Food Hydrocolloids 43 (2015) 510–520

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Biological and physicochemical properties of bovine sodium caseinate hydrolysates obtained by a bacterial protease preparation

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ARTICLE INFO

Article history:
Received 22 October 2013
Accepted 7 July 2014
Available online 31 July 2014

Keywords:
Bacillus sp.P7
Bovine sodium caseinate
Hydrolysates
Bioactivity
Acid aggregation and gelation
Microstructure

ABSTRACT

In this work, we aimed at the production of bovine sodium caseinate (NaCAS) hydrolysates by means of an extracellular protease from *Bacillus* sp. P7. Mass spectrometry was carried out to evaluate peptide mass distribution and identified sequences of peptides with a signal/noise ratio higher than 10. Antioxidant and antimicrobial properties of hydrolysates were evaluated. An acid-induced aggregation process of the hydrolysates and their corresponding mixtures with NaCAS were also analyzed. The results showed that the enzymatic hydrolysis produced peptides, mostly lower than 3 kDa, with different bioactivities depending on the time of hydrolysis (t_i). These hydrolysates lost their ability to aggregate by addition of glucono-δ-lactone, and their incorporation into NaCAS solutions alter the kinetics of the process. Also, the degree of compactness of the NaCAS aggregates, estimated by the fractal dimension of aggregates, was not significantly altered by the incorporation of hydrolysates. However, at higher protein concentrations, when the decrease in pH leads to the formation of NaCAS acid gels, the presence of hydrolysates alters the microstructure and rheological behavior of these gels.

1. Introduction

Caseins (CN) are the main milk protein fraction (~80%) which occurs in micelles as large particles of colloidal size (Walstra, Jenness, & Badings, 1984). However, the micellar structure of CN is destroyed during the manufacture of sodium caseinate (NaCAS) (Mulvihill & Fox, 1989). NaCAS are extensively used in food industry because of their physicochemical, nutritional and functional properties, such as emulsifying and gelation capacities, thus contributing to food texture (Alvarez, Risso, Gatti, Burgos, & Suarez Sala, 2007; Nishinari, Zhang, & Ikeda, 2000).

A gel structure is formed during NaCAS acidification as a result of the dissociation and aggregation of CN fractions (α_{S1}-, α_{S2}-, β- and κ-). In the traditional process, NaCAS is acidified by bacteria which ferment lactose to lactic acid. However, direct acidification achieved by the addition of a lactone, such as glucono-δ-lactone (GDL), has gained the attention of the food industry, since this process avoids potential complications related to starter bacteria (variable activity and variations with the type of culture used). 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 (Braga, Menossi, & Cunha, 2006; de Kruif, 1997).

The high growth in consumer demand for healthy and nutritional food products has encouraged the food industry to carry out an improvement in the development of natural and functional food ingredients and dietary supplements. In the primary sequence of proteins there are inactive peptides that could be released by enzymatic hydrolysis *in vivo* or *in vitro*. These peptides acquire different biological activities, such as opioid, antihypertensive, immunomodulatory, antibacterial and antioxidant activities, among others, with potential applications in food science and

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http://dx.doi.org/10.1016/j.foodhyd.2014.07.009
0268-005X/

technology (FitzGerald, Murray, & Walsh, 2004; Haque & Chand, 2008; Phelan, Aherne, FitzGerald, & O'Brien, 2009; Sarmadi & Ismail, 2010; Silva & Malcata, 2005).

CN are considered important sources of bioactive peptides that could be released through different types of enzymatic hydrolysis using microbial or digestive enzymes (Korhonen, 2009; Silva & Malcata, 2005). Under moderate conditions of pH and temperature, it is possible to obtain components with biological activities that enhanced nutritional and functional properties such as gelation, emulsification and foam formation (Hartmann & Meisel, 2007; Silva & Malcata, 2005).

It is known that commercial proteases have been employed in the production of protein hydrolysates with bioactives properties such as antioxidant activity (Rival, Boeriu, & Wichers, 2001; Saiga, Tanabe, & Nishimiura, 2003; Zhu, Zhou, & Qian, 2006). Microbial proteases are particularly interesting because of the high yield achieved during their production through well-established culture methods (Gupta, Beg, & Lorenz, 2002; Rao, Tanksale, Ghatge, & Deshpande, 1998). It have been reported that a proteolytic *Bacillus* sp. P7, isolated from the intestinal conduct of the Amazonian fish *Piaractus mesopotamicus*, produces high levels of extracellular proteases with biotechnological potential during submerged cultivations in inexpensive culture media (Corrêa et al., 2011).

