LWT - Food Science and Technology 47 (2012) 407–412

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

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

The pH-dependent thermal and storage stability of glycosylated caseinomacropeptide

Nadja Siegert [a,*], Alexander Tolkach [b], Ulrich Kulozik [a]

[a] Technische Universität München, Chair for Food Process Engineering and Dairy Technology, Z I E L Research Center for Nutrition and Food Science, Weihenstephaner Berg 1, D-85354 Freising, Germany

[b] Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, 5 Sal, 1958 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 15 February 2011
Received in revised form
6 September 2011
Accepted 1 February 2012

Keywords:
Glycoprotein
Caseinomacropeptide
N-acetylneuraminic acid
Thermal treatment
Storage stability
Bovine milk

ABSTRACT

Bioactive properties of bovine glycosylated caseinomacropeptide (gCMP) like antibacterial effects are reported to be closely associated with their structure in terms of attached glycans. Technological properties are also influenced by the carbohydrate side chains. However, during product manufacturing gCMP can be modified due to processing. Processing conditions, which influence the degree of glycosylation of gCMP lead to alterations of bioactivity and techno-functional properties of gCMP and accordingly gCMP-containing products. Hence, gCMP was studied for its glycan stability during heat treatment and storage under different pH values. Process stability (preservation of native protein structure in terms of attached glycans) was analysed by quantifying the release of the terminal carbohydrate, N-acetylneuraminic acid (Neu5Ac), from gCMP. The results clearly showed that the thermal stability of gCMP is strongly influenced by pH. When the pH was decreased from 7 to 2, reduced stability was found even at low heating temperatures. Minimal destabilisation effects were found at neutral pH. Similar observations were found during storage of gCMP. Neu5Ac was released after six days of storage, with a maximum release of 30% at pH 2. Acidic pH conditions were responsible for the hydrolysis of the glycans from the peptide backbone during heat treatment and storage.

1. Introduction

Bovine caseinomacropeptide (CMP), the hydrophilic fragment *f*(106–169) of κ-casein released by the action of chymosin possesses interesting nutritional and technological properties such as antibacterial and antiadhesive effects as well as foaming and emulsifying properties (Janer, Pelaez, & Requena, 2004; Kawasaki et al., 1992; Kreuss, Krause, & Kulozik, 2009; Kreuss, Strixner, & Kulozik, 2009; Marshall, 1991). Therefore, it is a potential ingredient for food applications such as infant formula and/or as prebiotics in adult nutrition (Fox, 2001; Korhonen, 2002; Steijns, 2001). However, CMP shows a high degree of heterogeneity due to the genetic variations of κ-casein and mainly due to post-translational glycosylation, since all sites for O-glycosylation of κ-casein are found in the C-terminal domain *f*(106–169) (Holland, Deeth, & Alewood, 2005; Saito & Itoh, 1992). Glycosylation of CMP differs in the numbers of occupied glycosylation sites, and further due to variability in size and structural composition of each of those glycans, which may be composed of N-acetyl galactosamine (GalNAc), galactose (Gal) and N-acetylneuraminic acid (Neu5Ac) (Holland, Deeth, & Alewood, 2006; Kawasaki et al., 1992; Saito & Itoh, 1992).

Since the attached glycans affect physicochemical properties like the net charge, conformation, solubility, in addition to protecting the protein from proteolytic degradation (Kundra & Kornfeld, 1999; Parodi, 2000; Sinclair & Elliott, 2005; Sola & Griebenow, 2009), differences in functionality occur between the glycosylated (gCMP) and non-glycosylated (aCMP) fractions of CMP. While aCMP possesses stronger emulsifying activity and foaming properties (Kreuss, Krause et al., 2009; Kreuss, Strixner et al., 2009), biofunctional effects are reported to be strongly mediated by the attached carbohydrates in gCMP (Byrne, Donohoe, & O'Kennedy, 2007; Kawasaki et al., 1993, 1992; Lemeste et al., 1990). Pathogenic organisms and toxins bind to cell surface carbohydrates to gain access to the mucosal membrane (Arnold, Wormald, Sim, Rudd, & Dwek, 2007; Newburg, 1999; Varki, 1993). Oligosaccharide sequences on soluble glycoconjugates such as gCMP can serve as cognate receptors for the encounter of rotavirus (Varki, 1993), cholera toxin (Kawasaki et al., 1992) or *Escherichia coli* strains (Parkkinen, Finne, Achtman, Vaisanen, & Korhonen, 1983). Upon

* Corresponding author. Tel.: +49 8161 71 3534; fax: +49 8161 71 4384.
E-mail address: Nadja.Siegert@wzw.tum.de (N. Siegert).

0023-6438/$ – see front matter © 2012 Published by Elsevier Ltd.
doi:10.1016/j.lwt.2012.02.001

binding to these glycoconjugates microorganisms, parasites or toxins are swept out leaving the mucosal cell untouched (Wang & Brand-Miller, 2003). The monosaccharide Neu5Ac is highly involved in this activity due to its location at the terminus of an oligosaccharide chain (Byrne et al., 2007; Schauer, 2000; Wang & Brand-Miller, 2003). Based on its high negative charge, Neu5Ac furthermore affects the technological properties of glycoproteins to a great extent (Byrne et al., 2007; Schauer, 1982; Varki, 1993).

Functional properties of proteins or peptides such as CMP are widely utilised in the dairy industry and other foods (Fox, 2001; Korhonen, 2002; Walstra, 2003). However, CMP can be subjected to modifications during product manufacturing. On the on hand, this concerns processing steps, which are necessary during the isolation of CMP from bovine milk and during subsequent applications of CMP. It should be noted that bovine milk already undergoes several processing prior to CMP isolation. On the other hand, processing during the manufacture of dairy and other CMP-containing food products may alter it.

