International Dairy Journal 53 (2016) 51–59

Contents lists available at ScienceDirect

International Dairy Journal

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

Proteomic characterisation of heat-induced hydrolysis of sodium caseinate

Brian A. McGrath [a, b], Michael Kinsella [a, c], Thom Huppertz [d], Paul L.H. McSweeney [a], Alan L. Kelly [a, b, *]

[a] School of Food and Nutritional Sciences, University College Cork, Ireland
[b] Food for Health Ireland, University College Cork, Ireland
[c] Pharmaceutical and Molecular Biotechnology Research Centre, Waterford Institute of Technology, Ireland
[d] NIZO food research, Ede, The Netherlands

ARTICLE INFO

Article history:
Received 16 June 2015
Received in revised form
27 September 2015
Accepted 27 September 2015
Available online 26 October 2015

ABSTRACT

The hydrolysis of sodium caseinate during heating for up to 120 min at pH 7.0 and 130 °C was investigated. The formation of 2% trichloroacetic acid (TCA)-soluble peptides was studied using spectrofluorimetry and high resolution liquid chromatography-mass spectrometry (LC-MS), whereas sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to study hydrolysis of the caseins. LC-MS and spectrofluorimetry showed an increase in 2% TCA-soluble peptides over time, confirming that proteolysis had occurred, whereas SDS-PAGE showed a decrease in band intensity for the main caseins over time. In total, 1023 casein-derived peptides were identified. For eleven of the most abundant peptides, release and breakdown were modelled. Peptide bonds containing Pro, Ser, Asn and/or Asp were preferentially hydrolysed during heating. The production of peptides in this manner may represent a novel alternative to enzymatic hydrolysis strategies.

1. Introduction

Heat treatment of milk and dairy products is commonly used in the dairy industry, ranging from thermisation (65 °C for 15 s) to sterilisation (120 °C for 10–20 min) or ultra-high temperature (UHT) treatment (typically 135–145 °C for several seconds). The effect of heat on the milk protein system is an important consideration as it can affect both the functional properties and nutritional quality of milk. The protein system in milk is very heat-stable and can withstand heating at 140 °C for 15–20 min at pH 6.7 (McCrae & Muir, 1995; O'Connell & Fox, 2003; Singh & Creamer, 1992).

In milk, whey proteins are the proteins most susceptible to denaturation and aggregation, and retain their conformation only within limited temperature ranges (Anema, 2009). On the other hand, casein is a remarkably heat-stable protein system; sodium caseinate can be heated at pH 6.7 at 140 °C for at least 40 min before coagulation occurs (Fox & Hoynes, 1975). This high heat stability is due largely to the lack of secondary and tertiary structures of caseins.

However, under certain combinations of temperature and pH, the colloidal stability of the casein micelles may be lost, as evident by visible flocculation, gelation or protein aggregation (O'Connell & Fox, 2003). High heat treatment can lead to an increase in casein micelle size (McMahon, 1996; Mohammad & Fox, 1987), intermolecular cross-linking (Singh, 1995) and dephosphorylation of the caseins, subsequently disrupting the native micelle structure, which is largely held together by calcium phosphate links (Dalgleish, Pouliot, & Paquin, 1987; Singh, 2004). Calcium phosphate present in the serum phase of milk becomes less soluble during heat treatment (Holt, 1995) and it is thought that formation of insoluble calcium phosphate and its deposition on casein micelles is largely responsible for the instability of casein micelles in UHT-treated milks (Dalgleish, 1992; Wahlgren, Dejmek, & Drakenberg, 1990). Guo, Flynn, and Fox (1999), who studied the effect of heat on the functional properties of sodium caseinate, found that, with heating, solubility increased and viscosity decreased, as did its foaming and emulsifying capacity, while the foam stability increased.

* Corresponding author. Tel.: +353 21 4903405.
E-mail address: a.kelly@ucc.ie (A.L. Kelly).

http://dx.doi.org/10.1016/j.idairyj.2015.09.006
0958-6946/

Severe heat treatment of milk has also been shown to cause proteolysis, but little detailed study has been carried out in this area. White and Davies (1966) showed that 10–20% of the total protein nitrogen in milk is converted to non-protein nitrogen (NPN) when heated at 135 °C for 60 min. Similarly, Saidi and Wrathesen (1993) found an 18% increase in the NPN content of milk following typical sterilisation conditions (120 °C for 20 min). Hustinx, Singh, and Fox (1997) found a linear increase in pH 4.6- and 2% trichloroacetic acid (TCA)-soluble peptides on heating a sodium caseinate solution at 140 °C and pH 7.0 for 100 min; fifteen of the pH 4.6-soluble peptides were isolated and identified. Gaucheron, Molle, Briard, and Le (1999) related an increase in the NPN content of casein micelle suspensions in a salt solution, in milk ultrafiltrate and in milk during sterilisation (120 °C for 10, 20 or 30 min) to an increase in proteolysis, and subsequently characterised ten peptides. In a later study, Gaucheron, Mollé, and Pannetiter (2001) studied the effect of pH on the proteolysis of sodium caseinate induced by heating at 120 °C and observed an increase in soluble nitrogen content with increasing pH (6.0, 7.0, 8.0, 9.0); eighteen low molecular mass peptides found in the soluble nitrogen fraction were subsequently characterised. Van Boekel (1999) studied the kinetics of deamidation, dephosphorylation and protein breakdown in heated sodium caseinate solutions; the extent of deamidation was related to the level of amide present in asparagine residues, while protein breakdown and dephosphorylation increased with increasing heating temperature and time.