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 & Britten, 2009). The aims of this work were to obtain protein hydrolysates of bovine NaCAS with a protease preparation from *Bacillus* sp. P7, determine the peptide mass distribution, identified peptide sequences and evaluate their different bioactivities (antioxidant, antimicrobial, reducing and chelating power). Also, the effects of the presence of these bioactive peptides on acid aggregation and gelation properties of NaCAS were studied.

2. Materials and methods

2.1. Materials

Bovine NaCAS powder, azocasein, the acidulant GDL, tris(hydroxymethyl)aminomethane (Tris), 8-anilino-1-naphthalene-sulfonate (ANS) as ammonium salt; 2,4,6-trinitrobenzene sulfonic acid (TNBS); 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS); ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine) were commercially acquired from Sigma-Aldrich Co. (Steinheim, Germany). Other chemicals employed were of analytical grade and were provided by Cicarelli SRL (San Lorenzo, Argentina).

2.2. Bovine sodium caseinate (NaCAS) preparation

NaCAS solutions were prepared by dissolving the commercial powder in distilled water. CN 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

Bacillus sp. P7, which secretes the extracellular proteases, was maintained in Brain-Heart Infusion (BHI) agar plates. The strain was cultivated 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, 0.5 g L^{-1} NaCl) for 48 h at 30 °C in a rotary shaker (125 rpm) (Corrêa, Daroit, & Brandelli, 2010). Culture was centrifuged (10,000 × *g* for 15 min at 4 °C) and the supernatant, which contained the extracellular proteolytic enzymes, was submitted to a partial purification.

2.4. Protease partial purification

The proteases were precipitated from culture supernatants by the gradual addition of solid ammonium sulfate to achieve 60% saturation, in an ice bath with gentle stirring. This mixture was allowed to stand for 1 h, centrifuged (10,000 × *g* for 15 min at 4 °C), and the resulting pellet was dissolved in 20 × 10^{-3} mol L^{-1} Tris-HCl buffer pH 8.0. The concentrated enzyme samples were applied to a Sephadex G-100 (Pharmacia Biotech, Uppsala, Sweden) gel filtration column (25 × 0.5 cm) previously equilibrated with the above mentioned buffer, and elution was performed using the same buffer at a flow rate of 0.33 mL min^{-1}. Thirty fractions (1 mL) were collected and submitted to the proteolytic activity assay. Fractions showing enzymatic activity were pooled to will be use in NaCAS hydrolysis.

2.5. Proteolytic activity assay

Proteolytic activity was determined as described by Corzo-Martinez, Moreno, Villamiel and Harte (2010), using azocasein as substrate. The reaction mixture contained 100 μL enzyme preparation, 100 μL of 20 × 10^{-3} mol L^{-1} Tris-HCl buffer pH 8.0, and 100 μL of 10 mg mL^{-1} azocasein in the same buffer. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 500 μL of 0.10 g mL^{-1} trichloroacetic acid (TCA). After centrifugation (10,000 × *g* for 5 min), 800 μL of the supernatant was mixed with 200 μL of 1.8 mol L^{-1} NaOH, and the absorbance at 420 nm was measured (Corzo-Martínez et al., 2010). One unit of enzyme activity (U) was considered as the amount of enzyme that caused a change of 0.1 absorbance units under the above assay conditions. Fractions showing proteolytic activity on azocasein were pooled and employed as a P7 protease preparation (P7PP) for NaCAS hydrolysis.

2.6. Hydrolysis of NaCAS

Samples of 0.01 g mL^{-1} of NaCAS in Tris-HCl buffer 20 × 10^{-3} mol L^{-1}, pH 8 were subjected to hydrolysis with the addition of 1 mL of P7PP (enzyme:substrate 1:50 ratio) at 45 °C. The hydrolysis reaction was stopped at different times (t_i; $i = 0, 0.5, 1, 2, 3, 4, 6$ 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. Protein concentration of the supernatants was measured as previously described (Kuaye, 1994).

2.7. Degree of hydrolysis (DH)

DH of NaCAS hydrolysates was determined by the TNBS method (Adler-Nissen, 1979). Protein hydrolysate samples (250 μL) were mixed with 2 mL phosphate buffer (0.212 mol L^{-1}; pH 8.2) and 2 mL 1% TNBS, and incubated at 50 °C for 1 h. Then, 4 mL of 0.1 mol L^{-1} HCl was added, and mixtures were maintained for 30 min at room temperature before performing readings at 340 nm. Total amino groups in NaCAS was determined in a sample (10 mg) which was completely hydrolyzed in 4 mL of 6 mol L^{-1} HCl at 110 °C for 24 h (Li, Chen, Wang, Ji, & Wu, 2007).

2.8. Mass spectrometry

Peptide mass distribution of hydrolysates was determined by MALDI-TOF-TOF mass spectrometry, at the CEQUIBIEM proteomic