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The molecular characterisation and antimicrobial properties of amidated bovine β-lactoglobulin

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Abstract

Amidation of bovine β-lactoglobulin (β-Lg) imparted antimicrobial properties to this protein. Amidated β-Lg was strongly bactericidal against resting cells of *Pseudomonas fluorescens*, *Pseudomonas fragi* and *Bacillus subtilis*, but had a much weaker effect against *Escherichia coli*, *Enterococcus faecalis*, *Salmonella typhimurium* and *Listeria monocytogenes*. Neither native nor amidated β-Lg was effective against the yeast *Saccharomyces cerevisiae* and the mould *Penicillium candidum*. Mass spectrometric analysis demonstrated that amidation of β-Lg converted aspartyl and glutamyl residues to asparaginyl and glutaminyl residues, respectively, and that the amidation reaction did not occur to the same extent on every β-Lg molecule. The charge change was also confirmed by SDS–PAGE, ion exchange chromatography and the change in isoionic point of β-Lg. Reverse-phase chromatography showed that amidation also led to alterations in hydrophobicity of the β-Lg molecule. The antibacterial properties of the amidated β-Lg appear to be dependent on the net positive charge and charge distribution on the molecule.

Keywords: β-Lactoglobulin; Protein amidation; Antimicrobial effects

1. Introduction

With the emergence of antibiotic-resistant strains of pathogenic microorganisms, there has been a revival of interest in antimicrobial properties of certain proteins as well as peptides liberated from them. It has been shown that the biological activity of proteins and peptides, including their antimicrobial properties, can be enhanced by chemical modification. In general, increasing the positive charge on the protein and peptide molecules enhances their antimicrobial, and more specifically, antibacterial effects. For example, amidation improved the effectiveness of bovine lactoferrin (LF) against a range of Gram-positive and Gram-negative bacteria, including a common dairy spoilage psychrotroph *Pseudomonas fluorescens* (Pan et al., 2005, 2007). These effects are dependent on the interactions of the antimicrobial proteins or peptides with structural elements of the bacterial cell wall and membranes (Hancock, 2004; Mäntylä et al., 2005).

While native LF has antimicrobial properties that can be enhanced by chemical modification, it is also of interest to see if chemical modification of protein molecules that are not antimicrobial in their native state, would impart such properties to these proteins. It is known that proteins that do not have intrinsic biological properties, may acquire such properties upon chemical modification. For example, β-lactoglobulin (β-Lg), the most abundant of bovine whey proteins with molecular mass of 18 kDa and p*I* 5.4 (Sawyer, 2003), acquires antiviral properties upon acylation (Jiang, Lin, Strick, Li, & Neurath, 1996; Neurath et al., 1996; Neurath, Strick, & Li, 1998). The antiviral effect increases with the increase in introduced negative charges, particularly against enveloped viruses (Swart et al., 1996, 1999). As β-Lg is represented in bovine milk at a far higher concentration than LF, the economy of any

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potential practical applications of modified β-Lg could be expected to be more favourable than that of LF. β-Lg occurs in bovine milk at an average concentration of approximately 3.2 g L^{-1} (Swaisgood, 1995), compared to 30–490 mg L^{-1} of LF, depending on the stage of lactation (Ahonen, Korhonen, & Antila, 1978).

Amidation converts aspartate and glutamate residues to asparaginyl and glutaminyl residues, respectively, resulting in an increase in the net positive charge on the protein (Nakai & Li-Chan, 1989). It has been shown that amidation of bovine β-Lg markedly changed its physico-chemical properties (Mattarella, Creamer, & Richardson, 1983; Mattarella & Richardson, 1982, 1983). However, no information appears available on biological properties of amidated β-Lg, specifically on its antimicrobial effects.

The aim of this study was to assess the antimicrobial effectiveness of amidated β-Lg and, by examining the effect of amidation on electrophoretic mobility, isoionic points and mass spectra of β-Lg, to gain an understanding of its mode of action.

2. Materials and methods

2.1. Amidation of β-lactoglobulin

Bovine β-Lg (Danisco USA, Inc., New Century, KS, USA) was modified by amidation. All reagents used for chemical modification and analysis were of analytical or electrophoresis grade. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

Amidation of β-Lg in this study was based on the method of Mattarella et al. (1983), also described in Pan et al. (2007), in which carboxyl groups of aspartyl and glutamyl residues react with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), in the presence of ammonium ions. The molar concentrations of both EDC and ammonium ions were in excess of the carboxyl residues present in β-Lg, at the molar ratios of 45:1 and 2.5:1, respectively.

The β-Lg was dissolved in 5.5 mol L^{-1} NH_4Cl (pH 4.75) to a final concentration of 20 mg mL^{-1}. Solid EDC (0.273 g) was slowly added to the protein solution (10 mL) with continuous stirring and the pH of the reaction mixture adjusted to 4.75 with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH. Following 60 min incubation at room temperature, the reaction mixture was dialysed overnight against distilled water (pH 5.0, adjusted with HCl). The final product was clarified by centrifugation at 8000 × *g* for 5 min, passed through a 0.2 μm filter and kept at −80 °C.

2.2. Characterisation of amidated β-lactoglobulin by mass spectrometry

The native and amidated β-Lg were characterised by liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) using the method described by Shiell, Beddome, and Michalski (2002), which was also applied to analysis of LF (Pan et al., 2007). Protein samples (500 μg mL^{-1}) were dissolved in 100 mmol L^{-1} NH_4HCO_3 pH 8.5 and digested with trypsin (proteomics sequencing grade; Sigma, St. Louis, MO, USA) at a ratio of 50:1 (w/w) at 37 °C for 20 h.

Reverse-phase chromatography (RPC) of digests was performed on a ProteCol C18 column (3 μm, 0.3 mm × 150 mm; SGE, Ringwood, Australia) using a Surveyor-MS HPLC system (Thermo, San Jose, CA, USA). Mobile phases were 0.2% (v/v) formic acid in water (A) and 0.2% (v/v) formic acid in 80% acetonitrile (B), run at a flow rate of 4 μL min^{-1} with a linear gradient of 5–100% B over 20 min, held at 100% B for 10 min and finally 100–5% B over 2 min. The effluent from the column was connected directly to the nanospray ion source of a LCQ Classic quadrupole ion-trap mass spectrometer (Thermo). Mass spectral data were acquired in a data-dependent mode known as the 'triple play' where the most intense ion in each full scan (m/z 400–2000) was automatically selected and subjected to a high-resolution zoom scan followed by a tandem mass spectrometry (MS/MS) product ion scan. The zoom scan allowed determination of the mass/charge state of the selected ion and hence an accurate mass measurement of the selected ion. The MS/MS product ion scan provided detailed confirmatory information on the amino acid sequence of the selected ion. The MS/MS scans were performed with normalised collision energy of 35%. Nanospray ion source settings were as follows: nanospray tip voltage 1.8 kV, capillary temperature 150 °C, capillary voltage 24 V and tube lens offset 5 V. Raw mass spectral data were analysed using the QualBrowser tool from Xcalibur Core Data System software version 1.3 (Thermo) to compile a list of monoisotopic peptide masses for each trypsin-digested protein. The peptide masses obtained were compared with theoretical peptide masses expected from tryptic cleavage of the target proteins, which were determined using the PeptideMass program from ExPASy tools (http://kr.expasy.org/tools/peptide-mass.html) (Wilkins et al., 1997). To further verify the identity of amidated peptides, the MS/MS data obtained were compared with the theoretical MS/MS pattern derived using the MS-Product program (http://prospector.ucsf.edu/ucsfhtml4.0/msprod.html) from the Protein Prospector site of MS analysis tools (http://prospector.ucsf.edu/). The SwissProt primary accession number for 162 amino acid bovine β-Lg is P02754 (http://au.expasy.org/uniprot/P02754).

2.3. Electrophoretic analysis

For denaturing polyacrylamide gel electrophoresis in the presence of ionic detergent (referred to as SDS-PAGE), all samples were separated on NuPAGE (Invitrogen, Carlsbad, CA, USA) 4–12% Bis-Tris precast gels used with 2-(*N*-morpholino) ethane sulphonic acid (MES) running buffers at pH 7.3. Protein samples (1 μg μL^{-1}) were mixed