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A comparison among β-caseins purified from milk of different species: Self-assembling behaviour and immunogenicity potential

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ABSTRACT

Caseins are a family of proteins constituted by α-caseins (αs-1 and αs-2 caseins), β-caseins and κ-caseins. β-caseins, in particular, show a temperature and concentration-dependent self-assembling behaviour. Recently, β-casein micelles have been proposed as natural nanocarriers for the delivery of hydrophobic compounds, promoting their bioavailability. Until now, all studies regarding both chemical-physical characterization and applications of β-caseins have employed the protein of bovine origin. However, it could be interesting to exploit the use of β-caseins from other milk sources for their potential encapsulation ability and immunogenicity but, at present, no information on the self-assembling behaviour is available for β-caseins from the milk of species different from bovine. In this work, for the first time, β-caseins from human milk and from donkey, goat, and sheep milk were purified and their self-assembling behaviour was compared to that of a commercial bovine β-casein, the only one for which the concentration and temperature aggregation behaviour is known. Furthermore, a preliminary evaluation of the immunogenicity potential of β-casein from other milk sources has been performed by cross-reaction experiments using anti-β-casein antibodies from bovine origin. The results indicated a similar self-assembling profile among all β-caseins examined compared to the bovine β-casein, suggesting the possible use of β-casein from other milk sources as nanocarriers. Since donkey and human β-casein do not cross-react with bovine anti-β-casein antibodies, they could be particularly interesting for the development of self-assembling systems with lower hypoallergenic potential.

1. Introduction

Caseins represent the most abundant protein fraction in milk, amounting to approximately 50–80% of its total protein content. Specifically, they are a family of proteins constituted by α-caseins (αs-1 and αs-2 caseins), β-caseins and κ-caseins, which differ each other for the amino acid sequence and the type and extent of the post-translational modifications [1]. They are present in milk as large colloidal aggregates, resulting from the supramolecular assembling of the different casein components, which are known as casein micelles with a mean size of 120 nm [2].

The biological function of caseins is to carry large amounts of the insoluble salt calcium phosphate in an aqueous environment (as in milk), providing nutritional support to the infants [3]. Among all caseins, β-casein is the only showing a temperature and concentration-dependent self-assembling behaviour. Native bovine β-casein is a single polypeptide chain with a molecular mass of 24 kDa, which is able to self-assemble into micellarstructures in aqueous solutions thanks to its amphiphilic primary structure [4]. It has a critical micellar concentration (CMC) ranging from 0.05 to 0.2% (w/v) as a function of temperature, pH, solvent composition and ionic strength [5]. Particularly, the effect of temperature on bovine β-casein self-assembling has been largely investigated in terms of micellization thermodynamics (e.g critical micelle temperature; CMT) and kinetics [6].

Recently, the β-casein micelles have been considered as a valid alternative to synthetic copolymers for the drug delivery and

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encapsulation of active compounds. Some studies have proposed the use of β-casein micelles as natural nanovehicles for the oral administration of hydrophobic compounds (including some chemotherapeutic drugs) promoting their bioavailability [7,8].

Furthermore, β-casein micelles can act as a transporter of hydrophobic nutraceutical substances such as vitamins, antioxidants, fatty acids, probiotics. These bioactive molecules are usually very susceptible to degradation, but once encapsulated, they can increase their stability and bioavailability [9–11].

Nevertheless, some concerns related to the immunogenicity and the role of β-caseins and their by-products still remain, in order to broaden their field of applications. In fact, bovine β-casein is considered one of the major responsible of the Cow Milk Protein Allergy (CMPA) [12], as suggested by the identification of multiple epitopes in both N- and C-terminal sequences of the protein [13,14].

The self-assembling behaviour and immunogenicity aspect have been mostly elucidated for bovine β-casein but they represent an interesting field of research as regards β-casein obtained from the milk of different animal species.

Based on these assumptions, we have decided to isolate and purify the β-caseins from donkey, goat, sheep, and human milk. The self-assembling behaviour of isolated β-caseins from the milk of different mammalian species was investigated in terms of size and critical micellization temperature (CMT) by dynamic light scattering (DLS) measurements and in terms of critical micelle concentration (CMC) by spectrofluorometric analyses and the results were compared with the ones obtained by using commercial bovine β-casein. Furthermore, the cross-reactivity of anti β-casein bovine antibodies against all β-caseins purified from the milk of different sources has been evaluated, as a preliminary screening of their immunogenicity potential.

2. Materials and methods

2.1. Milk samples preparation

Donkey, goat, sheep and bovine milk were obtained from local farms (10 pluriparous animals at mid-stage of lactation for each kind of milk). Human milk was given from three volunteer women (two months of lactation). In each case, skimmed milk was prepared from 10 mL of fresh milk by centrifugation at 3000*g* (30 min; 15 °C). A 10% (v/v) acetic acid solution was added to the skimmed milk until reaching a pH value of 4.6 and, subsequently, samples were centrifuged at 3000*g* for 10 min to precipitate isoelectrically the total caseins fraction. Each total casein sample from donkey, goat, sheep and human milk was resuspended in 10 mL of 50 mM ammonium acetate, pH 5.5 containing 8 M urea (Buffer A) and filtered using membranes (Millipore HAWP/D1300; 0.45 μm) before HPLC purification. The protein concentration of each resuspended casein sample (from donkey, goat, sheep, human and bovine milk) was determined according to the Bradford method [15]. Lyophilized bovine β-casein, used as reference, was purchased from Sigma Aldrich (St. Louis, MO).

