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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

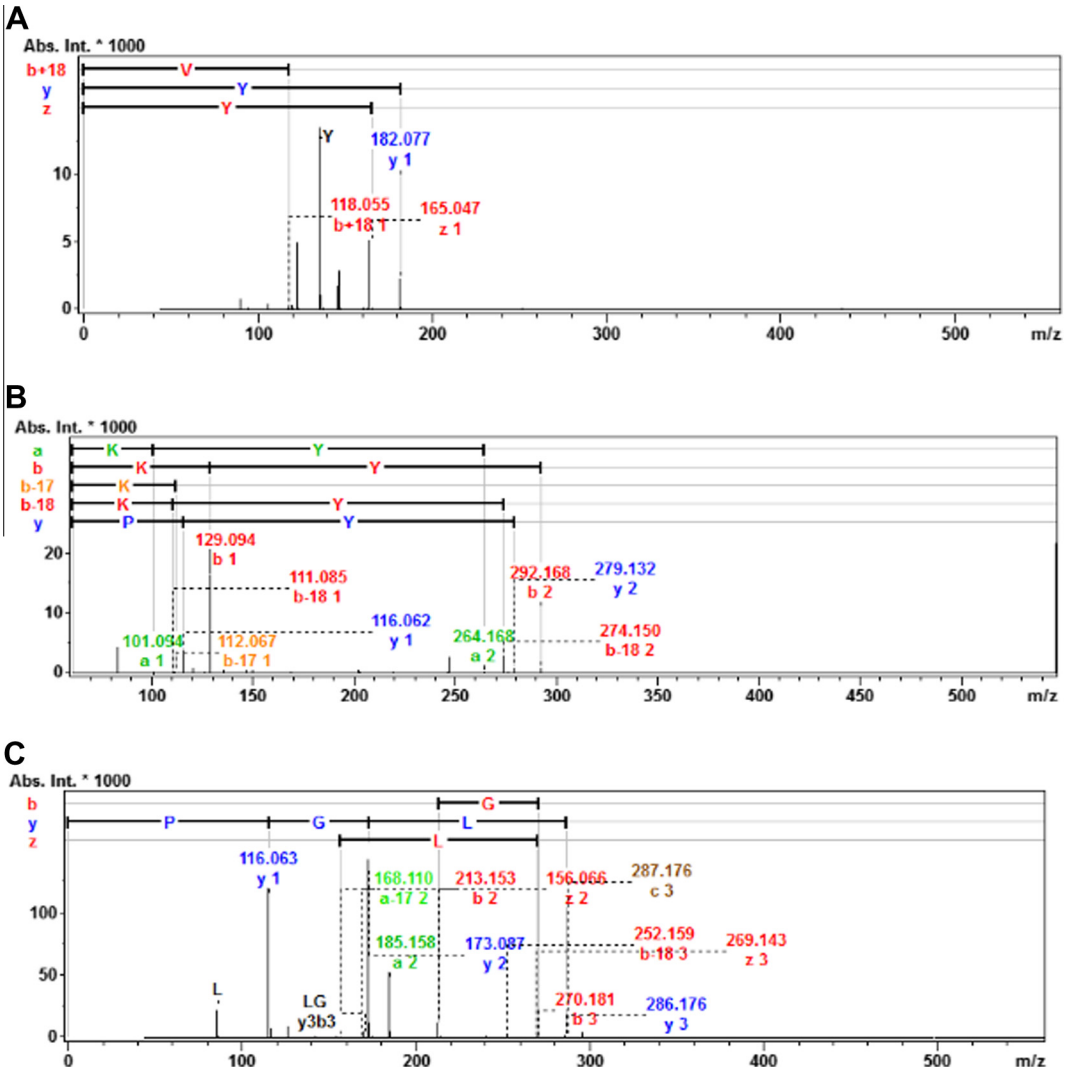


**Fig. 1.** Mass spectrometry fragmentation spectra for synthetic (A) Val-Tyr, (B) Lys-Tyr-Pro and (C) Val-Leu-Gly-Pro. The *x*-axis represents the *m*/*z* of the fragment ions while the *y*-axis represents the intensity of the detected ions.

for short peptide identification or alternatively it may be that there were few short peptides in the mixture.

The specific objective of this study was to investigate the potential of a direct UPLC MS/MS approach to generate sufficient fragment ions allowing for accurate identification of short peptides in (a) synthetic peptide samples and (b) short peptides in defined and in (c) complex milk protein hydrolysates.

## 2. Materials and methods

### 2.1. Materials

Brewer's Clarex™ (*Aspergillus niger* prolyl endoproteinase, *An*-PEP, specific activity: $37 \times 10^{-3}$ U/mg) was kindly donated by Dutch State Mines (DSM, Heerlen, Netherlands). Corolase PP was a gift from AB Enzymes (Darmstadt, Germany). Acid caseinate (89% (w/w) protein) and sodium caseinate (87.5% (w/w) protein) were supplied by Kerry Ingredients (Kerry, Ireland). Acetonitrile, water and formic acid (all MS grade) were purchased from Sigma–Aldrich (Dublin, Ireland). Synthetic peptides Asn-Pro, Val-Glu-Pro, Lys-Tyr-Pro, Lys-His-Pro, His-Gln-Pro, Leu-Pro and Ile-Ile-Val, Val-Arg-Gly-Pro, Phe-Leu-Gln-Pro and Val-Leu-Gly-Pro were from Thermo Fisher Scientific (Ulm, Germany) while Val-Tyr and Val-Phe were from Bachem AG (Bubendorf, Switzerland).

### 2.2. Purification of β-casein from acid caseinate and hydrolysis of β-casein with An-PEP

β-Casein was purified from acid caseinate and subsequently hydrolysed using *An*-PEP as previously described (Norris, Poyarkov, O'Keeffe, & FitzGerald, 2014).

### 2.3. Hydrolysis of sodium caseinate using Corolase PP and ultrafiltration through a 5 kDa membrane

A 10% (w/v) aqueous solution of sodium caseinate was allowed to hydrate at 50 °C for 1.5 h and was then hydrolysed with Corolase PP at an E:S ratio of 0.62% (w/v) at 50 °C for 4 h. The pH was maintained at pH 7.0 using a pH stat (718 Stat Titrino, Metrohm, Herisau, Switzerland) as previously described (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). Following 4 h incubation, samples were heated to 80 °C for 20 min in order to inactivate the enzyme. Hydrolysate samples were processed through a 5 kDa nominal molecular mass cut-off membrane using a bench-scale ultrafiltration system (Sartoflow Alpha, Sartorius AG, Goettingen, Germany). The 5 kDa permeate was freeze-dried (FreeZone 18L, Labconco, Kansas City, USA) and then stored at −20 °C until use.

### 2.4. Identification of short synthetic peptides by UPLC–ESI-MS/MS

Synthetic peptides were reconstituted to a concentration of 0.05 mg/mL in mobile phase A (0.1% formic acid in MS-grade $H_2O$), passed through a 0.22 μm filter (Restek Ireland, Belfast, N. Ireland) and 7 μL was injected onto an Acquity BEH 300 C18 column (2.1 × 50 mm, 1.7 μm; Waters, Dublin, Ireland). Separation was achieved using an Acquity UPLC (Waters, Dublin, Ireland) at a flow rate of 0.2 mL/min with isocratic elution for 5 min at 100% mobile phase A followed by gradient elution to 80% mobile phase B (0.1% formic acid in 80% acetonitrile) from 5 to 30 min. Column temperature was maintained at 25 °C. Detector response was monitored at 214 nm.

The micrOTOF QII (Bruker Daltonics, Bremen, Germany) was calibrated using sodium formate (10 mM NaOH, 0.2% formic acid in isopropanol). Mass spectra were acquired in positive ion mode and scans were performed for Auto MS/MS between 50 and 600 *m*/*z* (during optimisation studies the lower and upper mass ranges were 50 and 2500 *m*/*z*, respectively). The analysis was performed under the following conditions; capillary voltage was 4500 V; collision energy was 10 eV; collision cell radio frequency (RF) was 140 Vpp; transfer time was 70 μs; nebulizer pressure was 1.8 bar; drying gas flow rate was 8 L/min and the dry heater temperature was 220 °C. During optimisation of the MS operating conditions the range of collision cell RF varied from 100 to 600 Vpp, while the transfer time was varied between 50 and 120 μs. MicrOTOF Control (version 2.3, Bruker Daltonics) was used to control experiments. Peptide sequences were determined by *de novo* sequencing using Data Analysis (version 4.0, Bruker Daltonics) and Biotools (version 3.2, Bruker Daltonics) software. Specifically, the Annotate tool of Data Analysis was used to assign peptide sequence; the peptide sequence was then confirmed using the Biotools Sequence Editor tool whereby all matched fragments were highlighted. The MS/MS tolerance was set to 0.1 Da.

### 2.5. Identification of short peptides in a β-casein An-PEP hydrolysate

The β-casein *An*-PEP hydrolysate (7 μL of 1 mg/mL, passed through a 0.22 μm filter) in mobile phase A (0.025% formic acid in MS-grade $H_2O$) was separated on UPLC using the same gradient as that employed in Section 2.4. Mobile phase B was 0.025% formic acid in 80% acetonitrile. The MS and MS/MS conditions were as in Section 2.4 except that the masses of the short peptides predicted to be generated during *An*-PEP digestion of β-casein were placed on an 'include' list in micrOTOF Control (Bruker Daltonics) such that these masses were preferentially fragmented.

### 2.6. Identification of short peptides in sodium caseinate hydrolysed with Corolase PP

The Corolase PP sodium caseinate hydrolysate (7 μL of 1 mg/mL, passed through a 0.22 μm filter) in mobile phase A (0.1% formic acid in MS-grade $H_2O$) was separated and analysed on the UPLC–ESI-MS/MS according to the conditions outlined in Section 2.4.

**Table 1**
Short peptides (⩽4 amino acid residues) theoretically predicted to be generated through digestion of bovine β-casein with *Aspergillus niger* derived prolyl endoprotease (*An*-PEP) and actual peptides detected in a 24 h hydrolysate. The amino acid residues (R) adjacent to the C- and N-terminal residues are shown in brackets.

| Location in β-casein | (R)-peptide sequence-(R)[a] | Peptide detected[b] | Experimental mass | Error (Da) | Rt (min) |
|---|---|---|---|---|---|
| f(52–53) | (P)-FA-(Q) | No | – | – | – |
| f(62–63) | (P)-FP-(G) | Yes | 263.1401 | −0.0011 | 11.5 |
| f(64–65) | (P)-GP-(I) | No | – | – | – |
| f(66–67) | (P)-IP-(N) | Yes | 229.1505 | 0.0042 | 9.6 |
| f(68–71) | (P)-NSLP-(Q) | Yes | 430.2388 | −0.0092 | 10.5 |
| f(87–90) | (P)-FLQP-(E) | Yes[c] | 504.3054 | −0.0238 | 12.5 |
| f(102–103) | (A)-MAP-(A) | Yes | 318.1464 | 0.0018 | 9.2 |
| f(111–112) | (P)-FP-(K) | Yes | 263.1401 | −0.0011 | 11.5 |
| f(113–115) | (P)-KYP-(V) | Yes[c] | 407.2369 | −0.0080 | 8.8 |
| f(116–118) | (P)-VEP-(F) | No | – | – | – |
| f(137–138) | (P)-LP-(L) | Yes | 229.1505 | 0.0042 | 9.6 |
| f(148–150) | (P)-HQP-(L) | Yes[c] | 381.1905 | −0.0024 | 1.2 |
| f(151–153) | (P)-LPP-(T) | Yes[c] | 326.2054 | 0.0020 | 9.6 |
| f(173–174) | (P)-VP-(Q) | No | – | – | – |
| f(175–177) | (P)-QKA-(V) | No | – | – | – |
| f(178–179) | (A)-VP-(Y) | No | – | – | – |
| f(187–189) | (P)-IQA-(F) | Yes | 331.1968 | 0.0008 | 9.1 |
| f(197–200) | (P)-VLGP-(V) | Yes[c] | 385.2473 | −0.0028 | 10.6 |
| f(201–204) | (P)-VRGP-(F) | Yes[c] | 428.2687 | −0.0071 | 8.8 |
| f(205–206) | (P)-FP-(I) | Yes | 263.1401 | −0.0011 | 11.5 |
| f(207–209) | (P)-IIV | Yes | 344.2583 | −0.0039 | 12.1 |

[a] One letter amino acid code.
[b] Peptides detected in the 24 h hydrolysate of β-casein.
[c] Peptide also previously detected by Norris et al. (2014).

## 3. Results and discussion

### 3.1. Identification of synthetic peptides

The general MS/MS conditions used for the fragmentation of a wide range of peptide masses may result in the over-fragmentation (whereby there is little or no signal remaining for both precursor and daughter ions, leading to an inability to assign peptide sequence) of short peptides as an appropriate collision energy for parent ion fragmentation depends on the mass of the peptide (Haller, Mirza, & Chait, 1996), charge state (Smith, Loo, Edmonds, Barinaga, & Udseth, 1990) and structure (Tang, Thibault, & Boyd, 1993). Therefore, when analysing short peptide sequences, it is necessary to develop a set of MS/MS conditions suitable for their specific fragmentation. A range of di-, tri- and tetra- synthetic peptides were separated on UPLC and analysed by MS/MS in order to optimise the fragmentation conditions for short peptides. