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Original Research Article

Identification and quantification of native beta-casomorphins in Australian milk by LC–MS/MS and LC–HRMS

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ABSTRACT

Beta-casomorphin 5 (BCM5) and beta-casomorphin 7 (BCM7) in 14 commercial pasteurised milks from around Australia were investigated using liquid chromatography–tandem mass spectrometry (LC–MS/MS) and liquid chromatography–high resolution (Orbitrap) mass spectrometry (LC–HRMS). Collision induced dissociation HRMS was used to confirm the presence of BCM7 in milk extracts. The accurate mass-to-charge ratio (m/z) and relative abundance of BCM7 parent ion and fragments in milk extracts matched with those obtained from the analysis of a BCM7 standard solution. The deviation against the theoretical values of the measured m/z of BCM7 (parent ion and fragments) all gave results below 5 ppm (relative error). BCM5 was below limit of detection (LOD) for all milks, while BCM7 was between 0.13 and 2.38 ng/g in 10 milks and below LOD in the others. Pasteurised milks containing the highest and lowest BCM7 levels were produced in Western Australia.

1. Introduction

Casein is one of the major milk proteins, consisting of α_{s1}, α_{s2}, β and κ-casein and is a source of bioactive peptides that can be released by endogenous enzyme activity in milk, by digestive enzymes in the gastrointestinal tract or during food processing (Sienkiewicz-Szłapka et al., 2009). Several bioactive peptides with opioid properties, designated as beta-casomorphins (BCMs), originate from β-casein (β-CN).

Beta-casein of bovine milk has 209 amino acids with 13 variants, A1, A2, A3, A4, B, C, D, E, F, H1, H2, I and G (Kamiński et al., 2007). Among the variants, β-CN A1 and β-CN A2 variant are frequently present in bovine milk. Both variants differ only at amino acid position 67, where the histidine residue on β-CN A1 variant is replaced by a proline residue on β-CN A2 variant. Normal milk is usually found to contain both β-CN A1 and β-CN A2 variants and so is designated as A1/A2 milk (Nguyen et al., 2015). However, milk containing only the β-CN A1 or only the β-CN A2 variant is known as A1 milk or A2 milk (Woodford, 2007).

Epidemiologically, several studies have reported that BCM5 and BCM7 (Fig. 1), which are released from β-CN A1 variant, are risk factors for the development of type 1 diabetes, autism in children, sudden infant death and ischaemic heart diseases (Birgisdottir et al., 2006; Elliott et al., 1999; McLachlan, 2001; Wasilewska et al., 2011). However, other reports have concluded that there is no strong evidence for the relationship between BCM7 and other related BCMs and these human diseases (EFSA, 2009; Swinburn, 2004; Truswell, 2005). Claeys et al. (2013) suggested casein peptide fragments may have a positive role, such as antimicrobial and antiviral properties.

In vitro studies have demonstrated that BCM7 is released from β-CN A1 variant by digestion with gastrointestinal enzymes (De Noni, 2008; Haq et al., 2015; Jinsmaa and Yoshikawa, 1999). However, BCM5 is not formed from either β-CN A1 or β-CN A2

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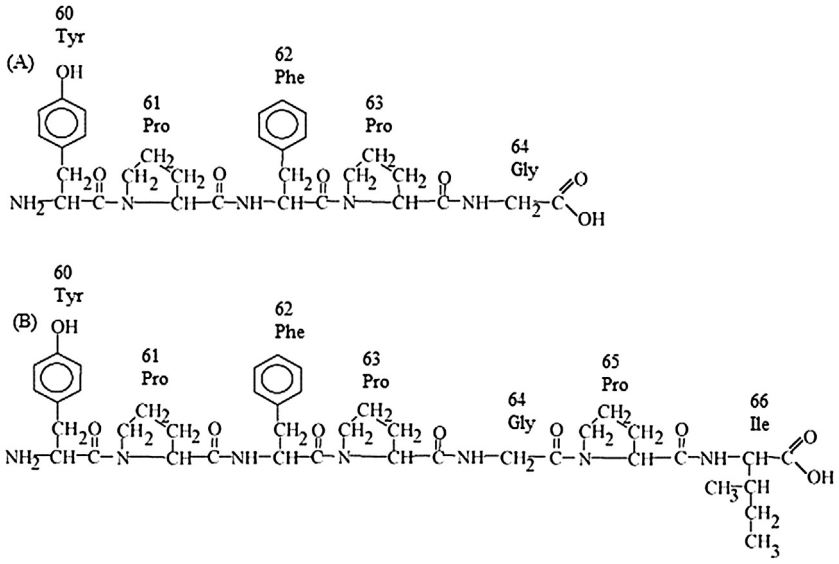

Fig. 1. Structure of beta-casomorphin 5 and beta-casomorphin 7 (Nguyen et al., 2014).

variant by digestion with these enzymes (De Noni, 2008; Haq et al., 2015). In a human study, BCM7 was identified in jejunal effluent after intake of bovine milk protein containing β-CN A2 variant (Boutrou et al., 2013).

Recently, investigations related to the presence of BCM5 and BCM7 in both milk and processed milk products such as yoghurt and milk powder have been a topic of great interest. According to De Noni and Cattaneo (2010), neither BCM5 nor BCM7 were identified in milk, yoghurt, milk powder, milk protein concentrate and infant formulas. In contrast, other studies have identified BCM7 in milk, yoghurt and milk powder (Cieślińska et al., 2012; Jarmolowska, 2012; Juan-García et al., 2009; Thiri et al., 2012) and BCM5 has been reported in infant formulas and high-protein concentrate (Jarmołowska et al., 2007; Wocior, 2008). However, none of these studies has reported an accurate molecular mass of the peptide BCM5 and BCM7, and confirmed their structure through high resolution fragmentation experiments to provide robust evidence of their identification.

In Australia consumer and media interest in A2 milk, a product rich in β-CN A2 variant, and other milk products containing natural A2 protein has increased in recent times. However, there has been no previous study in Australia reporting the concentration of BCM5 and BCM7 in these pasteurised milks as well as in normal milks.

The aim of the present study was to determine the levels of BCM5 and BCM7 in a range of pasteurised milks from around Australia. For this aim, highly sensitive isotope dilution LC–MS/MS and LC–HRMS were used for simultaneous identification and quantification of BCM5 and BCM7.

2. Materials and methods

2.1. Materials and chemicals

Fourteen pasteurised milks were purchased from supermarkets around Australia between May and July 2014. The products were randomly coded PM 1 to PM 14 (Table 1) and extracted immediately or stored at −80 °C until extraction. BCM5 (purity 97.8%), BCM7 (purity >98.7%) and their deuterated standards [^{2}H10] BCM5 (BCM5-d_{10}) and [^{2}H10] BCM7 (BCM7-d_{10}) (deuterium enrichment >99%, 2H enrichment at phenylalanine position) (Bachem, Bubendorf, Switzerland), methanol (MeOH) (Mallinckrodt Baker Inc., Phillipsburg, NJ), formic acid (purity 99%) (Ajax FineChem, Sydney, Australia) were of HPLC grade. Ultrapure

Table 1
Milk samples used for analysis of beta-casomorphin 5 and beta-casomorphin 7.

Sample code	Processing location	Specification	Protein content			Days to expiration[b] (days)
			Total[a] (mg/mL)	β-Casein A_2 variant[a] (mg/mL)	β-Casein A_1 variant[a] (mg/mL)	
PM 1	South Brisbane, QLD	Full cream milk	36	n.a	n.a	5
PM 2	South Brisbane, QLD	Full cream milk	32	n.a	n.a	5
PM 3	Wagga Wagga, NSW	full cream milk	34	n.a	n.a	4
PM 4	Harvey, WA	Full cream milk	33	n.a	n.a	12
PM 5	Margaret River, WA	Full cream milk	33	n.a	n.a	9
PM 6	Northcliffe, WA	Full cream milk	32	n.a	n.a	8
PM 7	Perth, WA	Reduced fat milk	31	10	n.a	13
PM 8	Perth, WA	Reduced fat milk	33	n.a	n.a	2
PM 9	South Brisbane, QLD	Full cream milk	36	n.a	n.a	3
PM 10	Manning Valley, NSW	Full cream milk	31	n.a	n.a	4
PM 11	Penrith, NSW	Full cream milk	34	7	4	4
PM 12	Docklands, Vic	Full cream milk	34	n.a	n.a	4
PM 13	Riverina, NSW	Reduced fat milk	31	10	n.a	7
PM 14	Perth, WA	Reduced fat milk	31	10	n.a	12

QLD, Queensland; NSW, New South Wales; Vic, Victoria; WA, Western Australia; PM, pasteurised milk; n.a., not available.
[a] Protein content calculated from data on the product label.
[b] Days to expiration on extraction.