Physicochemical properties and biological activities of ovine caseinate hydrolysates

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Dairy Sci. & Technol. (2012) 92:335–351
DOI 10.1007/s13594-012-0068-3

ORIGINAL PAPER

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Received: 18 November 2011 / Revised: 17 April 2012 / Accepted: 23 April 2012 /
Published online: 8 June 2012

Abstract Caseins are the precursors of bioactive peptides, which can be released through enzymatic hydrolysis and subsequently be incorporated into food products. This investigation evaluated the antioxidant and antimicrobial activities, as well as the physicochemical features, of ovine sodium caseinate (NaCAS) subjected to proteolysis. A *Bacillus* sp. P45 protease preparation was utilized in the hydrolysis of ovine NaCAS, for up to 7 h. Absorbance and intrinsic fluorescence spectra suggested that the hydrolysis of NaCAS exposed aromatic amino acids to the aqueous media. Following a slight decrease in the surface hydrophobicity (S_0) observed up to 2 h of hydrolysis, S_0 increased in the hydrolysates from 3 to 7 h, indicating exposure of hydrophobic groups on the surface of the generated peptides. An increase in the antioxidant properties, evaluated by the 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid method, was observed following hydrolysis up to 4 h. One-hour hydrolysates inhibited the growth of *Salmonella enteritidis*, *Escherichia coli*, *Corynebacterium fimi*, and *Listeria monocytogenes*, showing antifungal activity of the hydrolysates. The gelation ability of the hydrolysates was maintained, as evaluated

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through acid aggregation induced by glucono-δ-lactone (GDL). Although aggregation occurred at slower rates than that of nonhydrolyzed samples, the compactness of the aggregates formed from 1- to 2-h hydrolysates was comparable to that of nonhydrolyzed NaCAS. The final state of aggregates formed from NaCAS/hydrolysate mixtures (4:1) was not altered. Thus, these functional hydrolysates might be potentially added to dairy products manufactured through acid-induced aggregation, without significantly affecting this process.

绵羊酪蛋白酸盐水解物物化的特性和生物活性

摘要: 酪蛋白是生物活性肽的前体物质,生物活性肽可以通过酶水解酪蛋白释放出来继而成为食用级的产品。本文考察并评价了绵羊酪蛋白酸钠经蛋白酶水解后水解物的抗氧化和抑菌活性及其物化特性。芽孢杆菌属(*Bacillus* sp.) p45 蛋白酶被用来水解绵羊酪蛋白酸钠,水解时间为7h。吸光度和内源荧光光谱表明酪蛋白酸钠被水解后,一些芳香族氨基酸暴露于水相介质。当水解时间为2h,水解物表面疏水性缓慢下降,当水解时间为3-7h, 水解物疏水性有所增加,该结果表明,疏水性基团暴露在新产生的肽的表面。水解物抗氧化特性通过2 ,2-联氮-二(3-乙基-苯并噻唑-6-磺酸)二铵盐(ABTS)法来评价,水解时间为4h时, 其抗氧化特性增加。水解时间为1h时,水解物能够抑制肠炎沙门杆菌、大肠杆菌、棒状杆菌属 和单核细胞增生利斯特菌的生长,证明了水解物有抗真菌的能力。酪蛋白酸钠水解物的凝胶能力通过葡萄糖酸内酯酸聚合诱导来评价。虽然水解物凝聚发生的速率低于没有水解的酪蛋白酸钠,但是,水解时间为1和2h水解物的致密性与没有水解的酪蛋白酸钠是相似的。酪蛋白酸钠与水解物以4:1混合形成的凝聚物的最终状态保持不变。因此,这些功能性的水解物可以通过酸诱导凝聚,可潜在的添加到乳制品加工中,对乳制品的加工过程不会产生影响。

Keywords Ovine caseinate · Protein hydrolysates · Gelation · Acid aggregation · Bioactive peptides

关键词 绵羊酪蛋白酸盐 · 蛋白水解物 · 凝胶化 · 酸凝聚 · 生物活性肽

1 Introduction

Milk is considered as a rich source of various nutrients, including proteins, fats, and carbohydrates (Park et al. 2007). Caseins, the main fraction of milk proteins, and the caseinates obtained from combinations of caseins and cations such as sodium and calcium, besides nutritional roles, are largely employed in the food industry due to their functional properties, such as emulsifying and gelation capacities, thus contributing to food texture (Alvarez et al. 2007; Nishinari et al. 2000).

Proteins are usually stable in aqueous solutions; therefore, destabilization and/or denaturation steps are essential processes for gelation to occur (Nishinari et al. 2000). In milk and caseinate solutions, acidification causes the dissociation and aggregation of casein fractions, resulting in the formation of a gel structure. Commonly, acidification is the result of lactose fermentation to lactic acid through bacterial activity; however, direct acidification achieved by the addition of a lactone, such as GDL, has

gained increased attention by the food industry, since this process avoid potential complications related to starter bacteria (Braga et al. 2006).

Proteolytic enzymes from animal, plant, and microbial sources are widely employed in the food industry (Rao et al. 1998). Proteases have been investigated for the modulation of protein functional features, including solubility, foaming, and emulsifying properties (Sinha et al. 2007; Rabiey and Britten 2009). Additionally, proteases have been employed in the hydrolysis of food proteins with the aim of releasing bioactive peptides encrypted in the protein structure, which might exert antioxidant, antihypertensive, and antimicrobial activities, thus presenting a prospective utilization in food science, technology, and nutrition (Phelan et al. 2009; Sarmadi and Ismail 2010).