Thermal treatment is a fundamental operation, which is applied to improve food safety, sensory qualities and shelf life. Furthermore, it is well known that heat treatment of milk and milk protein solutions affect the functional properties of native proteins. Hence, milk proteins can be subjected to modification such as conformational changes due to denaturation, intermolecular reactions or Maillard reaction (Fenaille et al., 2006). In addition, unfavourable conditions are created by a low pH, promoting the hydrolysis of saccharides (Klewicki, 2007). As intrinsically disordered peptide without a secondary or tertiary structure, CMP is less vulnerable to denaturation. However, the cleavage of glycosidic linkages of gCMP, resulting to a loss of carbohydrate side chains, is likely to occur during processing. In that case gCMP would be altered, resulting in the acquisition of properties comparable to aCMP. This affects biological and technological properties, and therefore would also affect properties of food products containing gCMP.

However, the impact of manufacturing such as heat treatment and variations of environmental factors on the stability of gCMP is not yet well known. In order to preserve and utilize the original functionality of gCMP for nutritional purposes in consumer products, the native glycoprotein structure including the carbohydrate chains, is required (Abe et al., 1991; Arnold et al., 2007; Sola & Griebenow, 2009). Moreover, to manufacture consistent products, providing desired characteristics, it is essential to know the impact of manufacturing, process parameters and storage conditions on gCMP stability, especially when CMP would be deliberately used as biofunctional ingredient.

The aim of the present study was therefore to determine the effect of heat treatment on the stability of carbohydrate side chains of gCMP. Since it is desirable to have ingredients or foodstuffs that are stable over time, storage stability was additionally studied. Due to its important role within several biological functions and its terminal position within the glycoconjugate, Neu5Ac was used as an indicator molecule for the release of carbohydrates from gCMP during processing. Total and non-protein-bound Neu5Ac content of the samples was determined in order to quantify the stability of gCMP. The impact of heat treatment on the pH-dependent stability of gCMP was determined at conventional processing conditions applied to raw milk, and dependent on the heating time. The storage stability was studied with respect to the pH- and storage temperature dependence.

2. Materials and methods

2.1. Sample preparation

Caseinomacropeptide (CMP) was obtained from Arla Foods Ingredients, Viby J, Denmark (Lacprodan CGMP-10). Protein content is 857 mg g^{-1}, and content of major protein fractions are as follows: aCMP 369 mg g^{-1}, gCMP 454 mg g^{-1}, α-Lactalbumin 60 mg g^{-1}, β-Lactoglobulin 117 mg g^{-1} (Kreuss & Kulozik, 2009). CMP solutions of 10 mg mL^{-1} were prepared by suspending CMP-powder in double distilled water and solutions were stored at 4 °C over night prior to usage. The pH value of the solution was adjusted to 2, 3, 4, 5, 6, 7 and 9 with NaOH and/or HCl (0.1 and/or 1 mol L^{-1}).

2.2. Heat treatment

The solutions were filled in stainless steel tubes (internal diameter: 4.0 mm; length: 260 mm; capacity: 3.3 mL; wall thickness 1 mm) and heated at conventional conditions applied to raw milk (Table 1). Heating times varied between 2 and 1200 s and heating temperatures between 65 and 135 °C. The impact of heating time was studied at 120 °C and heating times between 15 and 120 s. For heating temperatures below 100 °C a water bath (WB 22, Memmert GmbH + Co. KG, Schwabach, Germany) was used. For heating temperatures above 100 °C a pressure vessel with steam as described by Spiegel (1999) was used. Temperature was measured in a reference tube located in the middle of the vessel, which was connected with the vessel lid. The beginning of holding time was defined as the moment, when the temperature of the reference tubes reached the desired temperature. After the holding time had elapsed, the samples were immediately cooled in cold water to ambient temperatures (20 °C). Each determination was repeated in triple.

2.3. Storage

To exclude microbial growth 0.2 mg mL^{-1} sodium azide (Sigma–Aldrich, C-No. S2002-25G) was added to the solutions. Solutions were stored at 4, 10 and 20 °C for 12 days in closed plastic cups.

2.4. Determination of N-acetylneuraminic acid

The thermal and storage stability of gCMP solutions was measured by quantitatively assessing the release of Neu5Ac. Released Neu5Ac was measured in 5 kDa permeate (see Filtration) of the samples.

2.4.1. Filtration

To distinguish between protein-bound and non-protein-bound Neu5Ac, all samples were ultrafiltrated (UF) prior to hydrolysis. Centrifugal filters (Amicon Ultra 5 kDa nominal molecular weight cut-off, NMWCO, Millipore, Carrigtwohill, Ireland) were used for UF of the samples under the following centrifugation conditions: 2000 *g*, 10 min at 20 °C (Heraeus Multifuge 1 S-R, Berlin, Germany). Free Neu5Ac, Neu5ac released from gCMP during processing or Neu5Ac bound to other saccharides were expected to be in the UF-permeate. Neu5Ac content was analysed from both the total sample (total Neu5Ac amount) prior to UF and from the UF-permeate (non-protein-bound Neu5Ac amount).

Table 1
Heat treatment conditions applied to CMP solutions.

Heat treatment	Temperature/time
Untreated	–
Low-temperature long-time (LTLT)	65 °C/30 min
High-temperature short-time I (HTST I)	72 °C/30 s
High-temperature short-time II (HTST II)	85 °C/4 s
High pasteurisation (ESL)	127 °C/2 s
Ultra-high temperature (UHT)	135 °C/5 s
Sterilisation (S)	110 °C/20 min