International Dairy Journal 47 (2015) 86–93

Contents lists available at ScienceDirect

International Dairy Journal

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

Characterisation of a whey protein hydrolysate as antioxidant

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ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form
25 February 2015
Accepted 26 February 2015
Available online 20 March 2015

ABSTRACT

A whey protein hydrolysate was fractionated by size-exclusion chromatography to investigate the effectiveness of individual peptide fractions as (i) deactivators of hypervalent haeme iron, (ii) as radical scavengers, and (iii) as iron chelators. The reduction of ferrylmyoglobin, MbFe(IV)=O, to metmyoglobin, MbFe(III), by the peptide fractions was found to follow first-order kinetics for excess peptide. The fractions with the highest rate of reduction of ferrylmyoglobin also exhibited most efficient radical scavenging as determined for the stable Fremy's salt radical. Inactivation of catalytic free ion by peptide fractions, as examined by their ability to inhibit formation of the ferrozine-Fe^{2+} complex, showed highest inhibition for the fractions with least scavenging capacity of the Fremy's salt radical. Radical scavenging of 1-hydroxylethyl radicals generated in Fenton reactions showed antioxidant activity for low peptide concentration, but prooxidative activity for increasing concentration. Specific whey peptide fractions, may be combined as a food additive to optimise antioxidative activity.

1. Introduction

Whey is an abundant side-stream of the dairy industry containing valuable proteins such as β-lactoglobulin and α-lactalbumin. Enzymatic hydrolysis of whey proteins, which requires only mild conditions, is generally used in the production of whey protein hydrolysates (WPHs) with excellent nutritional quality (Foegeding, Davis, Doucet, & McGuffey, 2002; Marshall, 2004). WPHs are often part of protein based formulas for infants intolerant to bovine milk proteins as well as to beverages due to their health benefits and good digestibility (Sinha, Radha, Prakash, & Kaul, 2007). Antioxidant activity of whey components is an important biological value of whey proteins and should also be considered when selecting ingredients for processed foods (Allen & Wrieden, 1982a, b; Ostdal, Daneshvar, & Skibsted, 1996). Milk proteins and peptides are known to provide antioxidative protection of lipids and proteins in food and beverages (Elias, Kellerby, & Decker, 2008; Pena-Ramos & Xiong, 2001; Peng, Xiong, & Kong, 2009; Pihlanto, 2006; Xiong, 2010).

The oxidative decomposition of unsaturated fatty acids and proteins, and subsequent chain reactions occurring in food products during processing and storage, are some of the main deterioration processes affecting food quality. Therefore, delay of these oxidative processes through the use of antioxidants, especially from natural sources, is of great interest in food industry. Generally, the ability of protein hydrolysates to inhibit lipid oxidation is related to amino acid composition and their sequence in individual peptide fractions and to the size of the peptides and their conformation (Pena-Ramos & Xiong, 2001; Pena-Ramos, Xiong, & Arteaga, 2004; Pihlanto, 2006). However, antioxidant mechanisms of whey also include chelation of transition metals by serum albumin (Meucci, Mordente, & Martorana, 1991) and by lactoferrin (Gutteridge, Paterson, Segal, & Halliwell, 1981). Several amino acids, such as cysteine, tyrosine, methionine, histidine, lysine, tryptophan and prolin have been shown to hold antioxidant activity as radical scavengers, although under certain conditions they may also possess prooxidant activity (Jung, Kim, & Kim, 1995). Functional ingredients, such as WPHs seem to have great potential as effective antioxidants (Gad et al., 2011; Peng et al., 2009). Therefore, the inclusion of relatively high amounts of WPH in products has the potential to enhance the product stability by preventing radical formation and oxidative deterioration during digestion.

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http://dx.doi.org/10.1016/j.idairyj.2015.02.012
0958-6946/

Addition of WPH to, e.g., meat products should, accordingly, be explored for preventing iron induced formation of free radicals during meat digestion especially for salted and cured meat products. It has been shown that WPH applied to cooked pork patties suppressed lipid oxidation (Pena-Ramos & Xiong, 2003), but it still remains to be shown that this depends on deactivation of iron species. Both β-lactoglobulin and α-lactalbumin may be good sources of hydrolysates with antioxidative activity, which should be explored for production of safer food products (Hernandez-Ledesma, Amigo, Recio, & Bartolome, 2007; Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005). In general, use of WPH with good nutritional and biological properties hold great potential in health-promoting foods (Athira et al., 2015). A better understanding of the mechanism by which WPHs inhibit lipid and protein oxidation can contribute to a better understanding of WPHs as beneficial food ingredients.

In the present study we screened a number of commercial whey hydrolysates; the WPH that showed the highest activity in reducing ferrylmyoglobin was investigated further to uncover the antioxidative potential and mechanism of the product. WPHs are complex mixtures of different products of protein hydrolysis, and to examine potential antioxidant activity, the selected WPH was fractionated by size exclusion chromatography (SEC) to separate it into peptide fractions. Identification of the potentially antioxidant peptides within the WPH fractions was performed by liquid chromatography coupled with mass spectrometry (LC-MS). Furthermore, mechanisms for their scavenging of free radicals and binding of ferrous iron were investigated in model systems. Among different methods, antioxidant activity of the peptide fractions was evaluated by studying reduction of the prooxidative ferrylmyoglobin, MbFe(IV)=O, ferrous ion chelating capacity, and capability to scavenge 1-hydroxylethyl radicals as generated in the Fenton reaction in presence of ethanol. Antioxidant capacity of the peptide fractions was examined by the reduction of the stable radical, Fremy's salt, in a quantitative assay.

