 7 and 9 with HCl (0.1 mol equi/L) or NaOH (0.1 mol equi/L), magnetically stirred at ambient temperature during 30 min. Protein content in supernatants obtained after centrifugation at 12,000 $\times$ *g* for 10 min were determined according to the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (BSA) as the standard. Protein solubility was calculated as the percentage of proteins present in supernatant.

2.3.3. Emulsifying properties

Emulsifying properties were measured according to the method of Yasumatsu et al. (1992). Briefly, 25 mL of sunflower oil (Cocinero, Molinos S.A., Argentina) were added to 25 mL of protein hydrolysate and the pH adjusted to 4, 6 or 10 with HCl (0.1 mol equi/L) or NaOH (0.1 mol equi/L). The mixtures were blended in for 3 min with a Minipimer 600 W (Philips, Argentina). The blending was transferred immediately to a 10 mL glass tube and centrifuged for 5 min at 1027 $\times$ *g* (Model 2036, Rolco S.R.L., Buenos Aires, Argentina). The emulsion was expressed as percentage, measuring the height of the emulsifying layer respect on the total liquid height.

2.3.4. Foaming properties

Foaming ability and foam stability of protein hydrolysates were tested according to the method of Puski (1975). Twenty millilitres of hydrolysed protein solution (1 g/100 mL) at pH 7 were homogenized at 2862 $\times$ *g* for 1 min to incorporate the air. The bender contents were immediately transferred to a 50 mL graduated cylinder and the foam volume was registered. The cylinder was placed at room temperature for 2 h and the residual foam volume measured. Foam ability was expressed as foam expansion at 0 min, while foam stability was expressed as foam expansion during 120 min. Foam expansion was expressed as the percent loss of foam volume, according to the following equation:

$$\text{Foam\_expansion}(\%) = \left[\frac{(A-B)}{B}\right]*100 \quad (5)$$

Where *A* is the volume after whipping (mL) at different times and *B* is the volume before whipping (mL).

2.3.5. Water holding capacity

Water holding capacity was determined using the method outlined by Beuchat (1977). One gram of hydrolysate sample was weight into a pre-weighed 15 mL centrifuge tubes. For each sample, 10 mL of distilled water was added and mixed using a vortex (Decalab S.R.L., Buenos Aires, Argentina) at highest speed for 2 min. After the mixture was thoroughly wetted, samples were allow to stand at room temperature for 30 min, then centrifuged at 1027 $\times$ *g* for 20 min. The supernatant was decanted and the centrifuged tube containing sediment was weighed. Water holding capacity (WHC) (grams of water per gram of sample) was calculated as:

$$\text{WHC} = \frac{(W_2 - W_1)}{W_0} \quad (6)$$

Where W_0 is the weight of the dry sample (g), W_1 is the weight of the tube plus the dry sample (g) and W_2 is the weight of the tube plus the sediment (g).

Triplicate samples were analysed for each hydrolysate.

2.3.6. Oil holding capacity

Oil binding capacity was determined following the Chakraborty method (1986). One gram (dry weight) (W_0) of hydrolysate was weighted into pre-weighted 15 mL centrifuge tubes and thoroughly mixed with 10 mL (V_1) of vegetable edible oil (Cocinero, Molinos S.A., Argentina) using a vortex mixer. Samples were allowed to stand for 30 min. The mixture was centrifuged at 1027 $\times$ *g* for 20 min. Immediately after centrifugation, the supernatant was carefully poured into a 10 mL graduated cylinder, and the volume was recorded (V_2). Oil absorption capacity (OAC) (millilitre of oil per gram of dry weight hydrolysate) was calculated as:

$$\text{OAC} = \frac{(V_1 - V_2)}{W_0} \quad (7)$$

Triplicate samples were analysed for each hydrolysate.

2.3.7. Gelation capacity

Gelation was determined by the method of Obatolu and Cole (2008). Sample dispersions (obtained after enzymatic hydrolysis and heating at 95 °C for 5 min) of 4, 8, 12, and 16 g/100 mL were prepared in 10 mL distilled water. The test tubes containing the suspensions were heated for 1 h in a boiling water bath followed by rapid cooling under ice bath. The least gelation concentration was determined when the sample from the inverted test tube did not fall or slip.

