Food Research International 51 (2013) 899–906

Contents lists available at SciVerse ScienceDirect

Food Research International

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

Structural consequences of dry heating on alpha-lactalbumin and beta-lactoglobulin at pH 6.5

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ARTICLE INFO

Article history:
Received 12 December 2012
Accepted 9 February 2013

Keywords:
Alpha-lactalbumin
Dry heating
Dehydration
Pyroglutamic acid
Protein cross-linking

ABSTRACT

In the present work, we investigated the structural modifications occurring during the dry heating of model whey proteins, β-lactoglobulin and α-lactalbumin. Samples were adjusted to pH 6.5, water activity $a_w = 0.23$ and dry heated at 100 °C for up to 24 h, and the structural modifications followed by gel permeation chromatography, reverse phase-HPLC, SDS PAGE and mass spectrometry (LC–MS/MS). The dry heating treatment traps a fraction of the proteins into covalently linked soluble aggregates. Moreover, a high proportion of non-aggregated α-lactalbumin (about 73%) was converted into non-native forms. The characteristic of those non-native species was the loss of one or two water molecules per α-lactalbumin molecules. Using tandem mass spectrometric peptide mapping, these chemical modifications were found to be attributed to (i) the formation of a pyroglutamic acid from the N-terminal glutamic acid and (ii) the formation of an internal cyclic imide at position Asp_{64}. The non-native species were not favored in the case of β-lactoglobulin as they represented less than 18% of non-aggregated proteins.

1. Introduction

Proteins are widely used as ingredients in food products due to their ability to generate large diversity of textures (e.g. gels, foams, emulsions). Modification of protein structures strongly affects this ability and strategies have been developed to identify safe conditions for generating protein structures with improved functional properties. Dry heating is used in food industry as a mean to modify protein structures leading to improved functional properties (Aoki et al., 1997; Ibrahim, Kobayashi, & Kato, 1993; Kato, Ibrahim, Watanabe, Honma, & Kobayashi, 1989, 1990; Matsudomi, Ishimura, & Kato, 1991). During dry heating, only minor modifications in secondary structures of proteins and a slight increase of accessibility of internal amino acids are observed. In addition dry-heated proteins usually form small soluble aggregates in which proteins are linked together by intermolecular disulfide bonds and also other covalent bonds (Ibrahim et al., 1993; Matsudomi, Takahashi, & Miyata, 2001; Mine, 1996, 1997). It was hypothesized that improved functional properties can be usually obtained for dry heating conditions leading to aggregates with specific size, structure and reactivity (Kato et al., 1990; Matsudomi et al., 2001; Mine, 1996). However, improved foaming properties have been reported for the dry-heated lysozyme despite the absence of protein aggregation, which in turn may question the contribution of the aggregated forms (Desfougeres, Lechevalier, Pezennec, Artzner, & Nau, 2008). The racemization of amino acids, the formation of internal cyclic imide, the deamidation of amino acid side chains were all identified as putative structural changes that can explain the enhanced functional properties (Desfougeres, Jardin, Lechevalier, Pezennec, & Nau, 2010; Matsudomi et al., 2001; Mine, 1997). Dry-heated proteins are less stable (Ibrahim et al., 1993; Desfougeres et al., 2010), which can explain their higher ability to form textures than corresponding native proteins. These structural modifications were mainly observed for egg white proteins dry-heated either under acidic (pH 3.5) or alkaline (pH 8–9) conditions.

Even if dry heating was shown to modify the functional properties of individual whey proteins, beta-lactoglobulin (β-Lg) and alpha-lactalbumin (α-La) (Ibrahim et al., 1993), dry heating of whey proteins under natural pH conditions, i.e. close to neutral pH, was only slightly studied. Particularly, the molecular basis of the modified functional behavior has not been specifically investigated in that work. More recently, we showed that the gelation property of dry-heated commercial whey protein isolate (WPI) is correlated to the presence of specific type of aggregates (Gulzar, Lechevalier, Bouhallab, & Croguennec, 2012). However, the complexity of the system (mixture of β-Lg + α-La + traces of other proteins) makes a detailed analysis of the structural changes on individual proteins difficult. Our research hypothesis is that understanding the structural and chemical changes that occur during dry heating is the basis for better controlling the functional properties and the reproducibility of dry-heated proteins. In the present study, we focused on the structural modifications that occur during