The composition of milk is widely variable among species, and such peculiarities are attracting the attention of food processors with the hope of manufacturing dairy products with specific tastes and textures. In this sense, the protein content of ovine milk is almost twofold higher than that of bovine milk (Park et al. 2007), representing a valuable source of caseins and caseinates, which could be utilized as functional ingredients in the food industry. Although the functional properties of ovine NaCAS have been demonstrated (Nespolo et al. 2010), studies on the acid aggregation, as well as the antioxidative and antimicrobial properties of ovine NaCAS hydrolysates, have rarely been reported when compared to bovine caseins and caseinates (Corrêa et al. 2011).

The abundant possibilities opened up by protein hydrolysis have further driven interest on the search for novel proteases suitable for particular applications. The use of proteolytic enzymes from microbial sources offer advantages for large-scale production in a relative short time, providing a regular and abundant supply of enzymes (Rao et al. 1998). *Bacillus* sp. P45, an effective feather-degrading strain, produces extracellular proteases during growth on inexpensive liquid media, which might have a potential biotechnological relevance, particularly for casein hydrolysis (Daroit et al. 2010). In this context, ovine sodium caseinate was treated with a protease preparation from *Bacillus* sp. P45. The obtained protein hydrolysates were then characterized, including the evaluation of antioxidant activity, antimicrobial potential, and acid aggregation properties.

2 Materials and methods

2.1 Materials

The acidulant GDL, imidazole, and 8-anilino-1-naphthalene sulfonate (ANS) as ammonium salt were acquired from Sigma-Aldrich Co. (Steinheim, Germany). HCl, NaOH, acetone, and chloroform were provided by Cicarelli SRL (San Lorenzo, Argentina).

2.2 Ovine sodium caseinate (NaCAS) preparation

Ovine casein (CN) was prepared from sheep milk of the Lacaune breed from southern Brazil. The milk was centrifuged (10,000*g* for 10 min at 4 °C) to remove as much fat

as possible, and the skim milk was acidified to pH 4.5 with 1 mol.L^{-1} acetic acid under continuous stirring at 25 °C to cause isoelectric precipitation of caseins. After 30 min at 40 °C, the mixture was filtered through Whatman no. 40 paper using a vacuum pump. Precipitated casein was washed with distilled water, dissolved with 10 g.L^{-1} NaOH until it reached pH 7.0, and precipitated again. Four successive cycles of precipitation and washing were carried out, and the final precipitate was washed with acetone and chloroform to remove the residual fat globules (Moatsou et al. 2004).

Ovine sodium caseinate (10 g.L^{-1}) was prepared from ovine casein by dissolving 1 g of casein in 50 mL of 0.1 mol.L^{-1} NaOH. The dissolution of ovine casein was adjusted to pH 6.8 by adding 0.1 mol.L^{-1} HCl and made to a final volume of 100 mL with distilled water. Casein concentration was measured according to the Kuaye's method, which is based on the ability of strong alkaline solutions (0.25 mol.L^{-1} 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.

2.3 Microorganism and protease preparation

For protease production, *Bacillus* sp. P45 was cultured in feather meal broth (10 g.L^{-1} feather meal, 0.3 g.L^{-1} Na_2HPO_4, 0.4 g.L^{-1} NaH_2PO_4, and 0.5 g.L^{-1} NaCl) for 48 h at 30 °C in a rotary shaker at 125 rpm (Daroit et al. 2010). Cultures were centrifuged (10,000×*g* for 15 min, at 4 °C), and the supernatant, which contained the extracellular proteolytic enzyme(s), was subjected to a partial purification protocol. Initially, ammonium sulfate was added to the supernatant, in a stirred ice bath, until 60 % saturation was reached. After standing for 1 h, this mixture was centrifuged (10,000×*g* for 15 min at 4 °C), and the pellet was dissolved in 20 mmol.L^{-1} Tris–HCl buffer (pH 8.0). The concentrated supernatant containing the proteolytic enzyme (s) was loaded into a gel-filtration Sephadex G-100 column (25×0.5 cm; Pharmacia Biotech, Sweden), equilibrated with Tris–HCl buffer (20 mmol.L^{-1}, pH 8.0). The column was eluted with this buffer at a flow rate of 0.33 mL.min^{-1}, and 30 fractions of 1 mL were collected. Fractions showing proteolytic activity on azocasein (Daroit et al. 2010) were pooled and employed as a protease preparation (142 U.mL^{-1}; 9.16 U mg.$protein^{-1}$) for protein hydrolysis.

2.4 NaCAS hydrolysis

Samples of 0.5 % (*w*/*v*) ovine NaCAS in Tris–HCl buffer (20 mmol.L^{-1}, pH 8) were subjected to hydrolysis with the protease preparation (2 %, *v*/*v*) 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 to 100 °C for 15 min. After centrifugation (10,000×*g* for 15 min), the supernatants were recovered, lyophilized, and kept at −18 °C until further use. Protein concentration of the supernatants was measured as previously described (Kuaye 1994). The protein qualitative composition of the supernatants was analyzed by urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (urea-SDS-PAGE), using low molecular weight markers (Sigma Chemical Co., St. Louis, USA).

2.5 Degree of hydrolysis

DH of ovine NaCAS hydrolysates was determined by quantifying the free amino groups in supernatants following their reaction with 2,4,6-trinitrobenzene sulfonic acid, as previously described (Adler-Nissen 1979). The total amino groups were determined in protein samples (10 mg) completely hydrolyzed in 4 mL of 6 mol.L^{-1} HCl, at 110 °C for 24 h.

2.6 Intrinsic fluorescence spectra

Excitation and emission spectra of the hydrolysates (0.1 %*w*/*v*) were obtained to detect any spectral shift and/or changes in the relative intensity of fluorescence (FI). Previously, the excitation wavelength (λ_{ex}), and the range of concentration with a negligible internal filter effect was determined. Samples (3 mL) for spectral analysis and FI measurements were poured into a fluorescence cuvette (1-cm light path) and placed into a cuvette holder maintained at 35 °C. Values of FI (n=2) were registered within the range of 300–430 nm using a λ_{ex} of 286 nm.

2.7 Absorption spectra

Absorption spectra of the hydrolysates (0.1 %*w*/*v*) were obtained using a diode array spectrophotometer (Spekol 1200, Analytikjena, Belgium). Samples (3 mL) were poured into a 1-cm cuvette and placed into a cuvette holder maintained at 35 °C by water circulation. Values of absorbance (A) were registered within the range of 250–320 nm (n=2).

2.8 Surface hydrophobicity (S_0)

S_0 was estimated using the ammonium salt of amphiphilic ANS as a fluorescent probe (Haskard and Li-Chan 1998), in an Aminco Bowman Series 2 spectrofluorometer (Thermo Fisher Scientific, USA). Measurements were carried out using λ_{ex} and emission wavelengths (λ_{em}) set at 396 and 489 nm, respectively, at constant temperature (35 °C). As mentioned above, both wavelengths were obtained from emission and excitation spectra of protein–ANS mixtures.

FI of samples containing ANS (6 mmol.L^{-1}) 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*/*v*) plot.

2.9 Acid aggregation and size variations of particles during acidification

The aggregation kinetics of hydrolysates (0.35 %*w*/*v*) and ovine NaCAS/hydrolysates mixtures (0.5 %*w*/*v*, 4:1 ratio), induced by acidification with GDL, was analyzed by measuring turbidity (τ) in the range of 450–650 nm, in a Spekol 1200 spectrophotometer with a thermostatized cell (35 °C). The amount of GDL added was calculated using the following relation (R): R=GDL mass fraction/protein mass fraction.

Acidification was initiated by adding solid GDL to 5 g of each sample. Absorption spectra (450–650 nm) and absorbance at 650 nm (A_{650}) were registered as a function of time until a maximum and constant A_{650} was reached; the pH decrease was simultaneously measured. Assays were performed, at least, in duplicate. Values of parameter β were calculated as presented below.

Changes in the average size of particles were followed by the dependence of turbidity (τ) on wavelength (λ) of the suspensions and determined as: β=4.2+ [d(logτ)]/[d(logλ)]. The β parameter has a direct relationship with the average size of the particles and can be used to detect and follow rapid size changes. It is obtained from the slope of logτ vs logλ plots, in the 450–650 nm range, where the absorption due to the protein chromophores is negligible, allowing then to estimate τ as absorbance in 400–800 nm range (Camerini-Otero and Day 1978). 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 (Risso et al. 2007). τ was measured as absorbance using a Spekol 1200 spectrophotometer, with a diode arrangement. Determinations of β were the average of at least duplicate measurements.

2.10 Antioxidant activity of ovine NaCAS hydrolysates

Scavenging of the 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical was determined by the decolorization assay described by Re et al. (1999). ABTS radical cation ($ABTS^{\bullet+}$) solution was prepared by reacting 5 mL of ABTS solution (7 mmol.L^{-1}) with 88 μL of 140 mmol.L^{-1} K_2SO_4. This mixture was allowed to stand in the dark at room temperature for 12–16 h before use. For the assay, the $ABTS^{\bullet+}$ solution was diluted with 5 mmol.L^{-1} phosphate-buffered saline (pH 7.4) to an absorbance of 0.7 (±0.02) at 734 nm. A 10 μL (15 mg.mL^{-1}) of sample was mixed with 1 mL of diluted $ABTS^{\bullet+}$ solution, and absorbance (734 nm) was measured after 6 min. Trolox was used as a positive control.

2.11 Antibacterial activity of ovine NaCAS hydrolysates

Antibacterial activity was tested against *Listeria monocytogenes* ATCC 15131, *Bacillus cereus* ATCC 9634, *Corynebacterium fimi* NCTC 7547, *Staphylococcus aureus* ATCC 1901, *Salmonella enteritidis* ATCC 13076, and *Escherichia coli* ATCC 8739. A 10^8 CFU.mL^{-1} of bacteria in NaCl solution (0.85 %*w*/*v*) was inoculated with a swab onto brain–heart agar plates, and then 15 μL of hydrolysates (250 mg mL^{-1}) was spotted. Plates were incubated at 37 °C for 24 h to verify possible inhibition zones (Motta and Brandelli 2002).

2.12 Antifungal activity of ovine NaCAS hydrolysates

To determine the antifungal activity of the hydrolysates, the filamentous fungi *Fusarium oxysporum* f. sp. *lycopersici*, *Aspergillus fumigatus*, *Penicillium expansum*, and the yeast *Candida tropicalis*, belonging to the Mycological Collection of the Laboratório de Bioquímica e Microbiologia Aplicada, were utilized as indicators. The fungi were cultivated in potato dextrose agar (PDA) plates for 5 days at 30 °C.

Conidia suspensions of the filamentous fungi were prepared and then added to PDA at 50 °C in a sufficient volume to provide a final concentration of 10^6 conidia.mL^{-1}. The medium was poured onto plates and, after solidification, 15 μL of the hydrolysates (250 mg.mL^{-1}) was spotted. Cell suspensions of the yeast were prepared following the same method used for antibacterial activity determinations. The plates were incubated at 30 °C for 48 h and subsequently observed for inhibitory activity against the fungal indicator (Corrêa et al. 2011).

2.13 Statistical analysis

Data presented are average values±standard deviations. Statistical analysis was performed with Sigma Plot 10.0 software. Relationship between variables was evaluated by correlation analysis, using Pearson correlation coefficient (r). Means comparisons were performed using Tukey's test. Differences were considered statistically significant at $P<0.05$ values.

3 Results

NaCAS hydrolysis, initiated by the addition of a protease preparation from *Bacillus* sp. P45, was carried out for up to 7 h. The degree of hydrolysis (DH), which measures the number of peptide bonds cleaved in the protein substrate, was determined in the hydrolysate supernatants. DH increased almost linearly from t_0 to t_2 (3.8 %), and the hydrolysis rate decreased thereafter, reaching 5.5 % after 7 h (Fig. 1a). Urea-SDS-PAGE was performed on the hydrolysates (Fig. 1b), and the disappearance of the major band (around 25 kDa, t_0) at t_1 was observed, with the concomitant appearance of lower MW bands. As the hydrolysis time progressed, the generated peptides had molecular masses <6,500 Da.

Fluorescence emission spectra of NaCAS hydrolysates are presented in Fig. 2a. Hydrolysis caused a fluorescence red shift as well as a decrease in the fluorescence intensity, which might be due to conformational changes in the surroundings of fluorophore groups in the peptides. Loss of protein fluorophores during proteolysis was excluded since resolubilized precipitates separated by centrifugation showed negligible fluorescence intensity (data not shown). An increase in the absorbance of intrinsic chromophores was also observed with the progression of hydrolysis time (Fig. 2b). S_0, determined for the NaCAS hydrolysates, remained practically constant for hydrolysates obtained up to 2 h of hydrolysis; however, S_0 increased with longer hydrolysis times (Table 1).

The acid aggregation of NaCAS hydrolysates was evaluated by variations on the average size of the particles (following by parameter β) as a function of time (Fig. 3a) and pH (Fig. 3b). The results show that the hydrolysates conserved the capability to aggregate and that the acidification process revealed two well-defined aggregation steps: (1) a slower phase with a decrease in β and (2) a rapid increase in β due to the formation of colloidal aggregates, which was initiated at a determined time (t_{ag}) and pH (pH_{ag}). Aggregation profiles show that t_{ag} for hydrolysates is higher than t_{ag} for NaCAS without hydrolysis (t_0), and that the t_{ag} of hydrolysates t_3 and t_4 was lower than hydrolysates t_1 and t_2. The pH_{ag} of hydrolysates t_{1-4} is higher than

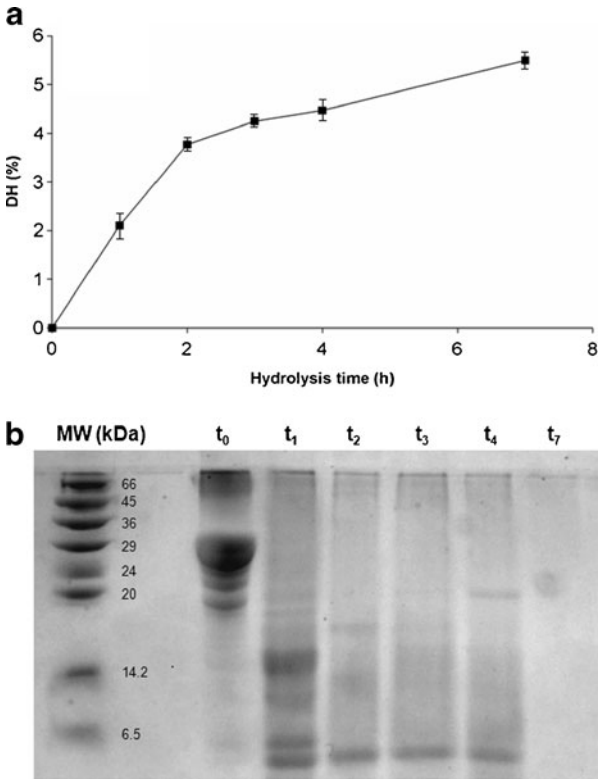


Fig. 1 **a** Degree of hydrolysis (DH, %) determined in supernatants of ovine sodium caseinate (NaCAS) hydrolysates obtained with the *Bacillus* sp. P45 protease preparation. Determinations were performed in triplicate. **b** Urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (urea-SDS-PAGE) of ovine NaCAS hydrolysates (t_0–t_7), and molecular weight markers (MW)

nonhydrolyzed NaCAS (Fig. 3b), and a reduction of the rate of pH decrease was observed for hydrolysates $t_{1–4}$ (Fig. 3c). The aggregation continued until β reached a limiting value, which corresponds to the D_f. The D_f values of aggregates formed at the end of the aggregation process are shown in Table 2. The degree of compactness of aggregates from t_1 and t_2 samples was similar to those obtained from NaCAS without hydrolysis, but diminishes for longer hydrolysis times, especially at t_4.

On the other hand, the average size of initial particles, estimated by β values before GDL addition, decreased as hydrolysis times increased, according to a decrease in molecular mass observed in Fig. 1b. Measurements of the hydrodynamic diameters by dynamic light scattering, performed to verify the correlation between the parameter β and the average particle size, showed a good linear correlation (r=0.9082, P<0.0018) (data not shown), allowing us to corroborate that a change of 0.1 in the parameter β corresponds to a variation of 16 nm in average size.

Acid aggregation of NaCAS/hydrolysates mixtures (4:1) was evaluated by following how the parameter β is modified as a function of time and pH after adding GDL (Fig. 4). The same two-step behavior observed for the hydrolysates aggregation (Fig. 3) was observed for NaCAS/hydrolysates mixtures (Fig. 4). In the presence of hydrolysates, changes on the t_{ag} can only be observed for the mixture NaCAS/

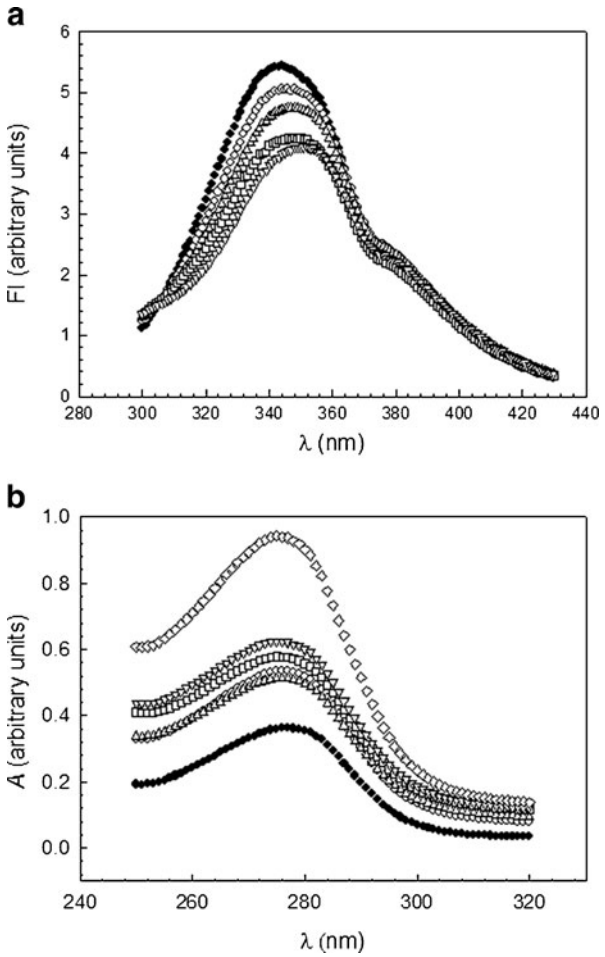


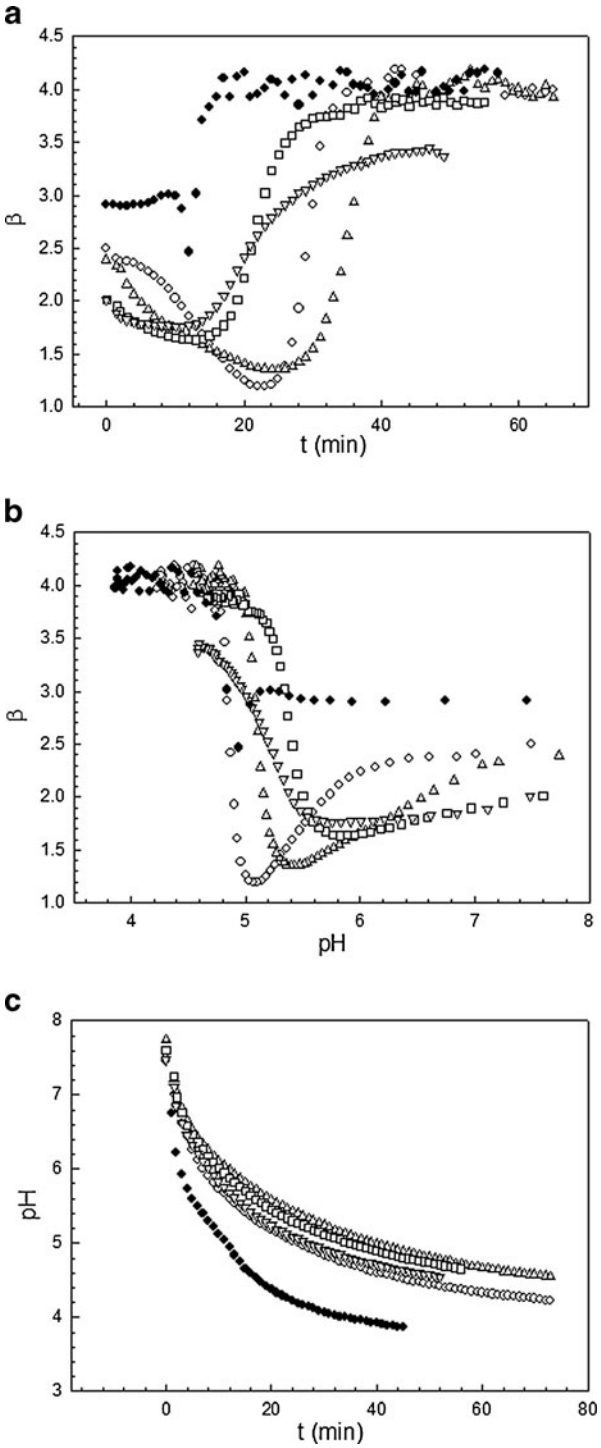
Fig. 2 Fluorescence emission (**a**) and absorption (**b**) spectra of the ovine sodium caseinate (NaCAS) hydrolysates obtained at different times (t): t_0 (*filled circle*), t_1 (*empty circle*), t_2 (*empty triangle*), t_3 (*empty square*), t_4 (*empty inverted triangle*), and t_7 (*diamond*). Assays performed in triplicate at 35 °C; hydrolysate concentration, 0.1 %*w/v*. *FI* fluorescence intensity, *A* absorbance, λ wavelength (nm)

hydrolysate t_1, probably associated with a decreased pH_{ag}, whereas no variations in the rate of pH decline were detected (data not shown). No significant changes were

Table 1 Surface hydrophobicity (S_0) of ovine sodium caseinate (NaCAS) hydrolysates obtained at 35 °C

Hydrolysate	S_0 $[(\%w/v)^{-1}]$[a]
t_0	5.5±0.8
t_1	4.7±0.7
t_2	4.2±0.5
t_3	10.7±0.2
t_4	23.3±0.6
t_7	39.0±0.3

[a]Values are the means±standard deviations of S_0 for triplicate determinations. Significance level α=0.01


a
β
t (min)
b
β
pH
c
pH
t (min)

◀ **Fig. 3** Variations of parameter β, proportional to the average size of particles, as a function of time (**a**) and pH (**b**), and changes of pH as function of time (**c**), after glucono-δ-lactone (GDL) addition, during the acid aggregation of ovine sodium caseinate (NaCAS) hydrolysates t_0 (*filled circle*), t_1 (*empty circle*), t_2 (*empty triangle*), t_3 (*empty square*), and t_4 (*empty inverted triangle*). Assays performed at 35 °C; GDL mass fraction/protein mass fraction (R): 1.5; hydrolysates concentration, 0.35 %*w*/*v*

observed in the degree of compaction of the aggregates formed at the end of the acidification process (D_f).

The antioxidant activity of ovine NaCAS hydrolysates obtained with a protease preparation from *Bacillus* sp. P45 was evaluated with the ABTS radical scavenging assay. Sample t_0, representing the nonhydrolyzed ovine NaCAS, showed antioxidant activity (Table 3). The radical scavenging ability of hydrolysates increased up to t_3, coinciding with a higher DH of the samples (Fig. 1a); however, further hydrolysis resulted in a slight decrease in antioxidant activity (t_4; Table 3).

The antibacterial activity of ovine NaCAS hydrolysates (t_0–t_4) was then investigated. The growth of *S. enteritidis*, *E. coli*, *C. fimi*, and *L. monocytogenes* was inhibited by the t_1 hydrolysate (Fig. 5); however, similar antibacterial activity was not observed for the other hydrolysates (t_0, t_2–t_4). Regarding antifungal activity, only the t_1 hydrolysate showed a partial inhibition of fungal growth (data not shown). The phytopathogenic fungi *F. oxysporum* and *P. expansum* and also *A. fumigatus* were inhibited by the hydrolysate.

4 Discussion

In this investigation, ovine NaCAS was hydrolyzed with a protease preparation from *Bacillus* sp. P45. The hydrolysis rate of ovine NaCAS reduced over time (Fig. 1a), indicating the decreased availability of cleavable peptide bonds within the substrate, which is directly affected by enzyme specificity. The DH after 7 h of hydrolysis is comparable to the DH of yak milk casein treated with papain and Neutrase (Jiang et al. 2007) and to that of bovine milk α-casein hydrolyzed with chymotrypsin or a *Streptomyces griseus* protease (Srinivas and Prakash 2010). The DH of t_3–t_4 resembled that of ovine caseinate hydrolyzed with a protease preparation from *Bacillus* sp. P7 (Corrêa et al. 2011).

Proteolysis promoted conformational modifications of NaCAS (Fig. 2a). Treatment with protease P45 resulted in the exposure of aromatic amino acid residues to the more polar aqueous environment (Fig. 2b), and, particularly, the absorbance peak seemed to correspond to the peak of tyrosine, which is located between 275 and

Table 2 Fractal dimension (D_f) values of ovine sodium caseinate (NaCAS) hydrolysates obtained at 35 °C

Hydrolysate	D_f[a]
t_0	4.10±0.10
t_1	4.10±0.08
t_2	4.00±0.10
t_3	3.88±0.03
t_4	3.42±0.02

[a]Values are the means±standard deviations of D_f for triplicate determinations. Significance level α=0.01

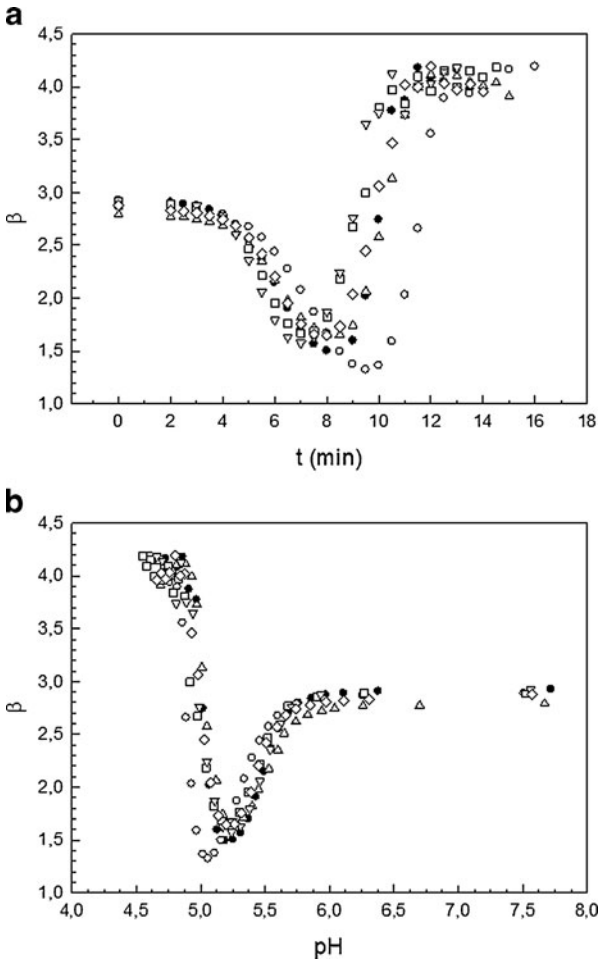


Fig. 4 Variations of parameter β, proportional to the average size of particles, as a function of time **(a)** and pH **(b)**, after glucono-δ-lactone (GDL) addition, during the acid aggregation of ovine sodium caseinate (NaCAS)/hydrolysates mixtures (4:1). Assays performed at 35 °C; GDL mass fraction/protein mass fraction (R): 2; NaCAS:hydrolysates total concentration: 0.5 %*w*/*v*, for hydrolysates t_0 (*filled circle*), t_1 (*empty circle*), t_2 (*empty triangle*), t_3 (*empty square*), t_4 (*empty inverted triangle*), and t_7 (*empty diamond*)

282 nm (Kelly et al. 2005). The evaluated surface hydrophobicity (S_0) indicated a higher exposure of hydrophobic groups in the surface of peptides from t_3 to t_7 hydrolysates (Table 1). In this sense, subtilisin-like proteases, usually showing a

Table 3 Antioxidant activity of ovine sodium caseinate (NaCAS) hydrolysates

Hydrolysate	ABTS radical scavenging activity (%)[a]
t_0	53.57±0.65
t_1	57.86±0.74
t_2	57.27±0.14
t_3	66.08±0.95
t_4	64.50±1.06

[a]Values are means±SEM of three independent determinations

preference for aromatic and/or hydrophobic residues at the P1 site of the cleavable peptide bond (P1′–P1) in the substrate, are among the proteolytic enzymes produced by *Bacillus* sp. P45 (Daroit et al. 2010).

The enzymatic hydrolysis of proteins prior to gelation is regarded as an innovative process that might be employed to modify and control the properties of acid-formed gels (Rabiey and Britten 2009). The two-step aggregation profiles of NaCAS hydrolysates (Fig. 3a), observed during the acidification process, were similar to those previously reported for nonhydrolyzed ovine NaCAS (Nespolo et al. 2010). In the first stage, a gradual increase in the turbidity (data not shown) and a decrease in the parameter β were observed during pH decrease as a function of time (Fig. 3). In aqueous solution, bovine NaCAS undergoes a considerable level of self-association, such as micelles or submicelles (Belyakova et al. 2003; Farrell et al. 1996). Other authors have suggested that bovine sodium caseinate associates into small well-defined aggregates with an aggregation number that depends on the environmental conditions such as temperature, pH, or ionic strength. It is probable that star-like aggregates are formed with a hydrophobic center and a hydrophilic (charged) corona (Pitkowski et al. 2008). It could be assumed that ovine NaCAS may exhibit a similar state in aqueous solution. Therefore, at the first aggregation stage, the decrease in the average diameters, estimated by β values, may be due to a dissociation of the pre-existing aggregates simultaneously with the formation of a large amount of new

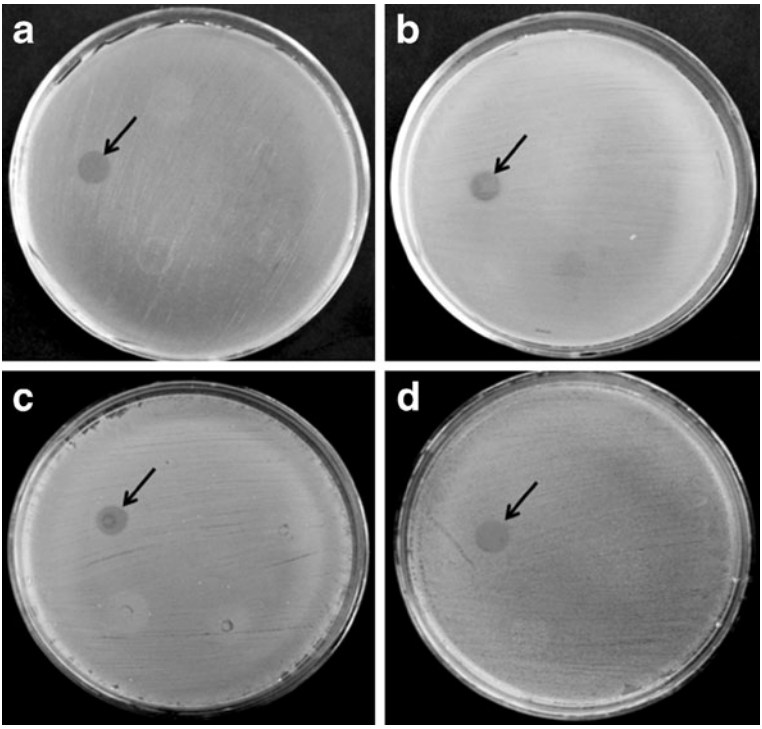


Fig. 5 Growth inhibition of *Salmonella enteritidis* (**a**), *Escherichia coli* (**b**), *Corynebacterium fimi* (**c**), and *Listeria monocytogenes* (**d**) by the 1-h ovine sodium caseinate (NaCAS) hydrolysate. *Arrows* indicate inhibition haloes

aggregates of smaller size due to a loss of the net charge of the particles, which reduces their electrostatic stability and made them more susceptible to flocculation. At pH values near to the isoelectric point, when the particles lose their electrostatic stability, the higher number of particles with electrostatic destabilization causes the formation of much larger particle size aggregates, resulting in the observed diameter increments (β values).

The higher pH_{ag} for hydrolysates $t_{1–4}$ (Fig. 3b) indicate that the hydrolysates are less stable in solution. In addition, the higher t_{ag} detected for hydrolysates when compared to nonhydrolyzed NaCAS are linked to the increased buffering capacity in the case of hydrolysates $t_{1–4}$ (Fig. 3c). On the other hand, the t_{ag} of hydrolysates t_3 and t_4 are lower than hydrolysates t_1 and t_2. This fact can be related to the increase of S_0 observed for hydrolysates obtained at longer proteolysis times (Table 1), which favors the hydrophobic attractive interactions and leads to particle aggregation.

For the NaCAS/hydrolysates mixtures, the acid aggregation (Fig. 4) and also the fractal dimension (D_f) were shown to be similar to that of nonhydrolyzed NaCAS, indicating that the hydrolysates could be included in a NaCAS matrix without significant alterations on the aggregation profile. Although the D_f could be related to rheological properties (Barrett and Peleg 1995), additional studies are needed to evaluate the effect of hydrolysate incorporation on gel structure and textural features.

In a previous work, comparing the results of acid aggregation of ovine NaCAS with those obtained for bovine NaCAS, similar profiles were observed, but D_f were always higher for ovine NaCAS (Hidalgo et al. 2011). Ovine NaCAS has a greater proportion of α_S- and β-CN, and lower κ-CN than bovine protein. Because of these features, the average sizes of the ovine colloidal particles in suspension were always bigger. These results could indicate that D_f also depends on the initial state of colloid particles of NaCAS in suspension, such as composition, average size, and level of self-association or the packing order of the primary particles, among others.

