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The effect of glycosylation on the interfacial properties of bovine caseinomacropeptide

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ABSTRACT

The two major fractions of bovine caseinomacropeptide (CMP), the glycosylated gCMP and non-glycosylated aCMP, were studied for their emulsifying properties. The main finding was that aCMP showed significantly better emulsifying properties than gCMP. While aCMP showed an emulsifying activity index (EAI) of 150.7 g/m^2, gCMP achieved a value of 98.5 g/m^2. Stability of emulsions was 1.4 times higher for aCMP as compared to gCMP. Droplet size measurements and creaming studies showed a strong influence of pH on both fractions with minimal emulsion stabilities at pH 4.1 (gCMP) and 4.9 (aCMP). Investigation of the flocculation behaviour and variations of the ionic strength indicate that the glycan side chains induce a combination of electric, steric and hydrophilic effects, preventing an ordered adsorption of gCMP molecules at the oil/water interface, while aCMP builds a stable network at the surface. For further elucidation, zeta potential measurements for both fractions were performed, resulting in isoelectric points of 3.15 for gCMP and 4.15 for aCMP in bulk solution. Finally, a hypothesis of surface adsorption of CMP is presented as a function of glycosylation and pH based on experimental data supported by an approach using protein modelling techniques.

1. Introduction

Glycoproteins/peptides are important compounds of numerous biological systems which play a major role in adhesion, cell/cell or cell/matrix interactions. Due to their omnipresence in biological systems, glycoproteins are found in numerous food raw materials.

The bovine glycopeptide caseinomacropeptide (CMP) is the hydrophilic part of κ-casein f(106–169) which is released upon renneting of milk by the endopeptidase chymosin. CMP is a random coil or intrinsically disordered peptide, a so-called IDP, which is significantly enriched in the amino acids Pro, Glu and Ser and depleted in Trp, Tyr, Phe, Cys and Leu as compared to globular proteins. This unfolded state without helix or sheet structures is ensured by a low mean hydrophobicity, high net charge and a high conformational flexibility (Uversky, 2002). Further on, CMP shows a high degree of heterogeneity due to genetic as well as post-translational modifications of the parental protein κ-casein, which are fully conserved in the CMP after hydrolysis. The heterogeneity due to genetic variation with two major variants A and B from a total of 11 genetic variants (Buchberger & Dovc, 2000) is amplified by a high degree of posttranslational modifications such as phosphorylation and glycosylation (Holland, Deeth, & Alewood, 2005; Holland, Deeth, & Alewood, 2006; Talbo, Suckau, Malkoski, & Reynolds, 2001). Five different *O*-glycans, which are covalently bound to threonine residues (T131, T133, T142 and T145) or serine (S165), have been identified so far (Holland et al., 2006). In most cases, the highly negatively charged sialic acid (N-acetyl neuraminic acid, NANA) is the terminal carbohydrate. On the basis of glycosylation, CMP can be classified in two major fractions: the glycosylated and phosphorylated glyco-CMP (gCMP) and the non-glycosylated, but phosphorylated aglyco-CMP (aCMP).

CMP has been the subject of growing interest throughout recent years due to its reported beneficial physiological properties (Anderson & Aziz, 2006; Brody, 2000; Burton-Freeman, 2008; Janer, Pelaez, & Requena, 2004; Lim, van Calcar, Nelson, C'Jleason, & Ney, 2007; Manso & Lopez-Fandino, 2004; Thomä-Worringer, Sorensen, & Lopez-Fandino, 2006). It therefore has a high potential for clinical and nutritional applications. Besides their biological activities, proteins or peptides can often be used as food product structuring agents. There are only a few studies reporting on the technological properties such as solubility, emulsifying properties or foaming abilities. Among the first investigations on the solubility and emulsifying properties of CMP and κ-casein was the study by Chobert, Touati, Bertrandharb, Dalgalarrondo, and Nicolas (1989). A

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good solubility over a wide range of pH with a minimum between pH 1 to 5 was found. A maximum emulsifying activity was observed at alkaline pH and a minimum in the pH range 4.5–5.5. However, the stability of heat-treated CMP stabilised emulsions deteriorated after 24 h of storage, particularly in the neutral and alkaline range of pH. Results published by Moreno, Lopez-Fandino, and Olano (2002) were in accordance with those results for the most part, except that the solubility was increased around pH 5.0. Both publications showed a strong pH dependence of the emulsifying properties and a high decrease of those properties around the isoelectric point with a minimum in the pH range 4.5–5.5. The influence of pH on the emulsifying capacity was assumed to be due to a decrease in voluminosity of the CMP molecules as the pH is reduced. This reduction in voluminosity can be explained by a reduction of electrostatic and steric repulsions within the molecules (Minkiewicz, Slangen, Lagerwerf, Haverkamp, Rollema, & Visser, 1996). Results by Martin-Diana, Frias, and Fontecha (2005) proved that the emulsifying activity index (EAI) shows a pH and protein concentration dependent behaviour for CMP, while the emulsion stability did not show a significant sensitivity with respect to pH. Also a dependence on ionic strength was reported. The authors suggested the utilisation of CMP as an emulsifier in foods, which undergo large pH variations during processing.

These studies were performed using whole CMP, which naturally consists of a aglyco- and glyco-fraction. The biological and technological functionalities as well as its stability in the gastrointestinal tract are thought to be mainly influenced by the properties of the glycan side chains of the gCMP (Byrne, Donohoe, & O'Kennedy, 2007). From a physiological or biofunctionality point of view, the role of the glycosylated fraction in the design of food products is of particular interest, also because the presence of a glycan shield enables an efficient protection against degradation by proteases (Balzarini, 2007) and some peptidases (Boutrou et al., 2008). On the other side, the presence of charged glycosidic side chains may exert an impact on the properties and stability of protein-stabilised foods like emulsions. Such a structure-function relationship concerning the impact of glycosylation has not been investigated so far. The aim of this study is an approach to bridge this gap. At first, a comparison of the emulsifying properties of aCMP versus gCMP with other protein emulsifiers is given. Thereafter, detailed results of aCMP/gCMP as emulsifiers as a function of concentration, pH and ionic strength are presented. Finally, a hypothesis on the different behaviour of surface adsorption of the two fractions is presented.

2. Experimental

2.1. Materials

Native CMP fractions (aCMP and gCMP) were prepared by a membrane-adsorption chromatography process as described by Kreuß, Krause, and Kulozik (2008). The gCMP had a purity of 97.1% and the aCMP of 89.3%. Sodium caseinate (purity: 91.4%), β-lactoglobulin (purity: 95.1%) and whey protein isolate (purity: 92.3%) were from Davisco Foods Int. (Le Sueur, USA) while egg white protein with a purity of 88.0% was from G.C Hahn & Co. (Lübeck, Germany). Sunflower oil was purchased from a local supermarket.

Analytical grade purity sodium acetate (NaAc), sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH) and sodium dodecyl sulphate (SDS) were purchased from Sigma (Taufkirchen, Germany). All samples were prepared with bidistilled water.

2.2. Preparation of emulsions

Peptide solutions were prepared by dispersing aCMP/gCMP powder in 0.02 M NaAc buffer and stirring for at least 2 h to ensure full hydration. The pH (from 2.0 up to 10.0) was adjusted using HCl or NaOH while the ionic strength was kept constant at 10 mM for all pH values adding NaCl. To asses the effect of concentration, aCMP/gCMP concentration was varied between 0.25% and 5% (w/w) (standard concentration for pH and ionic strength experiments was 1% peptide). Ionic strength experiments were performed by adding from 50 up to 750 mM NaCl at a constant pH of 6.0 and 1% peptide concentration.

Oil-in-water model emulsions containing 10% sunflower oil (w/w) were blended with 90% peptide solution with an Ultra Turrax (IKA Werke, Staufen, Germany) for 120 s at a rotation speed of 9000 rpm and a shear gap width of 0.5 μm. These coarse emulsions were immediately fed through an APV 1000 high-pressure homogeniser from Gaulin GmbH (Germany) at a pressure of 500 bar and an inlet temperature of 20 °C.

2.3. Emulsifying activity index (EAI) and emulsion stability index (ESI)

The emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by the turbidometric technique as described by Pearce and Kinsella (1978). Emulsions for the EAI/ESI experiments were prepared with 25% sunflower oil (w/w), while the aqueous phase consisted of 0.5% dispersions (w/w) of aCMP/gCMP, sodium caseinate, whey protein isolate, β-lactoglobulin or egg white protein in a 0.1 M phosphate buffer at pH 8.0. After preparing the emulsions, an aliquot of 50 μl was dispersed in 5 ml of a 0.1% SDS solution. The absorbance of the diluted emulsions was then measured by a Lambda 25 spectrophotometer from Perkin Elmer (Waltham, USA) at 500 nm in 10 mm glass cuvettes.

Turbidity was calculated using the following formula:

$$T_b = 2.303 \cdot AF \cdot 1/L \tag{1}$$

where T_b: turbidity, A: absorbance at 500 nm, F: dilution factor and L: path length of cuvette in metres.

The EAI (m^2/g) was then defined as:

$$\mathrm{EAI} = 2T_b/(\Phi \cdot c) \tag{2}$$

where Φ: oil volume fraction of the emulsion, c: weight of protein per unit volume (g/ml) of the protein aqueous phase before emulsion formation.

The ESI (h) was calculated by the turbidity directly after preparing the emulsions and after a time of 10 min using the following formula:

$$\mathrm{ESI} = \frac{T_b(0)\Delta t}{[T_b(0) - T_b(10\ \mathrm{min})]} \tag{3}$$

2.4. Particle size distribution and flocculation measurement

All particle size measurements were carried out using a laser diffraction spectroscope, model LS230 from Beckman–Coulter (Krefeld, Germany). The sample to be measured was dispersed in deionised water (also used to rinse the machine between different samples). For the determination of the real particle size distribution, a 1 ml aliquot of the homogeneous emulsion was dispersed in an excess of 0.5% SDS solution (40 ml) in order to dissociate flocculated droplets. The median oil droplet size of the volume based distribution was used to characterise the size of the droplets in a sample ($d_{4,3}$ (+SDS)). Signals typically obtained were quasi-Gaussian with a slight tailing at the right side of the size distribution (larger particles).

Measuring without SDS was performed for the determination of the flocculation factor F. This allows the measuring of a size