2.4. Thermal properties

Thermal characteristics of hydrolysate samples were determined with a Shimadzu DSC-50 instrument (Shimadzu Corporation, Kyoto, Japan). Lyophilized samples (1 mg each) were directly weighed into coated aluminium pans and 10 μL of water was added. The aluminium pans were hermetically sealed and heated from 30 to 120 °C at a rate of 10 °C/min. A sealed empty pan was used as reference. Thermal Denaturation or maximum temperature (T_{max}) and denaturation enthalpy were calculated from thermograms with the thermal analysis software (Thermal Analysis Instruments TA-60, MD, USA). The denaturation temperatures were assigned to

each thermal curve, based on the temperature at which the lowest value for the heat flow occurred. For the curves, the offset-temperature (T_{offset}; point where the extrapolated leading edge of the last endotherm intersects with the baseline) was also determined. The start of the transition, Ton, for all studies, was taken as the onset point of the transition that is the point at which the extrapolated baseline intersects the extrapolated slop in the transition state.

2.5. Rheological properties

Protein suspension containing 10 and 12.5 g of BSG hydrolysate/100 mL of distilled water were prepared and the pH of suspensions was adjusted to 7 with NaOH (0.1 mol equi/L) or HCl (1.0 mol equi/L). Suspensions were heated to 90 °C in a shaking water bath and kept 30 min, then cooled to room temperature without stirring. Gel viscosity (centipoises) of these protein suspensions was determined using a Brookfield VD-II+ Viscometer at different shear rates (1, 2.5 and 5 rpm) at room temperature. The results were means of triplicates of independent run.

2.6. Colour

Colour determination was carried out using a Minolta colorimeter (Model CR-300, Konica Minolta Sensing Americas Inc., New York, USA) with an 8 mm diameter measuring area. A white plate ($Y = 93.2$, $x = 0.3133$, $y = 0.3192$) was used to standardize the instrument. The colour of hydrolysate solutions (5 g/100 mL) in distilled water was measured by L^* (lightness), a^* (redness, + or greenness, −), and b^* (yellowness, + or blueness, −) chromaticity co-ordinates of the CIELab scale (1978). The parameter b^* was analysed, as it measures from blue to yellow (−60 = blue, +60 = yellow). The colour was evaluated as hue angle ($h = \tan^{-1}[b^*/a^*]$), that corresponded to the colour tone and is associated with the colour perceived by human eye, where 0° = red, 90° = yellow, 180° = green, and 270° = blue (McGuire, 1992); and chroma ($C = \sqrt{(a^{*2} + b^{*2})}$), or colour saturation, a measurement of the vividness of colour (higher values indicate a more vivid colour).

Measurements were performed in 5 different positions on the samples. Reported values correspond to the mean.

2.7. Antimicrobial activity

An agar well diffusion assay (AWDA) was used for detection of antagonistic activity (Ponce, Moreira, del Valle, & Roura, 2008). BHI (Brain and Heart Infusion, Britania, Buenos Aires, Argentina) agar (1.5 g/100 g agar) plates were inoculated with 100 μL of an overnight culture of indicator strain. The indicators used were: *L. monocytogenes* (Gram-positive indicator) provided from CERELA and *E. coli* O157:H7 isolated from meat (Gram-negative indicator).

Wells 5 mm in diameter were cut into these agar plates. Hydrolysate (30 μL) was placed into each well. The plates were then incubated aerobically for 24 h at 30 °C and the inhibition zones were subsequently examined (6 mm clear or larger zones around the well were scored as positive inhibition).

2.8. Statistical analysis

Each assay was performed by duplicate in 3 independent experimental runs and data were averaged. Standard deviation was also calculated. Data were analysed using SAS software version 8.0 (SAS 1999; PROC GLM, general linear model procedure). Mean differences were judged at the 5% significance level. The Tukey–Kramer multiple comparison test was used for pair-wise comparison when significant differences were found.