High D_f values are usually attributed to a diffusion limited particle–cluster aggregation (PCA) mechanism, though there is no "universal" agreement on the limits of the PCA mechanism (Brasil et al. 2001). Fractal dimensions higher than three have been reported in the literature (Meakin 1983; Jullien and Botet 1987). Higher dimensions are harder to visualize, but other explanations should be regarded to conveniently describe certain fractal geometries. In a previous study, we already reported $D_f>3$ for ovine acid aggregates in the presence of different concentrations of calcium (Nespolo et al. 2010). To our knowledge, other studies have not been reported for ovine caseinate.

Caseins have been reported as potential precursor proteins for the release of bioactive peptides (Phelan et al. 2009). Variability in milk caseins sequences, determined by the high genetic polymorphisms observed among species (Park et al. 2007), might affect the susceptibilities of these proteins to proteolysis and, consequently, the production of peptides with biological activities (Minervini et al. 2003). Therefore, the bioactivity of protein hydrolysates is dependent on the size and amino acid sequences of the generated peptides, which, in turn, depend on protease specificity, protein substrate and hydrolysis conditions (Sarmadi and Ismail 2010).

As previously reported (Gómez-Ruiz et al. 2008), nonhydrolyzed caseinate demonstrated antioxidant activity. Maximal antioxidant activity was observed for the t_3 hydrolysate, which showed a 12.5 % increment when compared to non-hydrolyzed ovine NaCAS (Table 3). Similarly, ovine NaCAS hydrolysates obtained with a protease preparation from *Bacillus* sp. P7 showed maximal antioxidant activity after 2 h of hydrolysis, resulting in a 12.8 % increment in comparison to the nonhydrolyzed counterpart (Corrêa et al. 2011). The general positive effect of proteolysis on the antioxidant activity of ovine NaCAS indicates the release of previously inactive peptide sequences from the primary protein structure (Phelan et al. 2009). Incorporation of such protein hydrolysates into foods and foodstuffs might serve diverse purposes, such as the increase in the nutritional value of food products, prevention of oxidation reactions during food storage, and delivery of antioxidant peptides through diet, protecting against oxidative damage (Gómez-Ruiz et al. 2008; Rossini et al. 2009).

The t_1 hydrolysate inhibited the growth of *S. enteritidis*, *L. monocytogenes*, and strains of *E. coli* (Fig. 5), which are important microorganisms related to foodborne diseases (Mor-Mur and Yuste 2010). Therefore, antimicrobial peptides produced through the hydrolysis of milk proteins might present useful applications due to the inhibition of pathogenic and also spoilage microorganisms, adding to the quality and safety of food products. Further interest is focused on caseins since these are safe food proteins abundantly available at low costs.

In a previous work, only the Gram-positive bacteria *B. cereus* ATCC 9634 and *C. fimi* NCTC 7547 were inhibited by ovine NaCAS hydrolysates (t_3) obtained with *Bacillus* sp. P7 protease (Corrêa et al. 2011). The higher susceptibility of Gram-positive microorganisms to casein-derived peptides, when compared to Gram-negative bacteria, might be attributed to the more complex cellular envelope of the latter (López-Expósito et al. 2006). The present investigation shows that both Gram-positive and Gram-negative bacteria were inhibited (Fig. 5), as reported for hydrolysates of bovine α-casein obtained with chymotrypsin (Srinivas and Prakash 2010). Specifically, hydrolysis products of ovine α_{s2}-casein were demonstrated to possess activity against various Gram-negative and Gram-positive bacteria, including *E. coli* and *Listeria innocua* (López-Expósito et al. 2006).

The inhibition of fungal strains by hydrolysate t_1 might also have practical applications, since some strains of *F. oxysporum* are responsible for severe vascular wilt or root rot diseases in many plants; for instance, *Fusarium* wilt caused by the soilborne fungus *F. oxysporum* f. sp. *lycopersici* is one of the most devastating diseases of tomato plants (Mandal et al. 2009). *P. expansum*, the agent of blue mould disease, is regarded as one of the main causes of postharvest spoilage of pears and apples, and is frequently isolated from a wide range of fruits (Snowdon 1990). In addition, the t_1 hydrolysate inhibited the clinically important pathogen *A. fumigatus*. Previously, t_3 ovine NaCAS hydrolysates obtained with *Bacillus* sp. P7 protease were demonstrated to inhibit *P. expansum* and *A. fumigatus*, but not *F. oxysporum* (Corrêa et al. 2011). The search for alternative fungicides is a topic of utmost interest because the disadvantages associated with synthetic chemical fungicides, such as high costs, environmental pollution, and induction of pathogen resistance (Wang et al. 2002). Therefore, due to scarce work in the area and the promising results obtained in the screening performed, such protein hydrolysate merits future studies on the identification of the compounds showing antimicrobial activity.

5 Conclusion

This investigation indicates that a protease preparation from *Bacillus* sp. P45 could be employed in the hydrolysis of ovine NaCAS to obtain peptides possessing antioxidant and antimicrobial activities. The aggregation profiles of the hydrolysates, and especially those of ovine NaCAS/hydrolysate mixtures, suggest their application in the food industry. Particularly, the hydrolysates might be incorporated into dairy products manufactured through acid-induced casein aggregation, showing minimal effects on the gelation process and potentially adding to food quality, functionality, and safety.

Acknowledgments This work was supported by grants from the Cooperation Program between Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) from Brazil and Technology and Productive Innovation Ministry (MINCyT) from Argentine, Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Edital PqG 02/2011), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

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