2. Materials and methods

2.1. Materials

Iron(II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), hydrogen peroxide (30%, v/v), ethylenediamine-tetraacetic acid disodium salt dihydrate were from Merck (Darmstadt, Germany). Metmyoglobin, MbFe(III), from horse heart (purity ≥ 90%) and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine monosodium salt (Ferrozine) were from Sigma (St. Louis, MO, USA). α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN), potassium nitrosodisulphonate (Fremy's salt), acetonitrile, β-lactoglobulin, and trifluoroacetic acid (TFA) were from Sigma–Aldrich (Steinheim, Germany). Ethanol (96%, v/v) was from Kemetyl A/S (Køge, Denmark). Peptigen IF-3080, Lacprodan α-10, Lacprodan DI-8095, Lacprodan DI-8090, Lacprodan CGMP-10, Lacprodan DI-9213, and Lacprodan MFGM-10 were the products of Arla Foods Ingredients (Viby J., Denmark). Water was purified using a Millipore Milli-Q purification system (Milli-Q Plus, Millipore Corporation, Bedford, MA, USA).

2.2. Fractionation of WPH by size exclusion chromatography

The selected WPH, Peptigen IF-3080, was fractionated by size exclusion chromatography using an FPLC system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) mounted with a column packed with Superdex™ 30 prep grade gel filtration resin (2.6 × 61 cm; Amersham Biosciences, Hillerød, Denmark). Five millilitres of a hydrolysate solution (50 mg L^{-1}) were injected and eluted with 30% (v/v) acetonitrile and 0.1% (v/v) TFA, at a flow rate of 2.5 mL min^{-1}. The eluted compounds were monitored at 214 nm and fractions of 10 mL were collected and subsequently pooled according to the elution profile and frozen until analysis. The peptide concentration in the fractions was determined spectrophotometrically at 280 nm using a β-lactoglobulin standard curve.

2.3. Peptide profiles

The peptide contents of the selected WPH were analysed using a uHPLC-Q-TOF system Agilent 1290 with a Agilent 6530 mass detector (Agilent Technologies, Palo Alto, CA, USA). The uHPLC system consisted of an autosampler with cooling (4 °C), a binary pump system, a column heater (45 °C) and a UV detector at 214 nm. The C18 column used was a XBridge™ BEH300 3.5 μm (2.1 × 250 mm) (Waters Corporation, Milford, MA, USA).

Elution was performed under the following conditions: injection volume of 5–20 μL, flow rate of 0.3 mL min^{-1}, mobile phase A: 0.05% trifluoroacetic acid (v/v) (TFA) (Fluka, St. Louis, MO, US) in ultra-pure 18 mΩ water (Evoqua Water Technologies, Alpharetta, GA, US), mobile phase B: 0.1% TFA (v/v) in acetonitrile (Rathbrun RH1015, Rathburn Chemicals Ltd, Walkerburn, UK). The samples were dissolved in ultra-pure water and eluted with a linear gradient from 0% B to 40% over 67 min; a UV absorption trace at 214 nm was recorded.

The liquid chromatography stream was going into the mass detector (QTOF) from 3 to 70 min at the rate of 0.3 mL min^{-1}. The QTOF measured 4 GHz and only in positive mode. The Jetstream interface (JSI) had a setting of 10 L min^{-1} in sheath gas flow at 300 °C. Drying gas was 325 °C, with a flow of 10 L min^{-1}. Voltage at capillary (VCap) was set to 2500 V, and fragmentor energy at 100 V. The instrument did auto MS/MS with fixed collision energies of 10, 20, 30, 50 and 75 with nitrogen as the collision gas. Three reference ions were calibrated during the analysis. It was chosen to use 2 precursors per cycle and an active exclusion after 3 spectra and/or 0.6 min. The precursor was made with a preference of singly, doubly and triply charged ions with an isolation width is ~1.3 amu.

All data were obtained by the Mass Hunter software LC/MS Data Acquisition Version B.05.01 build 5.01.5125 (Agilent Technologies, Santa Clara, CA, USA). All the data analysis were performed by MassHunter Qualitative B.06.00 with BioConfirm.

The interpretation of the MS/MS data was performed by MassAI software version 1.08 (MassAI Bioinformatics, Stenstrup, Denmark). Furthermore, the FASTA format from the whey proteins such as α-lactalbumin, β-lactoglobulin genetic variant A and B as well as from the α_S-casein, β-casein, and κ-casein was used for the interpretation. The mgf file from MassHunter was combined using a software tool in MassAI, the tolerance was set to 0.1 *m/z* and 30 s in retention time. The combined mgf files were investigated for the most abundant milk proteins using the Fasta format. The MS data tolerance was 10 ppm and MS2 tolerance was 0.1 *m/z*. The search was made without any use of modifications.

2.4. Reduction of Fremy's salt radical

The antioxidant capacity of the peptide fractions was evaluated by their ability to reduce the stable Fremy's salt radical as measured by electron spin resonance (ESR) spectroscopy. The peptide fractions were diluted in 5 mL of 25% ethanol, and 1.5 mL of the diluted solutions were mixed with 100 μL of Fremy's salt (160 μmol L^{-1}) dissolved in a 25% saturated sodium carbonate solution. The concentration of the Fremy's salt solution was adjusted based on spectrophotometric measurements at 270 nm. The mixture was left for 5 min before the ESR spectrum was recorded on a Jeol JES-FR30 ESR spectrometer (Jeol Ltd, Tokyo, Japan). The measurements were carried out at room temperature and the instrument settings were: