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ORIGINAL RESEARCH

Physicochemical and antioxidant properties of bovine caseinate hydrolysates obtained through microbial protease treatment

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It is currently possible to obtain bioactive nutritional components through enzyme hydrolysis under well-controlled and moderate conditions of pH and temperature. In this work, we aimed for the production and characterisation of bovine sodium caseinate (NaCAS) hydrolysates by means of an extracellular protease from Bacillus *sp. P45. Antioxidant properties were evaluated through 2,2-diphenyl-1-picrylhydrazyl and thiobarbituric acid reactive substances assays. A glucono-δ-lactone acid-induced aggregation and gelation processes of the hydrolysates and their corresponding mixtures with NaCAS were also analysed. Hereby, we demonstrate that the presence of hydrolysates affects the kinetics of NaCAS aggregation processes but does not significantly alters the final state of the acid aggregates obtained.*

Keywords Acidification, *Bacillus*, Proteolysis, Bovine caseinate, Caseinate gels, Antioxidant activity.

INTRODUCTION

Caseins (CN) represent the main protein fraction of bovine milk. Caseins precipitation may be achieved at pH 4.6 and may be subsequently resolubilised through an increase in pH (Walstra and Jenness 1984). If resolubilisation is carried out by the addition of sodium hydroxide (NaOH), it will lead to the formation of the corresponding sodium salt, sodium caseinate (NaCAS) (Mulvihill and Fox 1989). Both CN and NaCAS are extensively used in food industry because of their physicochemical, nutritional and functional properties that make them valuable ingredients in complex food formulations.

By means of chemical or enzymatic hydrolysis, the proteins are cleaved into free amino acids and/or peptides of different sizes. Enzymatic hydrolysis under moderate conditions of pH (6–8) and temperature (40–60 °C) makes it possible to obtain bioactive nutritional components with enhanced functional properties. Hydrolysis is targeted at optimising thermal stability, decreasing allergenicity, producing bioactive peptides, modelling the amount and size of the peptides for special diets, and modifying functional properties such as gelation, emulsification and foam formation(Silva and Malcata 2005; Hartmann and Meisel 2007). For instance, it has been recently demonstrated that the partial hydrolysis of whey protein may give rise to nanoparticles that allow the formation of translucid and reversible gels with capable of encapsulating specific molecules (Graveland-Bikker and de Kruif 2006). In addition, casein hydrolysates obtained by proteolytic treatment with trypsin and chymotrypsin contain a set of more than 200 peptides of different sizes, which have smaller molecular masses and less secondary structure than the native state of CN (FitzGerald 1998; Sakanaka *et al.* 2005).

The high-growth consumer demand for healthy and high nutritional-valued food products has encouraged the food industry to carry out a substantial improvement in the production of supplemented and functional formulations. Nowadays, there are several nutritional formulae available containing peptides; however, the types and amount of these peptides vary among different products offered to consumers, depending on the protein source (casein, lactalbumin, soya, meat) and on the

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hydrolysis treatment performed (Pintado *et al.* 1999; Zhong *et al.* 2007; Rossini *et al.* 2009). Indeed, the significance of such peptides is demonstrated by the diverse physiological effects obtained by varying the protein source (Korhonen and Pihlanto 2006; Hartmann and Meisel 2007).

Proteases are important hydrolytic enzymes widely employed in the food, feed, detergent, leather and pharmaceutical industries, accounting for approximately 60% of the global enzyme sales. In the food industry, they have been routinely and widely used for several purposes such as cheesemaking, baking, preparation of soya hydrolysates and meat tenderisation (Rao *et al.* 1998; Sumantha *et al.* 2006). Commercial proteases have been recently employed in the production of protein hydrolysates with promising bioactive properties including antioxidant activity (Rival *et al.* 2001; Saiga *et al.* 2003; Zhu *et al.* 2006). Consequently, to investigate novel protease preparations from different sources and deepen our understanding of their derivatives, products are indispensable to obtain protein hydrolysates with desired properties.

Microbial proteases are particularly interesting because of the high yields achieved during their production through well-established culture methods. Protease production is an inherent property of micro-organisms, and the exploitation of biodiversity is considered to be an auspicious alternative to provide industry with a greater variety of micro-organisms and proteases well suited for diverse applications (Rao *et al.* 1998; Gupta *et al.* 2002). Particularly, bacteria living in the intestinal tract of fishes are considered as an untapped bioresource for enzyme production (Esakkiraj *et al.* 2009). In this sense, a proteolytic *Bacillus* sp. P45, isolated from the intestinal conduct of the Amazonian fish *Piaractus mesopotamicus*, was reported to produce high levels of extracellular proteases with biotechnological potential during submerged cultivations in inexpensive culture media (Daroit *et al.* 2009, 2011).

Both dissociation and a further aggregation step of casein fractions because of caseinate acidification give rise to the formation of a gel structure. A possible explanation of this observation is that as pH is adjusted towards the isoelectric point, a decrease in the repulsive interactions takes place, resulting in an electrostatic destabilisation of the colloidal aggregates as pH is lowered slightly below 5 at a given temperature (Braga *et al.* 2006; Ruis *et al.* 2007). Nowadays, a process that has gained the attention of food industry is direct acidification by the addition of a lactone, such as glucono-δ-lactone (GDL), which overcomes some of the difficulties associated with the traditional process of using lactic bacteria. In fact, the final pH of the system bears a direct relation to the amount of GDL added, whereas starter bacteria produce acid until they inhibit their own growth as pH becomes lower (de Kruif 1997; Braga *et al.* 2006). Among other factors, enzymatic hydrolysis of proteins might be an alternative treatment to control the characteristics of acid-set gels and to confer desired rheological and organoleptic properties (Rabiey and Britten 2009).

In this work, the characterisation of bovine caseinate hydrolysates obtained using microbial proteases from *Bacillus* P45 was performed, and an acid aggregation process and antioxidant capability of these hydrolysates was evaluated.

MATERIALS AND METHODS

Materials

Bovine NaCAS powder, azocasein, the acidulant GDL, tris(hydroxymethyl)aminomethane (Tris), 8-anilino-1-naphthalenesulfonate (ANS) as ammonium salt; 2,4,6-trinitrobenzene sulfonic acid (TNBS); 2,2-diphenyl-1-picrylhydrazyl (DPPH); and thiobarbituric acid (TBA) were commercially acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals employed were of analytical grade.

Sodium caseinate solution was prepared by dissolving the commercial product in distilled water (pH 6.8). Caseins concentration was measured according to the Kuaye's method, which is based on the ability of strong alkaline solutions (0.25 M NaOH) to shift the spectrum of the amino acid tyrosine to higher wavelength values in the UV region (Kuaye 1994). All the values obtained were the average of two determinations.

Micro-organism and protease production

A protease-producing strain *Bacillus* sp. P45 was utilised (Daroit *et al.* 2009) and maintained in brain-heart agar (BHA) plates. For protease production, the strain was cultured in feather meal broth (10 g/L feather meal, 0.3 g/L Na_2HPO_4, 0.4 g/L NaH_2PO_4, 0.5 g/L NaCl) for 48 h at 30 °C in a rotary shaker (125 rpm). Cultures were centrifuged (15 min at 10,000 *g* and 4 °C), and the supernatant (crude protease) was submitted to a partial purification protocol.

Partial purification of crude protease

A concentration step was carried out over the crude protease supernatant through ammonium sulfate precipitation until 60% saturation was reached, in a stirred ice bath. This mixture was allowed to stand for 1 h and centrifuged (10 000 × *g* for 15 min at 4 °C), and the resulting pellet was resuspended in a minimal volume of 20 mM Tris-HCl buffer, pH 8.0. The concentrated enzyme samples were loaded into a size-exclusion column (25 × 0.5 cm) over Sephadex G-100 (Pharmacia Biotech, Stockholm, Sweden), previously equilibrated with Tris-HCl buffer (20 mM, pH 8.0). Elution was carried out using the same buffer with a flow rate of 0.33 mL/min, and 30 fractions of 1 mL were collected. Fractions showing proteolytic activity were pooled and employed as a protease preparation (protease P45) for protein hydrolysis.

Proteolytic activity assay

Proteolytic activity was determined as described elsewhere using azocasein as substrate (Corzo-Martínez *et al.* 2010). Briefly, the reaction mixture contained 100 μL of enzyme preparation, 100 μL of 20 mM Tris-HCl buffer (pH 8.0) and

100 μL of 10 g/L azocasein in 20 mM Tris-HCl buffer (pH 8.0). The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 600 μL of 10% (w/v) trichloroacetic acid. After centrifugation (10000 × *g* for 5 min), 800 μL of the supernatant was mixed with 200 μL of 1.8 M NaOH, and the absorbance at 420 nm was measured. One unit of enzyme activity (U) was considered as the amount of enzyme that caused a change in absorbance of 0.01 units at the above assay conditions.

NaCAS hydrolysis

Samples of 0.5% (w/w) NaCAS in buffer Tris-HCl 20 mM, pH 8, were submitted to hydrolysis by protease P45 (40 U/50 mL NaCAS 0.5%) at 45 °C. The hydrolysis reaction was stopped at different times (t_i; i = 0, 1, 2, 3, 4 and 7 h) by heating the samples at 100 °C for 15 min. When the samples reached room temperature, they were centrifuged for 15 min at 10 000 × *g*. The concentration of hydrolysates in the supernatants was measured according to the Kuaye's method, and their composition was analysed by SDS-urea-PAGE. In addition, degree of hydrolysis (DH), intrinsic fluorescence spectra, surface hydrophobicity (S_0) and antioxidant activity were evaluated.

Degree of hydrolysis

The DH, considered as the percentage of peptide bonds cleaved, was determined as the quantification of free amino groups following the reaction with TNBS as previously described by Adler-Nissen (Adler-Nissen 1979). In a few words, protein hydrolysate samples (250 μL) were mixed with 2 mL phosphate buffer (0.212 M; pH 8.2) and 2 mL 1% TNBS and incubated at 50 °C for 1 h. After this period, 4 mL of 0.1 M HCl was added, and the mixtures were allowed to stand for 30 min at room temperature before the absorbance at 340 nm was measured. The total number of amino groups was determined in a protein sample (10 mg), which was completely hydrolysed in 4 mL of 6 M HCl at 110 °C for 24 h (Li *et al.* 2007).

Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The qualitative composition of the hydrolysates was analysed by urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Urea-SDS-PAGE) using a vertical gel system, according to the method of Laemmli (Laemmli 1970). The protein bands were identified using commercial low molecular weight protein markers (Sigma Chemical Co., St. Louis, MO, USA).

Intrinsic fluorescence spectra

Excitation and emission spectra of the hydrolysates (0.1%) were obtained with the aim of detecting any spectral shift and/or changes in the relative intensity of fluorescence (FI). Previously, the excitation wavelength (λ_{exc}) and the range of concentration with a negligible internal filter effect were determined. The samples (3 mL) used for the spectral analysis and FI measurements were poured into a fluorescence cuvette with a light path length of 1 cm and placed into a cuvette holder maintaining temperature at 35 °C. Values of FI (N = 2) were registered within the range of 300–430 nm using a λ_{ex} of 286 nm.

Surface hydrophobicity (S_0)

S_0 was estimated according to Kato and Nakai method, (Kato and Nakai 1980; Haskard and Li-Chan 1998) using the ammonium salt of amphiphilic ANS as a fluorescent probe, in an minco Bowman Series 2 spectrofluorometer (Thermo Fisher Scientific, Milwaukee, Wisconsin, USA). The measurements were carried out using λ_{exc} and emission wavelength (λ_{em}) set at 396 and 489 nm, respectively, at a constant temperature of 35 °C. As mentioned earlier, both wavelengths were previously obtained from emission and excitation spectra of protein-ANS mixtures.

Intensity of fluorescence of samples containing ANS and different concentrations of NaCAS hydrolysates (FI_b) as well as the intrinsic FI without ANS (FI_p) were determined (N = 3). The difference between FI_b and FI_p (ΔF) was calculated, and S_0 was determined as the initial slope in the ΔF vs protein concentration (% w/w) plot.

2,2-diphenyl-1-picrylhydrazyl radical–scavenging assay

This method is based on the capture of the DPPH radical by antioxidants, producing a decrease in absorbance at 515 nm (Brand-Williams *et al.* 1995). DPPH was dissolved in methyl alcohol and used at a concentration of 60 μM. This solution was homogenised and transferred to a dark glass bottle. The prepared solution was used only in the day of analysis. In the dark, aliquots of 0.1 mL of protein hydrolysate samples were transferred to test tubes with 3.9 mL of radical DPPH (60 μM DPPH solution) and homogenised by shaking. Likewise, these same proportions (0.1 mL of sample and 3.9 mL of radical DPPH) were used as a control, and methyl alcohol was used as a blank. The standard curve was developed with DPPH in a range from 0 to 60 μM. The results were expressed as EC_{50} (μg/mL), which is the minimum antioxidant concentration required to reduce 50% of the initial DPPH reaction from the time the extract reached stability. The assay was performed in triplicate.

Thiobarbituric acid reactive substances

The TBA reaction was performed as previously reported (Ohkawa *et al.* 1979). Test tubes containing Milli-Q water and extra virgin olive oil were subjected to oxidation with 100 μM ferrous sulfate and incubated in a water bath at 80 °C for 10 min. Thereafter, the hydrolysate samples, 81 g/L sodium lauryl sulfate (SDS), buffered acetic acid (pH 3.44) and 6 g/L TBA were added to each tube. The reaction mixture was further incubated in a water bath at 100 °C for 1 h. For each sample tested, a blank and a standard control were used for all comparisons. The products of reaction were determined by measuring absorbance at 532 nm with a spectrophotometer. The concentration

of thiobarbituric acid reactive substances (TBARS) was calculated using a calibration curve obtained with a set of standard samples of known concentrations of 1,1,3,3-tetramethoxypropane, and results were expressed as nmol of malonaldehyde (MDA) per mL of sample. The experiment was performed in triplicate.

Size variations of particles

Changes in the average size of particles were followed by the dependence of turbidity (τ) on wavelength (λ) of the suspensions, determined according to:

$$\beta = 4.2 + \frac{\mathrm{d}(\log \tau)}{\mathrm{d}(\log \lambda)} \quad (1)$$

β is a parameter that has a direct relationship with the average size of the particles and can be used to easily detect and follow rapid size changes and was obtained from the slope of log τ vs log λ plots, in the 450–650 nm range, where the absorption owing to the protein chromophores is negligible allowing then to estimate τ as absorbance in 400–800 nm range (Camerini-Otero and Day 1978). On the other hand, it has been shown that β for a system of aggregating particles of the characteristics of caseinates tends, upon aggregation, towards an asymptotic value that can be considered as a fractal dimension (D_f) of the aggregates (Horne 1987; Risso *et al.* 2007).

τ was measured as absorbance using a Spekol 1200 spectrophotometer (Analytikjena, Suarlée, Belgium), with a diode arrangement. Determinations of β were the average of at least duplicate measurements.

Acid aggregation

Kinetics of NaCAS or hydrolysates (0.35%) and NaCAS/hydrolysates mixtures (4:1) aggregation induced by the acidification with GDL was analysed by measuring turbidity (τ) in the range of 450–650 nm, in a Spekol 1200 spectrophotometer with a thermostatised cell.

The amount of GDL added was calculated using the following relation:

$$R = \frac{\text{GDL mass fraction}}{\text{Protein mass fraction}} \quad (2)$$

R used for this experiment was 1.5, at temperature of 35 °C.

Acidification was initiated by the addition of solid GDL to 5 g of different samples. Absorption spectra (450–650 nm) and absorbance at 650 nm (A_{650}) were registered as a function of time until a maximum and constant value of A_{650} was reached; simultaneously, pH decrease was measured. The determinations were performed in duplicate. Values of parameter β were calculated using Equation 2.

Rheological properties of acid gels

Rheological properties of NaCAS, hydrolysate t_1 and NaCAS/hydrolysate t_1 mixtures (3.0% w/w) were determined in a stress- and strain-controlled rheometer TA Instruments, AR G2 model (Brookfield Engineering Laboratories, Middleboro, MA, USA) using a cone geometry (diameter: 40 mm, cone angle: 2°, cone truncation: 55 mm) and a system of temperature control with a recirculating bath (Julabo model ACW 100; JULABO Labortechnik GmbH, Seelbach, Alemania) connected to a Peltier plate. An amount of solid GDL according to an R of 0.5 was added to initiate the acid gelation. Measurements were taken every 20 s during 100 min with a constant oscillation stress of 0.1 Pa and a frequency of 0.1 Hz. The Lissajous figures at various times were plotted to make sure that the determinations of storage or elastic modulus (G') and loss or viscous modulus (G'') were always obtained within the linear viscoelastic region. A duplicate of each one of the determinations was also obtained. The G'–G'' crossover times (t_g) of acidified caseinate systems were considered here as the gel times, as most studies of milk or caseinate gelation have adopted this criterion (Curcio *et al.* 2001; Braga *et al.* 2006). pH at t_g was also determined considering the pH value at the G'–G'' crossover (pH_g).

Conventional inverted microscopy

The degree of compactness of gels was evaluated through digital image analysis. For this, image of bottom gel surface was obtained by conventional inverted microscopy. To obtain the microscopic images, 80 μL of each sample was placed in compartments of the LAB-TEK II cells. The samples were obtained as a duplicate under a constant temperature set at 35 °C. Transmission images of gels were obtained using a conventional inverted microscopy (Union Optical, Phoenix, AZ, USA) with an objective 100× and a digital camera (Canon PowershotA640, Buenos Aires, Argentina) with a zoom 7.1×.

Statistical analysis

The data are reported as the average values ± their standard deviations. The statistical analysis was performed with Sigma Plot 10.0 and OriginPro 8 softwares. The relationship between variables was statistically analysed by correlation analysis using Pearson correlation coefficient (r). Comparisons of the means were carried out using the Tukey's test. The differences were considered statistically significant at $P < 0.05$ values.

RESULTS AND DISCUSSIONS

NaCAS hydrolysis by protease P45

The extent of hydrolysis in the supernatant fraction obtained by centrifugation increased up to 2 h of incubation time reaching the highest molecular mass below 6500 Da (Figure 1).

The progression in DH during the hydrolysis of NaCAS by protease P45 is shown in Figure 2. The DH increased as the hydrolysis time became higher, reaching 9% after 7 h. This value resembles the DH obtained by hydrolysing yak milk casein with alcalase (from *Bacillus licheniformis*) or neutrase (from *Bacillus amyloliquefaciens*) for 7 h (Jiang *et al.* 2007).

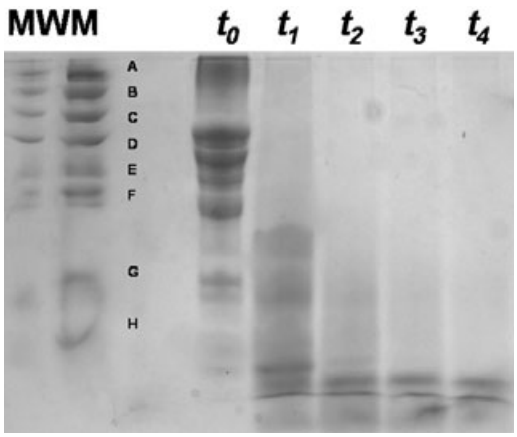

Figure 1 Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the hydrolysates obtained at different times of sodium caseinate hydrolysis with protease P45. Molecular weight markers (MWM): (A) 66 kDa; (B) 45 kDa; (C) 36 kDa; (D) 29 kDa; (E) 24 kDa; (F) 20 kDa; (G) 14.2 kDa; and (H) 6.5 kDa.

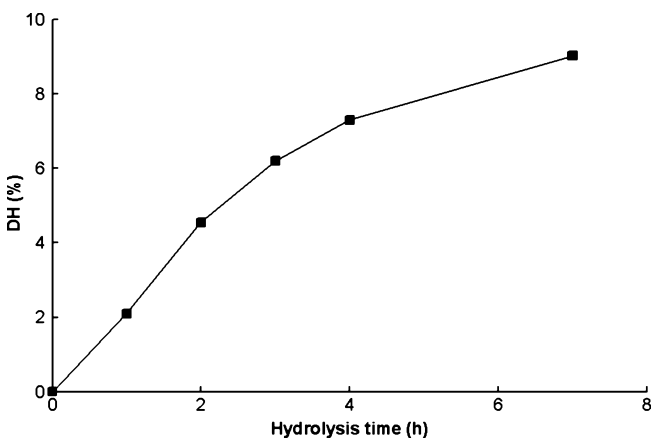

Figure 2 Degree of hydrolysis of sodium caseinate hydrolysates obtained with a protease preparation from *Bacillus* sp. P45.

Intrinsic fluorescence spectra and surface hydrophobicity

Emission spectra of NaCAS and the hydrolysates obtained at different times of hydrolysis (t_i) are presented in Figure 3. A fluorescence red shift as well as a decrease in the fluorescence intensity can be observed when hydrolysis occurs. It can be elucidated that this is because of a conformational change in the surroundings of fluorophore groups in the peptides. The loss of protein fluorophores during enzymatic proteolysis was ruled out owing to the negligible fluorescence intensity of resolubilised precipitates that were separated by centrifugation.

S_0 of NaCAS hydrolysates obtained at different t were determined, and they are listed in Table 1. S_0 decreased as t increased, especially after 2 h of hydrolysis, which would indicate a higher exposure of hydrophilic groups in the protein surface that protrude towards the aqueous environment.

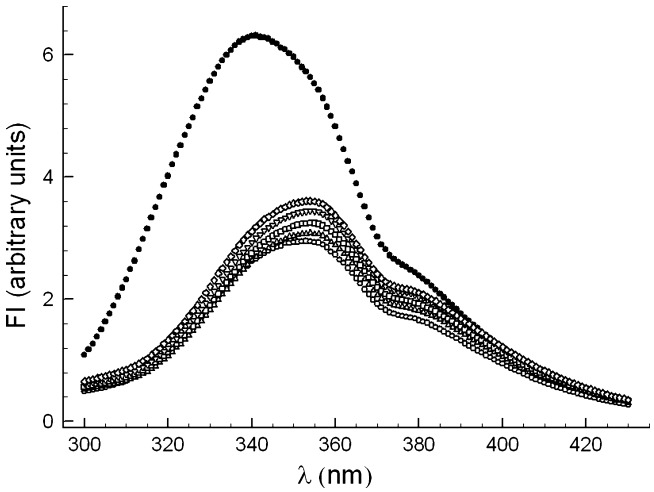

Figure 3 Fluorescence emission spectra of the hydrolysates obtained through proteolysis with protease P45 at different times (t): (●) t_0; (○) t_1; (Δ) t_2; (□) t_3; (∇) t_4; (◇) t_7. T 35 °C; hydrolysates concentration: 0.1% w/w.

Table 1 S_0 values of sodium caseinate hydrolysates obtained at 35 °C after hydrolysis with the protease P45[a]

Sample	S_0 (%w/w^{-1})
t_0	114 ± 8
t_1	89.7 ± 0.6
t_2	14 ± 2
t_3	18 ± 1
t_4	8.6 ± 0.1

[a]Errors are standard deviations of S_0. $\alpha = 0.01$.

Antioxidant activity of hydrolysates

The antioxidant activity of the peptide solutions generated by the hydrolysis of NaCAS with protease P45 was evaluated by DPPH and TBARS assays, which are considered as useful methods widely utilised for this purpose (Pihlanto 2006).

The DPPH method is based on the scavenging (reduction) and stabilisation of the free radical DPPH by proton-donating substances, such as antioxidants (Zhu *et al.* 2006). Figure 4 shows that the hydrolysis of NaCAS with P45 for 1 h (t_1) resulted in a significantly higher scavenging of the radical DPPH (lower EC_{50}) when compared to control and hydrolysate t_0. A higher hydrolysis time (2–7 h) did not increase the antioxidant activity, which was maintained at a level similar to that obtained at t_1. This could suggest that NaCAS hydrolysates contain peptides that act as electron donors and could react with the free radicals to convert them into more stable products and terminate the radical chain reaction (Wu *et al.* 2003; Wang *et al.* 2007). On the other hand, CN digested with various proteases showed lower DPPH activity than whole CN (Rival *et al.* 2001). Decreased DPPH scavenging was also observed

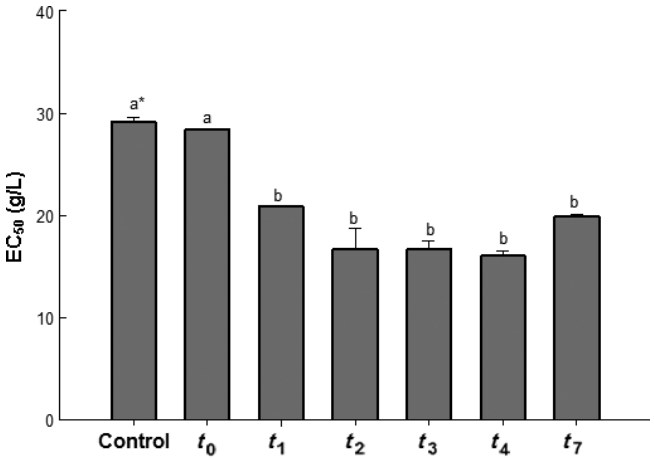

Figure 4 Determination of antioxidant activity of sodium caseinate hydrolysates, obtained with protease P45, using the 2,2-diphenyl-1-picrylhydrazyl radical–scavenging method. Results are means ± SEM. *Means with the same letter are not significantly different according to Tukey's test ($P < 0.05$).

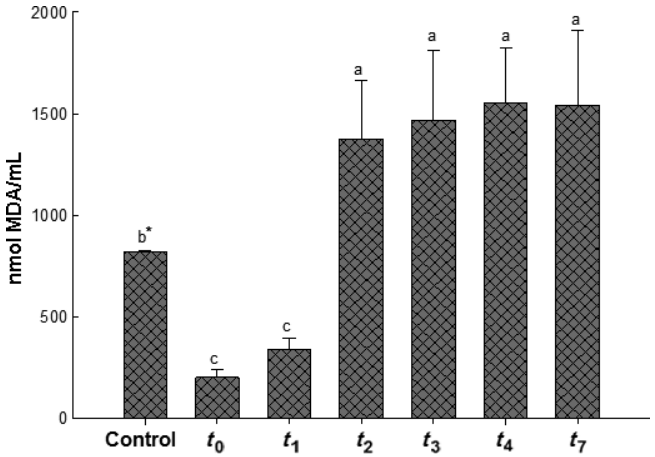

Figure 5 Antioxidant activity of sodium caseinate hydrolysates, obtained with protease P45, evaluated by the thiobarbituric acid reactive substances method. Results are means ± SEM. *Means with the same letter are not significantly different according to Tukey's test ($P < 0.05$).

after the hydrolysis of porcine haemoglobin by Flavourzyme; however, enhanced DPPH activity was observed when the hydrolysis was carried out with alcalase (Chang *et al.* 2007). The antioxidant activity of protein hydrolysates is related to the size and amino acid sequence of the generated peptides, which in turn are determined by the protease specificity (Pihlanto 2006). For instance, low molecular weight peptides tended to present a higher DPPH-scavenging activity than the high molecular weight counterparts (Chang *et al.* 2007; Li *et al.* 2007). In this sense, the DH also seems to affect the antioxidative potential of protein hydrolysates (Klompong *et al.* 2007; Thiansilakul *et al.* 2007).

Hydrolysates, t_0 and t_1, showed similar inhibition of lipid oxidation measured through TBARS assay (Figure 5); however, hydrolysis of NaCAS for periods in the range 2–7 h has negatively affected TBARS activity, which might be related to the type of peptides formed during hydrolysis (Chang *et al.* 2007). From these results, it is suggested that the hydrolysis of NaCAS with protease P45 for 1 h has a positive effect on the content of bioactive peptides with antioxidant properties. The substitution of synthetic antioxidants by natural ones is gaining interest because of the consumers' preferences and health concerns associated with the use of artificial food additives. The utilisation of protein hydrolysates in food products presents advantages over other antioxidants, because they also confer nutritional and functional properties, as small peptides are more easily absorbed in the intestinal tract than larger molecules (He *et al.* 2006; Moure *et al.* 2006). Particularly, hydrolysates from milk proteins could be used as natural antioxidants to prevent oxidation reactions that lead to deteriorations in food quality and to enhance the antioxidant properties of functional foods, which may limit the occurrence of free radical damage in human body (Saiga *et al.* 2003; Pihlanto 2006; Zhu *et al.* 2006).

Acid aggregation of hydrolysates

Figure 6 shows the acid aggregation of hydrolysates followed by the variations of A_{650}. Results showed that the capability to aggregate of hydrolysates decreased as hydrolysis time increased, and it is lost at times higher than 2 h. This fact can be related to the sharp decrease in the S_0 of hydrolysates. This behaviour does not favour the hydrophobic attractive interactions, which ultimately drives to particle aggregation.

The comparison on variations of parameter β as function of time and pH during the acidification process of hydrolysates t_0 and t_1 is plotted in Figure 7. Although the pHag of hydrolysates t_1 is higher than hydrolysates t_0 (Figure 7b), the time required

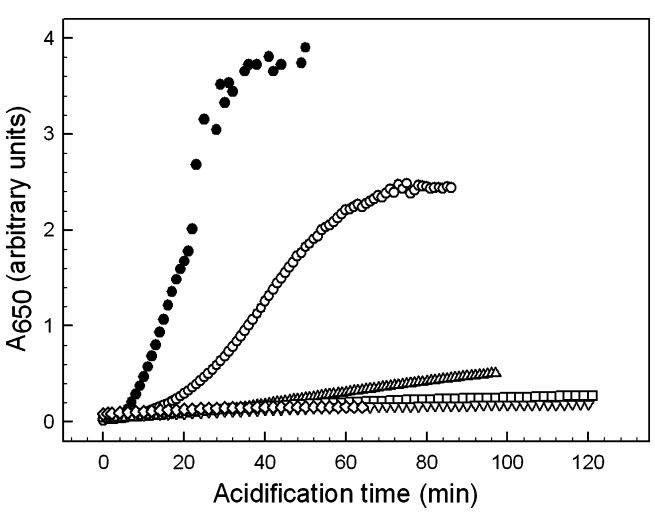

Figure 6 A_{650} variations ($N = 2$) as a function of the acidification time, after the addition of glucono-δ-lactone, during the acid aggregation of sodium caseinate hydrolysates (0.5%) obtained by protease P45 at different times (t): (●) t_0; (○) t_1; (Δ) t_2; (□) t_3; (∇) t_4; (◇) t_7. *R* 0.5; T 35 °C.

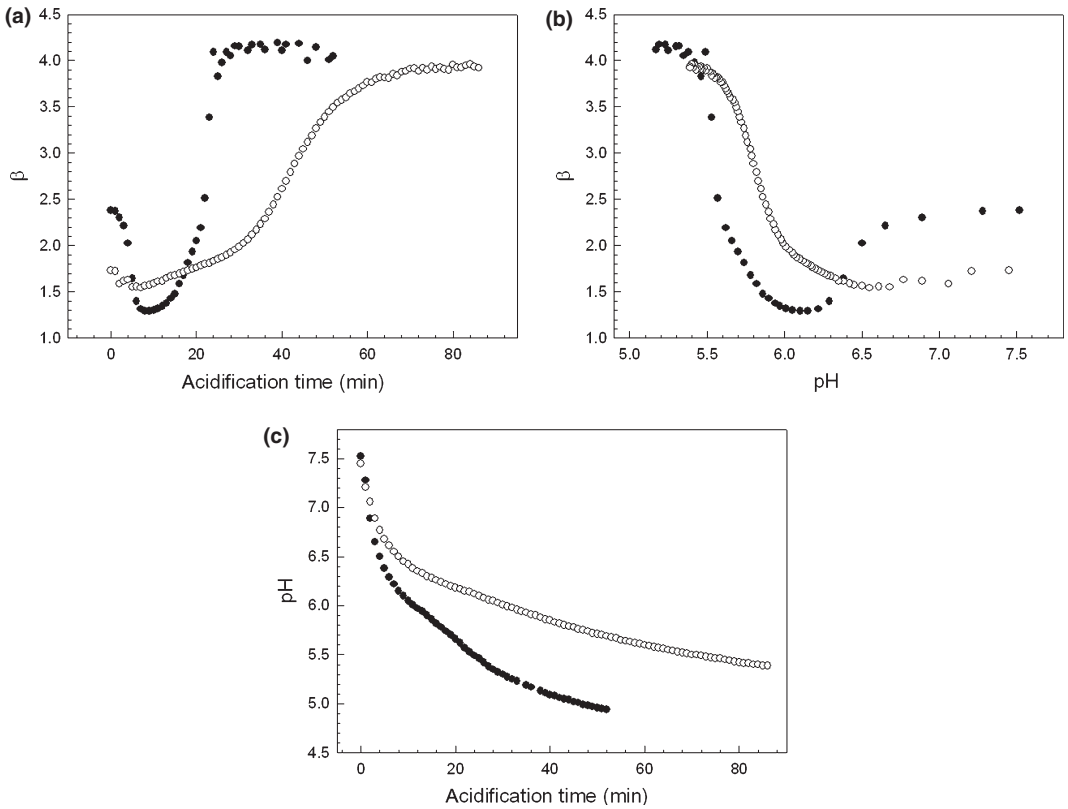

Figure 7 Variation of parameter β as a function of time (a) and pH (b), and variations of pH vs time (c) (N = 2), after the addition of glucono-δ-lactone during the acid aggregation of (●) sodium caseinate without hydrolysis (t_0) and (○) with 1 h of hydrolysis (t_1). Protein/hydrolysates concentration: 0.35%w/w; R 1.5; T 35 °C.

for the gel meshes to be constituted, i.e. to reach the D_f, is also higher (Figure 7a). This fact could be related to its decreased S_0 and to a diminished rate at which pH becomes lower. The second reason would be because of a higher efficiency of protonisation of NaCAS without hydrolysing (t_0), which leads the GDL hydrolysis reaction to the dissociation of gluconic acid.

The degree of compactness of aggregates formed at the end of the process (D_f) of hydrolysates t_1 is slightly lower than hydrolysates t_0. Therefore, the hydrolysates t_1 with antioxidant activity could form acid aggregates or gels, depending on the concentration assayed.

Acid aggregation of NaCAS/hydrolysates mixtures

The acid aggregation of NaCAS/hydrolysates mixtures (4:1) was evaluated by following how the parameter β is modified as a function of pH and time after adding GDL (Figure 8).

The aggregation process reveals two well-defined steps. The first stage, much slower, shows a decrease in the parameter β along of the time, while the pH decreases. It is known that bovine NaCAS in aqueous solution has a considerable level of self-association (like submicelles or micelles) (Farrell *et al.* 1996; Belyakova *et al.* 2003); therefore, these profiles suggest a slow dissociation of original caseinate submicelles to form a large number of small particles. The second step starts with a sharp increase in the average size of particles owing to formation of colloidal aggregates that grow until β reaches a limit value, i.e. D_f.

In the presence of hydrolysates, changes of the time at which the second step starts (t_{ag}) and the pH value observed at the t_{ag} (pH_{ag}) can be observed. The t_{ag} increased as times of hydrolysis are increased, especially for times ≥2 h. This might be partially associated with a reduction in the pH_{ag}. An increase in the hydrolysis time favours the electrostatic stability of the NaCAS/hydrolysates mixtures. It seems that the addition of these hydrolysates increases the net negative charge of caseinate colloidal particles. In the first stage, while the pH diminishes, the energetic barrier owing to electrostatic repulsion among similarly charged residues decreased and the collisions that lead to the formation and development of aggregates were affected. A larger surface potential, and therefore a higher energy barrier, will require a greater number of protons to neutralize this potential and a longer time to achieve it.

However, no significant changes were observed in the degree of compaction of the aggregates formed at the end of the process by this technique. Therefore, we had also evaluated if the hydrolysates can be included in the NaCAS acid gel matrix without significantly altering its texture.

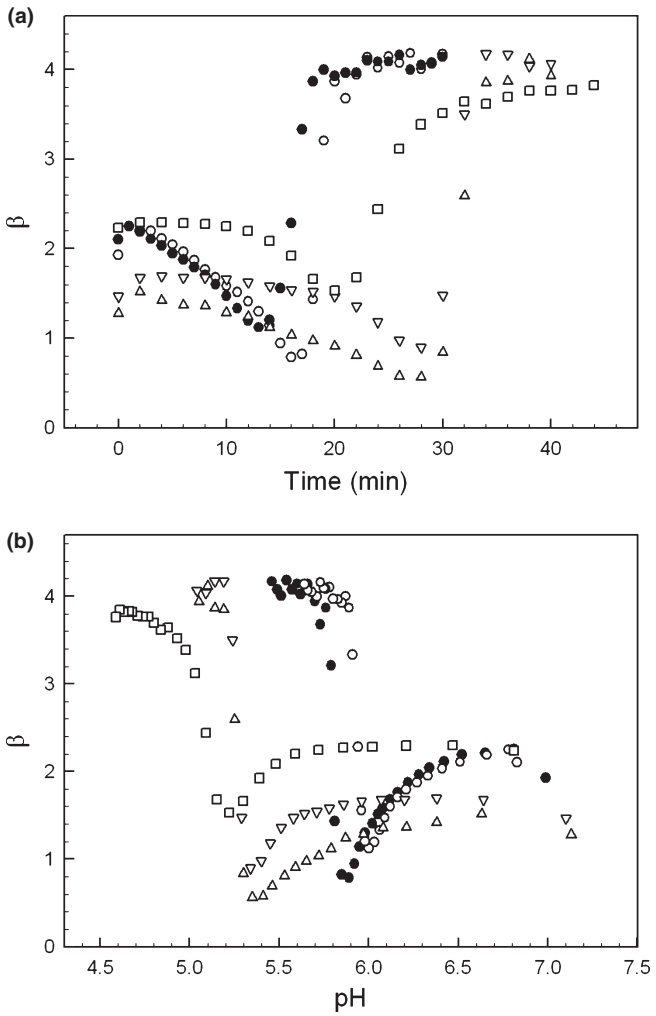

Figure 8 Variation of parameter β as a function of time (a) and pH (b), after the addition of glucono-δ-lactone during the acid aggregation of sodium caseinate/hydrolysates mixtures (4:1). Hydrolysis times (*t*): (●) t_0; (○) t_1; (Δ) t_2; (□) t_3; and (∇) t_4. Protein concentration 0.5%w/w; *R* 0.5; T 35 °C.

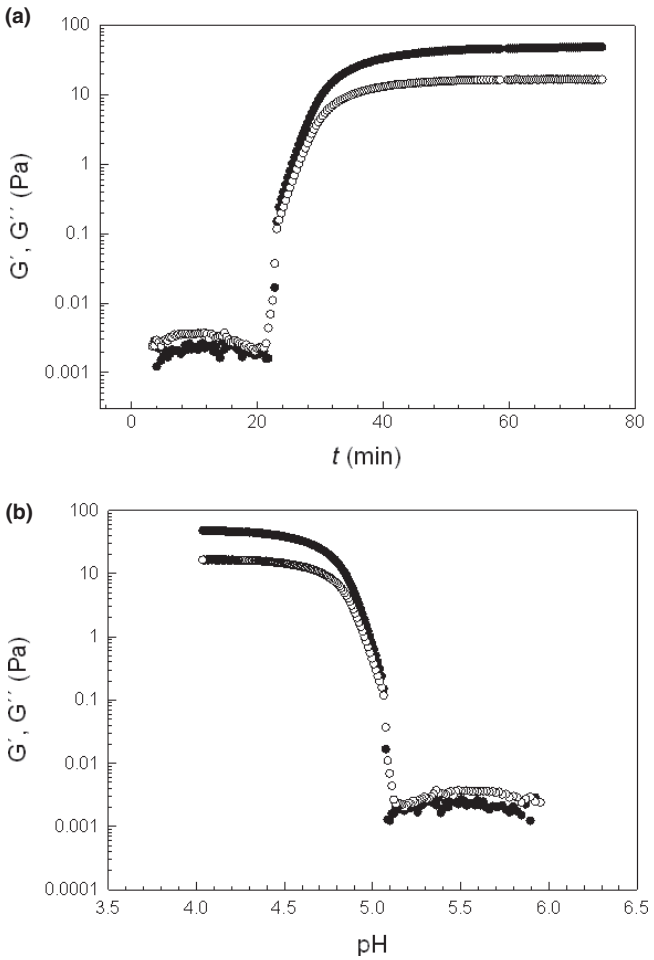

Figure 9 Example of G′ (●) and G″ (○) variations as a function of time (a) and pH (b) during the acidification process of sodium caseinate by the action of glucono-δ-lactone. Protein concentration 3.0%w/w; *R* 0.5; T 35 °C.

Rheological properties of acid gels

As the hydrolysate obtained at t_1 retained its ability to aggregate after adding GDL and showed antioxidant activity with both methods assayed, we only determined the rheological properties of NaCAS untreated as well as of the hydrolysate at t_1 and a mixture of both.

Figure 9 shows, as an example, how G′ and G″ are modified across the acidification process. There is a slow stage where both modules have very low values followed by a sharp increment of G' before G'–G'' crossing. Results of rheological properties during gelation of the samples (3.0% w/w) revealed that the t_g and pH_g were amended in line with those reported for the aggregation process at low protein concentrations (Table 2).

However, the maximum value of G' achieved (G'_{max}) of hydrolysates t_1 was much lower than the NaCAS without hydrolysis at the same concentration. Therefore, gels of hydrolysates of NaCAS obtained by 1 h of hydrolysis with protease P45 will be weak, and they could be used in the manufacture of spreads. However, the hydrolysates t_1/NaCAS mixture gels, to levels where hydrolysates t_1 present antioxidant activity, did not change significantly G'_{max} compared with NaCAS gels.

Table 2 Values of t_g, pH_g and G'_{max} of sodium caseinate (NaCAS), t_1 hydrolysates and their mixtures. Protein concentration 3.0%w/w; *R* 0.5, T 35 °C[a]

Sample	t_g *(min)*	pH_g	G'_{max} *(Pa)*
NaCAS	16.3 ± 0.2	4.89 ± 0.02	67 ± 8
Hydrolysate t_1	37 ± 2	4.94 ± 0.02	6 ± 1
NaCAS/hydrolysate t_1 mixture (4:1)	25 ± 2	5.06 ± 0.04	53 ± 5

[a]Errors are standard deviations. α = 0.01.

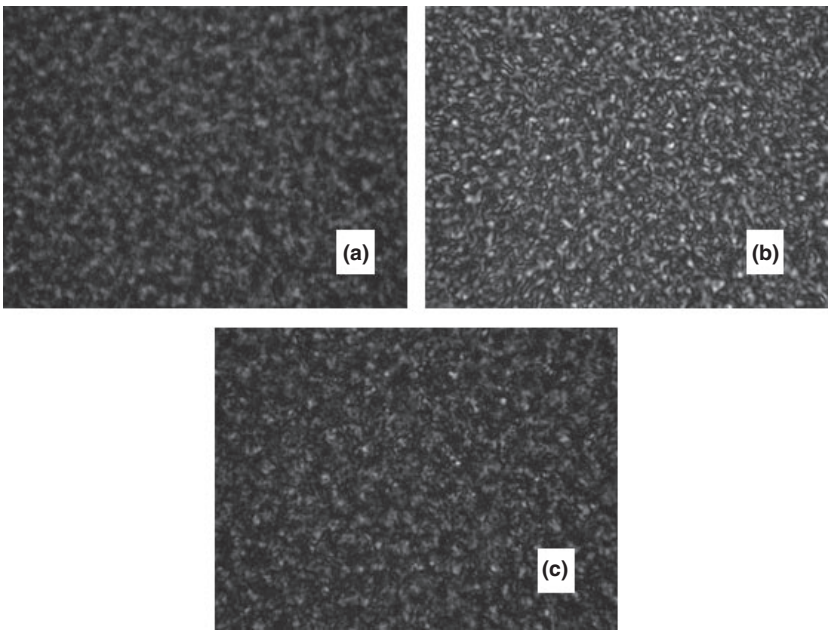

Figure 10 Digital images of gels from sodium caseinate (NaCAS) without hydrolysis t_0 (a), hydrolysate t_1 (b) and NaCAS/hydrolysate t_1 mixture (4:1) (c), which were obtained using a conventional inverted microscopy with an objective 100× and a digital camera with a zoom 7.1×. Protein concentration 3.0%w/w; *R* 0.5; T 35 °C.

Digital images of gels

In Figure 10, we show digital images of gels from NaCAS without hydrolysis (a), hydrolysate t_1 (b) and NaCAS/hydrolysate t_1 mixture (c), which were obtained using a conventional inverted microscopy. From these images, it is possible to observe differences in the internal microstructure of gels, i.e. in the structure compaction and the size of internal interstices. In the gels from hydrolysate t_1, there are small aggregates and free particles, and the interstices or pores are not well defined, resulting in a relatively uniform and homogeneous image. The microstructure of both gels a and c was similar, with a slightly higher degree of compaction of the gels obtained from NaCAS without hydrolysis. Their images show a network with well-defined pores.

CONCLUSION

Bacillus sp. P45, isolated from the intestine of the Amazon basin fish *Piaractus mesopotamicus*, produces extracellular protease (protease P45), which generates functional hydrolysates from bovine NaCAS. The peptides formed reach molecular mass below 6500 Da after 2 h of hydrolysis and also diminish its surface hydrophobicity. The antioxidant activity of the hydrolysates obtained from each hydrolysis period evaluated by DPPH assay was similar and higher than the sample without hydrolysis (t_0). However, only hydrolysates t_0 and t_1 showed inhibition of lipid oxidation measured through TBARS assay. Hydrolysates t_1 maintained the capability to aggregate under acid conditions when GDL was added, but the final gels obtained were less compact and structured. On the other hand, the incorporation of hydrolysates in NaCAS solutions modifies the kinetics of the acid aggregation process but does not alter significantly the degree of compactness of the aggregates formed. In particular, gels obtained from hydrolysates t_1/NaCAS mixtures showed similar elasticity and microstructure to the NaCAS gels. These results are promising regarding the use of these hydrolysates in the production of dairy products, e.g. yoghurt-style desserts, formed by the mechanism of acid–induced casein aggregation.

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