The relationships between different properties were also determined using Pearson correlation coefficients.

3. Results and discussions

3.1. Hydrolysates preparation

The extent of proteolysis was quantified as the degree of hydrolysis (DH), which refers to the proportion of cleaved peptide bonds in a protein hydrolysate (Rutherfurd, 2010). The hydrolysis process was carried out for 24 h and aliquots were withdrawn at 0, 2, 8 and 24 h. Different methods were used to evaluate the DH of the peptide bonds that depended on three essential principles: the amount of nitrogen released by the protein hydrolysis in the presence of a precipitation agent, the determination of free α-amino groups, and the titration of the released protons (Wang, Zhao, Zhao, Bao, & Jiang, 2007). In the present work, the DH was measured by the OPA method. Nielsen et al. (2001) determined that the DH of protein hydrolyses analysed by the OPA method is more accurate, is easier and faster to carry out, has a broader application range, and is environmentally safer than other methods.

As shown in Fig. 1, the hydrolysis proceeded at a low initially rate with DH reaching only 2.65 ± 0.04% after 2 h. According to Yalçin and Celik (2007), this slower initial hydrolysis for BSG protein can be attributed to its greater content of inter-chain disulfite bonds making it more resistant to proteases. After this initial induction period, the hydrolysis BSG protein likely unfolds and becomes susceptible to the action of proteases, leading to an increased hydrolysis rate. Nevertheless, *B. cereus* hydrolysis proceeded very slowly, with a DH of 3.81 ± 0.26% even after 8 h of hydrolysis. No significant difference in the DH value was apparent between HP_1 and HP_2 ($P < 0.05$). Thus, two kinds of hydrolysates of BSG were obtained under the present hydrolytic conditions. Finally, at 24 h hydrolysis time, the DH was 21.80 ± 2.32%. Similar DHs were obtained by Treimo, Aspmo, Eijsinkm, and Hornm (2008). Contrarily, this DH value was higher that the previously value achieved with BSG hydrolysates by Celus, Brijs, and Delucour (2009), who hydrolysed a chemically isolated protein concentrate.

3.2. Peptide chain length

The peptide chain length is related to the average molecular weight of peptides in hydrolysates. However, hydrolysates with similar PCL may have substantially different peptide molecular weight distributions.

Once determined the DH, the average peptide chain length of the hydrolysates could be estimated, assuming all hydrolysates are

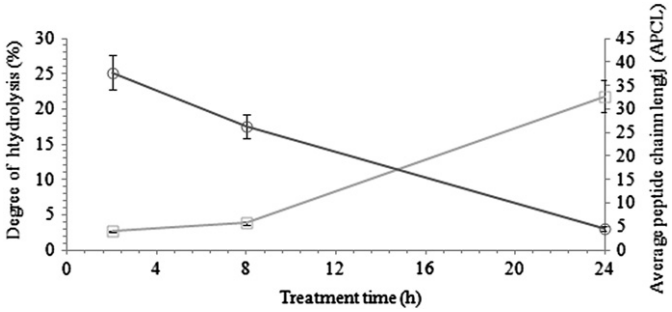

Fig. 1. Changes in degree of hydrolysis (□) and average peptide chain length (○) during enzymatic hydrolysis of BSG proteins.

soluble. This is related to the average molecular weight of peptides in the hydrolysates.

Fig. 1 shows also the changes in average peptide chain length (APCL) of the hydrolysates. The average peptide chain length of the hydrolysates was about 38 amino acid units after 2 h hydrolysis. It was reduced to 5 amino acid units at the end of the 24 h enzymatic hydrolysis. The results of APCL were in agreement with the model of DH change.

3.3. Functional properties

3.3.1. Clarity

Turbidity profiles, expressed as optical density (OD) of BSG hydrolysates at various pH values are shown in Fig. 2.

Turbidity was pH dependent, probably due to the hydrolysed polypeptides in solution. Solutions of HP_3 can be considered clear because their OD was under 0.1 at 660 nm (Desslie & Cheryen, 1988). HP_1 had turbidity over all the pH range; and HP_2 had only turbidity at pH 4.