2.2. β-Caseins purification from donkey, goat, sheep, human and bovine milk

Each aliquot of whole casein fraction from donkey, goat, sheep, human and bovine milk was subjected to cationic exchange chromatography on a HPLC ÄKTA Purifier (GE-Healthcare, Little Chalfont, UK). The column used was a Mono S HR 5/50 G L, 1.0 ml bed volume, 10 μg particle size (GE-Healthcare, Little Chalfont, UK). The purification was performed according to the protocol of Rasmussen et al. [16], developed for the separation of the different casein fraction from bovine milk. The column was equilibrated in buffer A at a flow rate of 0.5 mL/min and eluted by a linear gradient between buffer A and buffer B (1 M ammonium acetate, pH 5.5; 8 M urea). The gradient used was: %B = 0, time = 0 min; %B = 0, time 20 min; %B = 100, time = 140 min. The peak corresponding to β-casein was subsequently identified by a polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), followed by N-terminal analysis. SDS-PAGE was performed as described by Laemmli [17], under reducing conditions using a 15% acrylamide-bis acrylamide solution and the Mini-Protean III apparatus, gel size 7 × 8 cm × 0.75 mm (Bio-Rad, Hercules, CA). The markers used were Bio-Rad molecular weight standard, low range (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa). The proteins were visualized on the gel by Coomassie Blue staining (0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid). The N-terminal analysis was performed after electrophoresis: the polyacrylamide gel was equilibrated for 15 min in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 11.0, containing 10% (v/v) methanol, and blotted onto a PVDF membrane (Sequi-blot, Bio-Rad, Hercules, CA). The N-terminal amino acid sequence was determined by Edman degradation using an automatic protein sequencer (Protein SequencerG1000 A, Hewlett Packard) on passively transferred electrophoretic bands on PVDF membrane. The identification of proteins and peptides was performed by consulting the Swiss-Prot and TrEMBL protein databases available on the ExPASy (Expert Protein Analysis System) proteomic server (https://www.expasy.org) at the Swiss Institute of Bioinformatics. For each sample, the peaks identified as β-casein were collected, pooled and dialysed (Spectra/Por® dialysis membranes, MWCO = 3000 Da, Spectrum Lab. Inc., Phoenix, AZ) against 20 mM phosphate buffer, pH 7.0. After dialysis, the concentration of β-caseins was determined using the Bradford method [15]. Aliquots of 1.0 mg of β-caseins were dried using a Savant SpeedVac concentrator (Thermo Fischer Scientific, Waltham, MA, USA) and stored at -20 °C until use.

2.3. Evaluation of the cross-reactivity between the β-caseins from different sources and bovine anti-β-casein antibodies

In order to evaluate the cross-reactivity between the β-caseins obtained from the milk of different species and the anti-β-casein antibodies, 10μg of each β-casein sample purified from donkey, goat, sheep and human milk and 10 μg of lyophilized bovine β-casein, used as positive control, were firstly separated by a 15% SDS-PAGE and subsequently blotted onto a nitrocellulose membrane (0.45 μm pore-size nitrocellulose membrane, Thermo Scientific, Rockford, IL) using a Mini Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA). After 90 min at 50 V, the membranes were blocked for 30 min with a 1% w/v BSA (bovine serum albumin) in Tris buffer saline solution (TBS, 50 mM Tris − HCl, pH 7.5; 150 mM NaCl) and subsequently incubated overnight with the anti-β-casein antibodies from bovine milk (≥98%, Sigma Aldrich, Saint Louis, Mo). The cross-reactivity between anti β-casein antibodies from bovine milk and β-caseins from donkey, goat, sheep and human milk was evidenced by the colorimetric horseradish peroxidase substrate reagent HRP-Conjugate Substrate Kit (Bio-Rad, Hercules, CA).

2.4. Critical micelle concentration (CMC) determination by pyrene fluorescence emission

Steady-state fluorescence spectra of pyrene in the presence of different concentrations of β-caseins from milk of different sources (from 0.02 mg/mL to 3.0 mg/mL) in buffer (50 mM HEPES, 110 mM NaCl pH 7.3) were recorded at 37 °C using a spectrofluorimeter (LS-55, Perkin-Elmer) equipped with a thermostated cell (HAAKE C25 P thermostat). The fluorescence emission spectra (350–600 nm) were measured using an excitation wavelength λ_{exc} = 338 nm and 2.5 nm slits. The intensity ratio of the third (III) and first (I) vibronic bands of the emission spectrum of pyrene, at 383 nm and 372 nm, was plotted against the β-casein concentration. The critical micelle concentration (CMC) of β-caseins was determined by fitting the experimental data with the following equation (GraphPad Prism 6):