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http://dx.doi.org/10.1016/j.foodres.2013.02.025

the dry heating of the two main whey proteins, β-Lg and α-La. β-Lg is a protein of about 18.3 kDa organized mainly in β-sheets. Its structure contains two disulfide bonds and one free sulfhydryl group inaccessible to solvent under physiological conditions. α-La has a predominant α-helices structure. It contains 8 cysteine residues, all engaged in disulfide bonds that stabilize the tertiary structure of the protein. Changes in whey protein structure during the dry heating have been investigated at different levels: (i) protein aggregation state, (ii) modifications in secondary and tertiary structures but also (iii) chemical modifications in the primary sequence of the protein. Mass spectrometry (MS and MS/MS) has been used for tracking subtle chemical modifications in the structure of proteins.

2. Materials and methods

2.1. Materials

β-Lg and α-La were obtained from a confidential industrial source. β-Lg powder contained 93% protein (determined by Kjeldahl method) of which 90% of β-Lg (determined by SDS-PAGE), 4.3 ± 0.2% moisture and 0.031 ± 0.002% calcium, 0.757 ± 0.038% sodium, 0.014 ± 0.001% potassium, 0.001% magnesium, 0.066 ± 0.003% phosphate, 0.019 ± 0.001% citrate. α-La powder contained 94% protein (determined by Kjeldahl method) of which 82% of α-La (determined by SDS-PAGE), 3.8% moisture and 0.067 ± 0.011% calcium, 0.95% sodium, 0.045% potassium, 0.002 ± 0.001% magnesium. No lactose was detected in β-Lg and α-La powders. Glycine was from Acros Organics (Geel, Belgium) and all other chemicals were from Sigma Aldrich (Saint-Quentin-Fallavier, France).

2.2. Preparation of powders and dry heating treatment

β-Lg and α-La powders were dissolved in distilled water at a protein concentration of 15% and the protein solutions were adjusted to pH 6.5 prior to freeze-drying. The freeze-dried samples containing 10 g of proteins were placed for 2 weeks in a desiccator containing saturated salt (CH_3CO_2K) solution to reach an a_w of 0.23. The a_w of the freeze-dried protein powders was checked by a_w meter (Novasina RTD 200/0 and RTD 33, Pfäffikon, Switzerland). Values of pH and a_w were chosen close to those found in the commercial spray dried powders. Freeze-dried protein powders (a_w 0.23) were heated at 100 °C for 8, 16 and 24 h in hermetically sealed bottles. A sample was kept unheated (reference sample). Each sample was prepared in duplicate.

2.3. Samples preparation

2.3.1. Total protein

Powders (unheated and dry-heated) were reconstituted at 10 g/L in 0.12 M NaCl solution. The pH was adjusted to 7 by addition of 1 N NaOH.

2.3.2. Non-aggregated proteins

A volume of 200 μL of 0.5 M acetic acid/sodium acetate buffer (pH 4.5 or pH 4.7 for α-La and β-Lg respectively) was added to 1000 μL of total protein samples (Section 2.3.1). The mixture was placed in a water bath equilibrated at 30 °C for 1 min for precipitating aggregated proteins. The mixture was then centrifuged at 10,000 *g* for 15 min using an Eppendorf 5415C Micro Centrifuge (Scientific Support, Hayward, California). The supernatant containing non-aggregated proteins was recovered.

2.3.3. Protein hydrolysis

For tryptic digestion, a volume of 10 μL of 100 mM dithiothreitol (DTT [Pharmacia Biotech, France] in 50 mM Tris–HCl buffer pH 8.5 containing 6 M urea) was added to 1 mL of β-lg and α-La samples (unheated and dry-heated) prepared at 1 g/L in a 50 mM Tris–HCl buffer pH 8.5 containing 6 M urea. The reduction was carried out at 56 °C for 1 h. After reduction, 40 μL of iodoacetamide (IAA) at 2 M was added to the solution and the mixture was incubated at room temperature in the dark. The solutions were dialyzed overnight against a 10 mM sodium bicarbonate buffer at pH 8.5 to remove excess reagents. A stock solution of 1 g/L trypsin was added to the dialyzed sample to have an enzyme/protein weight ratio of 1:100. The solutions were hydrolyzed at 37 °C for 2 h. The hydrolysis was stopped by decreasing the pH below 3 with trifluoroacetic acid (TFA).

For peptic digestion, α-La (unheated and dry-heated) was diluted at 4 g/L in a 20 mM phosphate buffer containing 8 M urea (pH 4.5) and 4.2 mM tris(2-carboxyethyl)phosphine (TCEP) for reduction, which is an efficient reducing agent under acidic pH condition. After 1 h at room temperature, the solution was diluted 1/4 in a 100 mM HCl/KCl buffer pH 1.0. α-La proteolysis was triggered by adding 50 μL of pepsin (170 μM in a 100 mM HCl/KCl buffer pH 1.0) to 200 μL of protein solution. After 5 h a second addition of 50 μL pepsin was performed to complete the proteolysis.

2.4. Samples analysis

2.4.1. Gel permeation chromatography

Proteins were analyzed by High Pressure-Gel Permeation Chromatography (HP-GPC) using a TSK G3000 SWXL (300 × 7.8 mm i.d.) column (Phenomenex, Le Pecq, France) connected to a Waters chromatography system (Milford, USA), consisting of a Waters 2695 Separation Module, a Waters 2487 Dual λ Absorbance Detector and a Empower chromatography application software to acquire, process and report chromatographic information. A phosphate buffer 0.05 M, pH 7 containing 0.1 M NaCl was used to equilibrate the column and to elute the proteins at a flow rate of 0.8 mL/min. Proteins were detected at 214 nm. The percentage of non-aggregated proteins in the sample was calculated from the ratio between the chromatographic peak area of non-aggregated proteins and total chromatographic peak area; the percentage of aggregated proteins was calculated by difference.

2.4.2. Reverse phase chromatography

Proteins were analyzed by reverse phase-HPLC on a Vydac C4 connected to the HPLC system described above (Section 2.4.1). The column was equilibrated with 59% of solvent A (milli-Q water containing 1.06‰ (v/v) trifluoroacetic acid) and 41% solvent B (HPLC grade-acetonitrile:Milli-Q water (80:20, v/v) mixture containing 1‰ (v/v) trifluoroacetic acid). A linear gradient started with 41% of solvent B to reach 61% over 24 min was used for protein elution. Protein separation was carried out at 40 °C at a flow rate of 0.2 mL/min. Proteins were detected at 214 nm.

2.4.3. SDS-PAGE analysis

SDS-PAGE was performed under reducing (with DTT) and non-reducing conditions (without DTT) using a Mini Protean II system (Bio-Rad Laboratories, A Technologies, Dublin, Ireland) as described by Laemmli (1970) using 15% acrylamide separating gel and 4% concentration gel. Protein samples were diluted 10 folds with the denaturing buffer (77.975% 0.08 M Tris–HCl pH 6.8; 20% glycerol; 2% SDS; 0.025% bromophenol blue). 10 μg of proteins was loaded in the sample slots and was separated at 75 V for 30 min and then at 150 V for 60 min. Gels were stained with Coomassie Brilliant Blue G250. A low molecular weight marker kit (14.4–94 kg/mol, Amersham Biosciences, France) was used for molecular weight (MW) calibration. The gels were scanned by Image Scan II (Amersham, Bioscience). The percentage of β-Lg and α-La monomers in the samples was quantified from the SDS-PAGE performed in the absence of DTT using densitometry software, Image Quant TL 1D (Amersham, Bioscience).

2.4.4. Mass spectrometry

Mass spectrometry analyses were carried out on a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer QStar XL (MDS Sciex,