Turbidity of HP_0 was higher because it contains the less hydrolysed polypeptides, and increased turbidity was obtained with decreasing pH.

3.3.2. Solubility

Solubility is one of the most important physicochemical and functional properties of protein hydrolysates (Amiza, Kong, & Faazaz, 2012). Fig. 3 presented the solubility profile of the hydrolysates at various pHs (2, 4, 6, 8 and 10). As shown in Fig. 3, the enzymatic hydrolysis treatments resulted in a remarkable increase in the protein content. The pH had a significant effect on the solubility of BSG protein. The pH-protein solubility profiles of the hydrolysates were practically pH-dependent, the minimum protein solubility tested was observed at pH 4, which is the isoelectric point of barley proteins (Celus et al., 2007); and maximum solubility at pH 10 or higher. However HP_1 showed comparable solubility over the entire pH range.

As was mentioned, the lowest solubility in distilled water was observed at pH 4. However, the protein solubility values at pH 4 and PH 6 were close to each other.

The use of pHs higher than 10 was not desirable because of undesirable changes such as protein denaturation and discolouration which could affect the functionality and sensory quality (Yu et al., 2007).

As was shown in Fig. 3 the hydrolysis time significantly increased protein solubility in BSG across the pH range tested (pH 2–10), indicating that soluble peptides were produced by proteolysis.

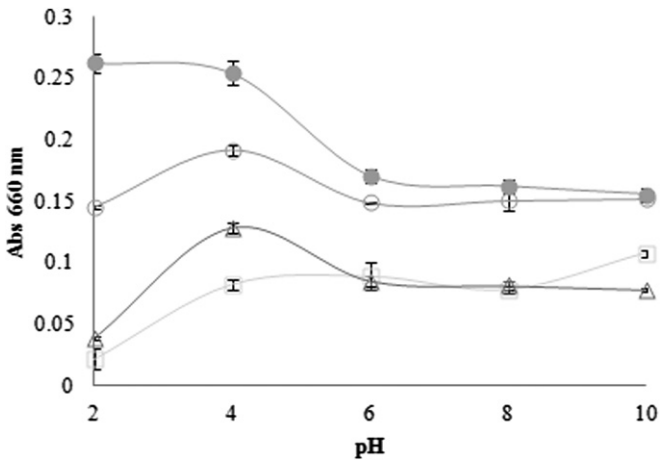

Fig. 2. Turbidity (as optical density at 660 nm) of BSG hydrolysates and control sample, at pH 2, 4, 6, 8 and 10. (●) Control, (○) HP1, (Δ) HP2 and (□) HP3.

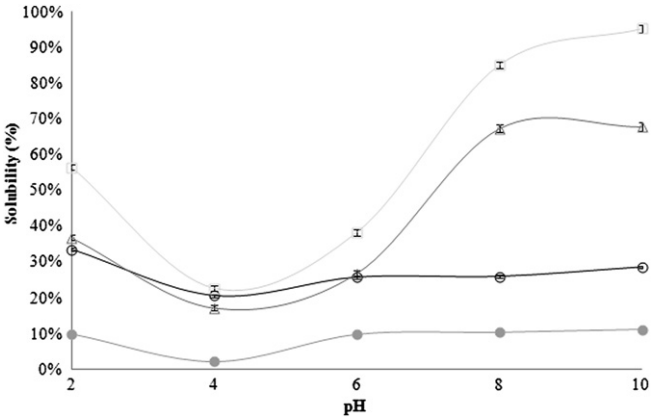

Fig. 3. Solubility profile of BSG hydrolysates and control sample, at pH 2, 4, 6, 8 and 10. (●) Control, (○) HP_1, (Δ) HP_2 and (□) HP_3.

Although no significant difference in the DH value was apparent between HP_1 and HP_2 ($P < 0.05$), in the basic region, the solubility of HP_1 was lower respect HP_2 and HP_3. On the other hand, the solubility values of the HP_1 and HP_2 were especially very close to each other in the strong acidic regions.

A good correlation between turbidity and solubility was also observed in this study ($R^2 = 0.986$). Turbidity of a protein solution is a functional property that is related to solubility and other physicochemical properties depending on its molecular size (Mahmoud, 1994).

Several researchers have shown that most vegetable proteins have the lowest protein solubility at their isoelectric point and then increased for basic pH values, this behaviours is similar to that of many other vegetable proteins reported earlier (Yalçin & Celik, 2007).

In general, BSG proteins are poorly water soluble (less than 10%) (Vassell, Stephens, & Celusta-Howard, 1949). The BSG pretreatment increased the solubility of the protein at acidic and alkaline pH values. Usually, solubility of the hydrolysates increased with increasing DH (Celus et al., 2007).

The high peptide solubility of protein hydrolysates indicates potential applications in food industry (Amiza, Amadou, Kamara, Zhu, & Zhou, 2011).

Researchers have studied the limited hydrolysis by using various proteases in order to increase the solubility properties of the plant proteins (Yalçin & Celik, 2007).

3.3.3. Emulsifying capacity (EC)

The ability of protein to help the formation and stabilization of the emulsion is particularly important in the manufacture of whipped products and emulsified sauces such as mayonnaise, frozen desserts and sausages (Granito, Guerra, Torres, & Guimand, 2004).

The EC was strongly affected by the pH (Table 1). The lower ECs were obtained in the isoelectric region. The low solubility of proteins in this region may decrease the EC since the proteins adopt a compact structure that prevents the breakdown and adsorption at the interface. The EC improves as they move away from the isoelectric region, which can be attributed to an increase in the electrical charge that increases the protein solubility (Ferreira, Kuskisku, Bordignon Luiz, Barrera Arellano, & Fett, 2007).

The emulsifying capacity (EC) was determined at three pH values (4, 6 and 10). In general hydrolysates showed improved EC compared to HP_0 at pH 4, 6 and 10.

Table 1
Emulsifying capacity (EC).

pH	EC (%)			
	HP_0	HP_1	HP_2	HP_3
4	$9 \pm 0.7^{c,B}$	$53 \pm 4.3^{a,B}$	$8 \pm 0.1^{c,C}$	$43 \pm 0.8^{b,B}$
6	$16 \pm 1.3^{c,A}$	$65 \pm 3.8^{a,A}$	$13 \pm 0.9^{c,B}$	$49 \pm 1.0^{b,A}$
10	$18 \pm 2.3^{c,A}$	$50 \pm 2.7^{a,B}$	$32 \pm 3.2^{b,A}$	$49 \pm 3.9^{a,A}$

Values with different lowercase letters in each row are significantly different ($P < 0.05$).
Values with different capital letters in each column are significantly different ($P < 0.05$).

The hydrolysates presented significant differences at pH 4 and 6 ($P < 0.05$), but no significant difference were observed among HP_1 and HP_3 at pH 10. HP_2 at pH 4 did not present practically EC (<10%) (Table 1).

After 2 h (HP_1) hydrolysis process, proteases degraded BSG proteins from polypeptides into oligopeptides, thus increasing the protein solubility and exposing more hydrophobic groups to water and oil interface, resulting in increased EC. The enhanced EC of this substrate by *B. cereus* hydrolysis is in agreement with the results of Amiza et al. (Amiza et al., 2011).

After 8 h (HP_2) incubation period, a drastically reduction in the EC was observed. This trend is expected because higher DH will lead to the presence of smaller peptides, which are less effective in stabilizing emulsions. The diminishing in EC with and extensively hydrolysis process is due to the reduction of hydrophobicity of the hydrolysate and the changes in the peptide size during hydrolysis (Souissi, Bougatef, Triki-Ellouz, & Nasri, 2007). At 24 h the EC increased again at all the studied pHs.

3.3.4. Water/oil holding capacity

Interactions of water and oil with proteins are very important in food systems because of their effects on the flavour and texture of foods (Amiza et al., 2011). Table 2 presents the water and oil holding capacities of the hydrolysates. There was no significant difference in WHC and OHC in all samples. The WHC and OHC ranged from 1.11 to 1.16 g water/g hydrolysate and 0.91 to 1.02 g oil/g hydrolysate, respectively. WHC and OHC values were also lower compared to lupin flour protein hydrolysates (Souissi et al., 2007), corn gluten hydrolysates, soybean hydrolysates and sunflower hydrolysates (Briones-Martínez, Juárez-Juárez, Oliver-Salvador, & Cortés-Vázquez, 1997).

3.3.5. Foaming properties

The formation of foam is analogous to emulsification process. In the case of foam, protein molecules, surround air droplets, are the polar phase, and air is the non-polar phase. Theoretically, the amphipathic character of protein makes them the good foaming agents that involved them at air–water interface to prevent bubble coalescence (Yu et al., 2007).

Foam expansion of control and hydrolysates samples (1 g/100 mL) at pH 7 was depicted in Fig. 4. Foam expansion at 0 min after whipping indicated the foam abilities of protein hydrolysates, which increased a 50, 250 and 300% respect to control sample.

Table 2
Water/oil holding capacity of BSG hydrolysates at pH 4, 7 and 10.

pH	HP_0	HP_1	HP_2	HP_3
WHC (g water/g sample)				
4	0.93 ± 0.02	1.14 ± 0.01	1.14 ± 0.04	1.14 ± 0.01
7	0.92 ± 0.02	1.14 ± 0.02	1.15 ± 0.02	1.11 ± 0.03
10	0.92 ± 0.01	1.12 ± 0.01	1.16 ± 0.02	1.13 ± 0.02
OHC (g oil/g sample)				
4	0.83 ± 0.02	0.93 ± 0.02	0.95 ± 0.02	0.97 ± 0.01
7	0.81 ± 0.02	0.94 ± 0.02	1.00 ± 0.02	1.02 ± 0.04
10	0.82 ± 0.03	0.91 ± 0.02	1.00 ± 0.03	0.98 ± 0.02

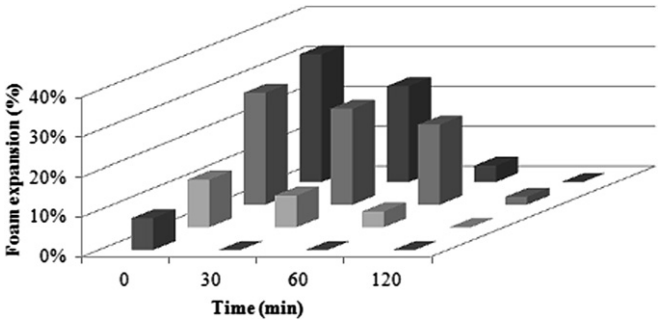

Fig. 4. Foam expansion of BSG hydrolysates at 0, 30, 60 and 120 min (■) control, (■) HP_1, (■) HP_2 and (■) HP_3.

Foam expansion after whipping was monitored for 120 min to indicate the foam stability of protein hydrolysates. Within the first 30 min, the HP_2 and HP_3 showed the highest foam stability. Thereafter, noticeable differences in foam expansion were observed among these samples with a major stability from HP_2. The decrease in the foaming expansion from HP_3 may be due to the small size of peptides produced by extensive hydrolysis that lower its surface activity. Thus the formation of a stable film around the gas bubble is hindered. This effect is also pronounced by the apparition of hydrophobic peptides during extensive hydrolysis. Pacheco-Aguilar et al. (Pacheco Aguilar, Mazorra Manzano, & Ramirez Suarez, 2008) suggested that the pH of hydrolysates greatly affect the foam stability. Also Lawal (2004) pointed out that an increase in foam stability with increasing concentration was a result of formation of stiffer foams.

3.3.6. Gelation capacity

For the studied hydrolysates concentrations no gel formation were obtained. The gelation of proteins is also affected by various environmental factors such as pH and salts (Granito et al., 2004). However, these were not studied in this work.

3.3.7. Viscosity of hydrolysate suspension

Viscosity is an important property of food that affects mouth feel, the texture of fluid such as beverage and proceeding such as pumping, extrusion and drying. If the concentration is high enough, the protein suspension can form gel upon heating follow by cooling.

Table 3 presents the viscosities of hydrolysate suspensions before and after heating.

Table 3
Viscosity (cp) of hydrolysate suspensions (10 and 12.5 g/100 mL) before and after heating at 1, 2.5 and 5 rpm.

Sample	Shear rate (rpm)	Hydrolysate concentration	
		10 g/100 mL	12.5 g/100 mL
HP_0	1	180[a]/719.8[b]	496.9/1020
	2.5	180/479.8	340/896.8
	5	120/320	239.9/479.8
HP_1	1	180/320	340/496.9
	2.5	120/239.9	180/239.9
	5	98/180	120/180
HP_2	1	120/216.8	239.9/320
	2.5	98/180	120/239.9
	5	60/96	98/120
HP_3	1	98/180	120/496.9
	2.5	48/120	60/239.9
	5	12/98	24/180

[a] Viscosity (cp) before heating.
[b] Viscosity (cp) after heating.

The apparent viscosity of the hydrolysates suspensions (HP_1, HP_2 and HP_3) was lower than that of control suspension. In general, low apparent viscosity is observed in proteins when their molecular mass is reduced by proteolysis (Tsumura et al., 2005). As shown in Table 3, viscosities of suspension before heating were lower that after heating.

The viscosities of the suspensions decrease with increasing shearing rate (rpm). Therefore, hydrolysates suspensions are shearing thinning.

Lower viscosity of protein suspension before heating is desirable during pumping and piping, and higher viscosity after heating is desirable for the thickening of soup, and production of sausage (Yu et al., 2007). Therefore, BSG hydrolysates exhibited these desirable rheological properties making their good candidate for many food formulations.

3.3.8. Colour

Table 4 shows the values for the Colour analysis of BSG hydrolysates. In terms of colour, the BSG presented light brown colouration. Subsequently, the pretreated BSG presented a dark brown colouration. HP_1 was discoloured during the hydrolysis process For HP_2 and HP_3 the results showed that the total colour (chroma value) increased compared to HP_0, which has the highest L^* value. Hydrolysis of BSG produced protein powers that were light yellow to brownish in Colour. L^* values of HP_1 solution was significantly different than that of HP_2 and HP_3 solutions ($P < 0.05$). However there was no significant different between L^* values of HP_2 and HP_3 solution. Meanwhile, the brownish colour of the hydrolysate solution significantly increased at 2 h of hydrolysis time; after 8 h, the solution colour was lighter and maintained constant till the end of the hydrolysis time, but no least brownish colour than HP_0. Enzymatic hydrolysis reactions are assumed to have contributed to reduction in the luminosity, given a darker appearance of the hydrolysates (Wasswa, Tang, Gu, & Yuan, 2007).

Furthermore, the decrease in the b^* value of HP_1 solution indicated a reduction in yellowness of the hydrolysate. This parameter increased significantly respect to the control sample, indicated an increase in the final yellowness of the hydrolysates.

The HP_2 and HP_3 solutions exhibited a significant lower greenness, while HP_1 solution presented a redness colour. HP_1 had also a less yellowness colour while HP_2 and HP_3 solutions colour exhibited a significantly higher yellowness. HP_1 exhibited the highest chroma value while HP_2 and HP_3 presented a lower chroma respect to the control solution. The Hue angle was significantly lower for HP_1, while HP_2 and HP_3 solution showed the higher hue angle and no significant differences between them was observed.

Table 4
The CIE L^*, a^* and b^* values, hue angle and chroma calculations for BSG hydrolysates solution (5 g/100 mL). Values with different letters in each column are significantly different ($P < 0.05$).

	L^{*a}	a^{*b}	b^{*c}	Hue angle[d]	Chroma[e]
HP_0	57.39 ± 0.08^x	-1.15 ± 0.02^z	6.09 ± 0.02^y	−1.38	6.20
HP_1	47.29 ± 0.10^z	0.83 ± 0.04^x	4.83 ± 0.03^z	1.40	4.90
HP_2	52.04 ± 0.42^y	-0.37 ± 0.02^y	6.69 ± 0.09^x	−1.52	6.70
HP_3	52.47 ± 0.19^y	-0.47 ± 0.03^y	6.97 ± 0.05^x	−1.50	6.99

[a] L^* values are a measure of lightness (higher value indicates a lighter colour).
[b] a^* values are a measure of redness (higher value indicates a redder colour).
[c] b^* values are measure of yellowness (higher values indicates a more yellow colour).
[d] Hue angle represents the change from the true red axis (a larger number indicates shift from red to yellow).
[e] Chroma is a measure of total colour (larger number indicates a more vivid colour).

Table 5
Data obtained from DSC curves.

Sample	T_{onset} (°C)	T_{max} (°C)	T_{offset} (°C)	ΔH (J/g)
HP_0	84.08^a	114.46^a	163.62^a	228.53^a
HP_1	82.24^a	109.64^b	161.48^a	170.72^b
HP_2	68.87^b	106.32^b	155.22^b	167.93^b
HP_3	66.20^b	101.74^c	153.06^b	127.17^c

Values with different letters in each column are significantly different ($P < 0.05$).

3.3.9. Differential scanning calorimetry

Hydrolysates exhibited an endotherm with T_{max} shifted to lower temperatures.

The DSC area of the control revealed one major broad endothermic peak, centred at around 110 °C, corresponded to the proteins denaturation, indicating that they are not completely denatured, probably because of its thermal stability. There were no differences among ΔH of HP_1 and HP_2, while ΔH of HP_3 was higher significantly ($P < 0.05$).

When the hydrolysis duration reach 24 h (HP_3), the T_{max} peak had shifted to 153.06 °C and the area had diminished by 44.35% (Table 5).

3.3.10. Antimicrobial activity

Antimicrobial inhibition zones against *E. coli* and *L. monocytogenes* are presented in Table 6. The diameters of inhibitory zones increased from approximately 12.5–27.0 mm for *E. coli* O157:H7 with increasing the proteolytic digests time. The results suggest that BSG biopeptides are effective in inhibiting the growth and survival of *E. coli*, and may be used as an integral component in foods to improve food safety.

According to the antibacterial assay, the Gram positive bacteria *L. monocytogenes* were found to be resistant against all tested hydrolysates (Table 6). Davies and Adams (1994) found that the mechanism of resistance of *L. monocytogenes* to nisin involves a reduction in the accessibility to suitable adsorption sites.

4. Conclusion

Brewer's present grain (BSG) is an important sources of protein and fibre. The present data focus on the BSG as sources of protein, and demonstrated that enzymatic hydrolysis is an efficient tool for preparing high quality protein hydrolysates of this byproduct with improved functional properties and strong antimicrobial activity.

Functional properties were greatly influenced by changes in pH of the medium. Protein hydrolysates made from BSG can be converted into a high value protein powder food ingredient.

It could also be environmentally friendly as it aims at diverting a potentially waste product from being an agent of pollution to a functional food component. If successfully implemented it could contribute to controlling protein energy-malnutrition that is prevalent in third world countries. The emulsifying properties of the hydrolysate obtained were improved significantly. Increased DH induced a decrease in the emulsion- and foam-stabilizing properties.

Table 6
Inhibition zone of hydrolysates and control samples against *E. coli* and *L. monocytogenes*.

	Halo diameter (mm)			
	HP_0	HP_1	HP_2	HP_3
E. coli O157:H7	<10	12.5 ± 1.91	15.17 ± 1.17	26.67 ± 2.94
L. monocytogenes	<10	<10	<10	<10

Additional nutritional evaluations using hydrolysates in formulation should be performed since there is an increasing demand for dietetic products for therapeutic use. Also, the bitterness of hydrolysates should be evaluated and the appropriate debittering should be applied in order to obtain a hydrolysate with the sensorial acceptability for further applications.

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