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Short communication

Identification of short peptide sequences in complex milk protein hydrolysates

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ABSTRACT

Numerous low molecular mass bioactive peptides (BAPs) can be generated during the hydrolysis of bovine milk proteins. Low molecular mass BAP sequences are less likely to be broken down by digestive enzymes and are thus more likely to be active *in vivo*. However, the identification of short peptides remains a challenge during mass spectrometry (MS) analysis due to issues with the transfer and over-fragmentation of low molecular mass ions. A method is described herein using time-of-flight ESI-MS/MS to effectively fragment and identify short peptides. This includes (a) short synthetic peptides, (b) short peptides within a defined hydrolysate sample, i.e. a prolyl endoproteinase hydrolysate of β-casein and (c) short peptides within a complex hydrolysate, i.e. a Corolase PP digest of sodium caseinate. The methodology may find widespread utilisation in the efficient identification of low molecular mass peptide sequences in food protein hydrolysates.

1. Introduction

There is currently a growing interest in high quality protein ingredients to meet the demand from an increasing global population. Enhancement of the functionality of such protein ingredients may be achieved through processing, fermentation or enzymatic hydrolysis, all of which may result in the production of short peptide sequences with various bioactivities. Milk protein hydrolysates having a range of bioactivities including immunomodulatory, mineral binding, anti-thrombotic, hypotensive, anti-diabetic, anti-obesity, anti-cancer, anti-microbial and opioid activities have been extensively reported in the literature (Clare & Swaisgood, 2000; FitzGerald & Meisel, 2003; Korhonen & Pilhanto, 2006; Meisel, 1997; Pihlanto, 2011). Low molecular mass BAPs have the potential to exert biological effects *in vivo* due to the increased likelihood of their surviving further hydrolysis by digestive enzymes and their increased permeability through intestinal cells (Foltz, van Buren, Klaffke, & Duchateau, 2009).

Food-derived peptides have classically been sequenced by Edman degradation, however, this approach generally requires extensive separation and isolation of peptides prior to sequencing. Liquid chromatography (LC) coupled to mass spectrometry (MS) and tandem MS (MS/MS) is now the preferred route for the separation and identification of peptides in complex bioactive peptide mixtures (Panchaud, Affolter, & Kussmann, 2012; Saavedra, Hebert, Minahk, & Ferranti, 2013; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013). During proteomic studies, proteins are generally specifically digested with enzymes of defined specificity (such as trypsin) to produce multiply charged ions that can be readily identified by MS and MS/MS. However, peptidomics is often complicated by the presence of short peptide sequences generated by a combination of different enzymes having poorly characterised specificities. This is particularly the case during the generation of food protein hydrolysates. Additionally, the resultant peptides may not be favourably charged for ease of subsequent MS detection.

A number of different approaches have been employed in the MS/MS-based sequence analysis of such peptides. These include (a) chemical derivitisation in order to increase peptide mass and thus assist in identification (Herregods et al., 2010), (b) partial sequences (sequence tags), along with knowledge of primary sequence (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004) and (c) the use of multiple reaction monitoring (MRM) which is performed to fragment specific masses of interest (Takahashi et al., 2012). Each of these approaches has their limitations/disadvantages when applied to the identification of short peptides within complex mixtures. Derivitisation, for example, adds another step in the process of peptide identification. Strategies requiring primary sequence knowledge have limited applicability in cases where prior knowledge of the primary sequence is unavailable.

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MRM requires knowledge of the masses of interest within a complex mixture or a prior MS run to determine the masses within the sample. Retention time prediction has been used as a tool to aid in the identification of short peptides in complex mixtures. This approach is based on the unique structure of a peptide and its characteristic elution properties under given chromatographic conditions (Kunda et al., 2012). However, the prediction is somewhat limited in that it only applies to specific separation conditions (mobile phase, stationary phase, temperature and pH) and therefore this technique may not be compatible with the need for continuous optimisation of chromatographic conditions due to differences in sample complexity. Furthermore, large numbers of synthetic peptides are necessary to establish a reliable standard curve. While retention time prediction generally may not be used in isolation for the identification of unknown peptide sequences, the technique may prove helpful in cases where there is insufficient fragmentation data to distinguish between two or more possible peptide sequences, particularly isobaric peptides, having the same mass but different amino acid composition and/or sequence (Le Maux, Nongonierma, & FitzGerald, 2015). Hydrophilic interaction chromatography (HILIC) coupled to MS has also been used with some success to efficiently separate and aid in the subsequent MS/MS identification of short peptides in complex mixtures (Harscoat-Schiavo et al., 2012; Le Maux et al., 2015).

While there are numerous reports in the literature on the bioactivity of short peptides, there is a limited amount of information on the application of direct MS/MS-based approaches (i.e. MS/MS on short peptides without prior derivitisation of the peptides) in the routine identification of short, non-tryptic peptide sequences in complex mixtures as occurs in food protein hydrolysates. This may be due to the fact that short peptides are often singly charged and are therefore more difficult to efficiently fragment during direct MS/MS sequence analysis. Short peptide identification is further complicated by the high probability of finding the peptide within a whole range of protein sequences leading to the redundancy of MS/MS database searching approaches. Therefore, a *de novo* sequencing approach is required, necessitating good fragmentation spectra in order to correctly assign peptide sequences. While there are some reports in the literature where di- and tripeptides have been identified within complex milk protein hydrolysates, the number of short peptides identified appears to be limited (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005; Hernández-Ledesma et al., 2004; Holder et al., 2013). This may be because the MS/MS method employed was not optimal

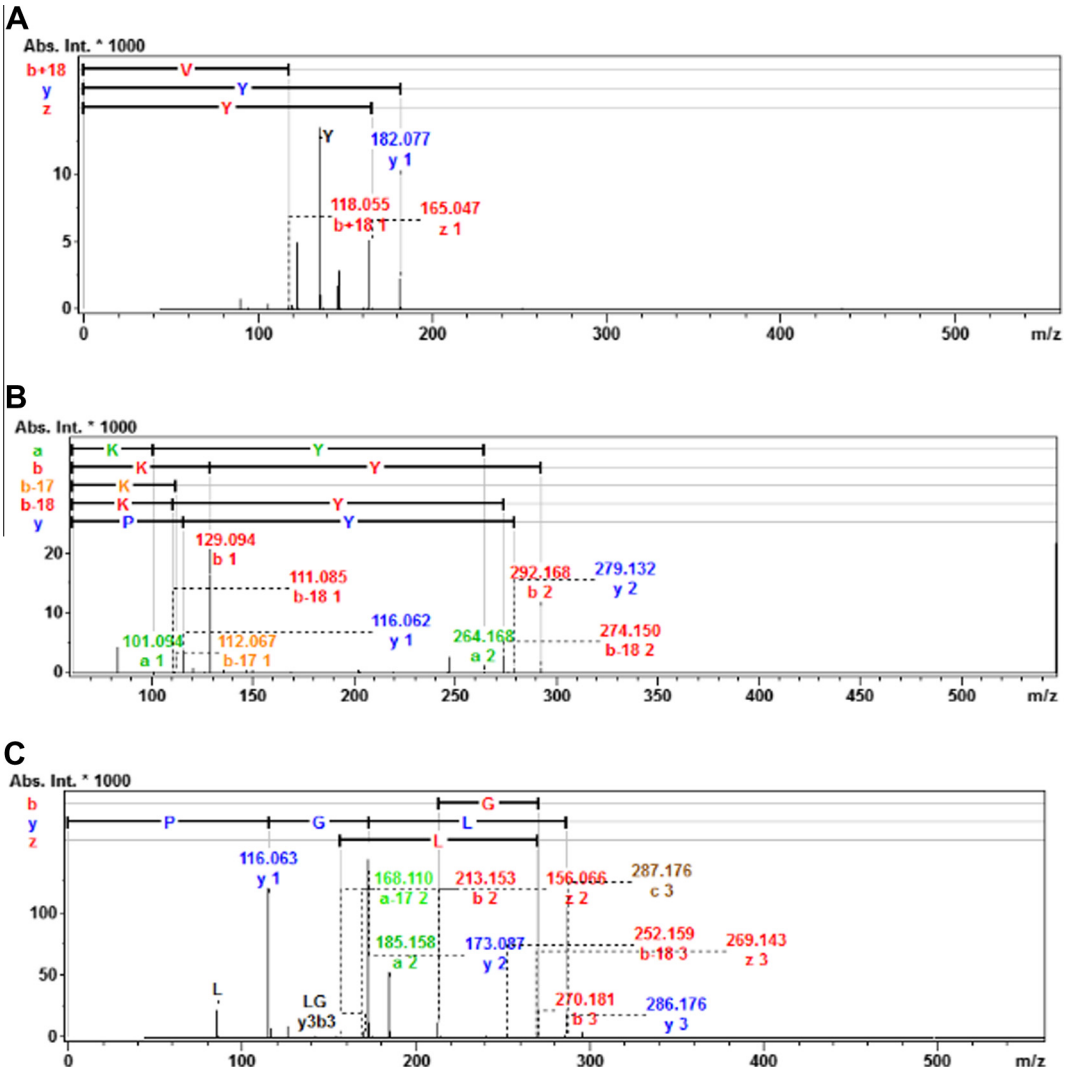

Fig. 1. Mass spectrometry fragmentation spectra for synthetic (A) Val-Tyr, (B) Lys-Tyr-Pro and (C) Val-Leu-Gly-Pro. The *x*-axis represents the *m*/*z* of the fragment ions while the *y*-axis represents the intensity of the detected ions.