The aim of the present study was to apply advanced proteomic techniques to the identification of the peptides produced during heat treatment of sodium caseinate at 130 °C over a 120 min period, with the aim of identifying the most heat-labile bonds in the caseins, and provide a far more detailed understanding of this phenomenon than previously known. Sodium caseinate was chosen to avoid numerous other changes that occur on heating the more complex milk system.

2. Materials and methods

2.1. Sodium caseinate preparation

Sodium caseinate (90.4%, w/w, protein), provided by Kerry Ingredients (Listowel, Ireland), was suspended in distilled water at a level of 2.5% (w/v) and allowed to hydrate overnight at 4 °C. Following hydration, the solution was adjusted to pH 7.0 using 1 N NaOH and dialysed for three 24 h periods against fifty volumes of distilled water using a 3.5 kDa dialysis membrane (Medicell International, London, UK), to remove any associated low molecular mass peptide material. Following dialysis, the pH was re-adjusted to pH 7.0 using 1 N NaOH.

2.2. Heat treatment

Aliquots of sodium caseinate (3 mL) were placed in glass tubes (length, 150 mm; diameter, 15 mm; wall thickness, 2 mm), sealed with silicone rubber stoppers. Tubes were heated while oscillating in an oil bath at 130 °C for up to 120 min. A 2 min equilibration time was also allowed. Following heating, the tubes were immediately cooled in ice water at 4 °C.

2.3. Preparation of the 2% trichloroacetic acid soluble fraction

The 2% TCA-soluble fraction of sodium caseinate was prepared by adding 166 μL 12% TCA to 833 μL sodium caseinate solution and vortexing the mixture; after 10 min, the mixture was centrifuged for 30 min at 18,000× *g* at 20 °C. After centrifugation, the supernatant was carefully removed and used for further analysis.

2.4. Spectrofluorimetric measurement

The relative fluorescence intensity of the 2% TCA-soluble fractions of sodium caseinate heated at 130 °C for 0–120 min was measured at 20 °C using a luminescence spectrometer (Varioskan Flash, Thermo Scientific, Waltham, MA, USA) at excitation and emission wavelengths of 280 and 340 nm, respectively, with slit widths of 5 nm. Under these conditions of excitation and emission, fluorescence measurement was used to evaluate the presence of peptides containing tyrosine and/or tryptophan in the 2% TCA-soluble fractions.

2.5. Reversed-phase ultra-performance liquid chromatography

The 2% TCA-soluble fractions were filtered through 0.22 μm pore filters (Millex, low protein binding durapore, syringe driven PVDF membrane; Millipore Ireland Ltd, Carrigtwohill, Ireland). The filtrate was analysed at room temperature (21 °C) by reversed-phase ultra-performance liquid chromatography (RP-UPLC) using a Waters Acquity UPLC H-Class Core System (Waters, Milford, MA, USA). The column used was an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm). The sample was eluted for 0.5 min with 100% solvent A [0.1% formic acid (sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Ireland) in deionised water (Milli Q System; Millipore Corp)], then with a linear gradient to 30% solvent B [0.1% formic acid in acetonitrile (HPLC far UV grade; Labscan Ltd, Dublin, Ireland)] over 9.5 min, followed by a linear gradient to 50% B over 2 min, and a linear gradient to 85% B over 0.5 min, followed by elution at 85% B for 0.5 min and finally with 0% B over 1.1 min. Eluate was monitored at 214 nm using a Waters Acquity UPLC TUV Detector (dual wavelength; Waters) interfaced with Empower 3 software. The flow rate was maintained at 0.46 mL min^{-1}.

2.6. Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to the method of Laemmli (1970) using a 15% (w/v) acrylamide separating gel and a 3% (w/v) stacking gel, both containing 0.1% (w/v) SDS. Sodium caseinate samples were prepared in 62.5 mM Tris–HCl buffer (pH 6.8) containing 2% (w/w) SDS, 5% (w/w) mercaptoethanol and 0.012% (w/w) bromophenol blue and heated at 95 °C for 4 min. Thirty micrograms of protein/peptide material was then loaded into each gel slot. Electrophoresis was carried out at a constant voltage (200 V) for 8 h using a Tris-glycine buffer (pH 8.3) containing 0.125% (w/w) SDS. The gel was removed from the electrophoresis unit, fixed in methanol: acetic acid: water (50:10:40, by vol) and stained with Coomassie Brilliant Blue R-250 to visualise the proteins. Images of gels were captured using the Epson Perfection 4180 Scanner (Epson, Suwa, Nagano, Japan) with Total Lab Quant software (Newcastle, UK); image resolution was set at 600 dots per inch.

2.7. Mass spectrometry

TCA-soluble peptides were identified using High Resolution Waters Xevo G2 Q-TOF liquid chromatography-mass spectrometry (LC-MS). This system consisted of a Waters Acquity UPLC system coupled to a quadrupole time-of-flight mass spectrometer. A sample (6.9 μL) was injected onto a Waters Acquity UPLC BEH C18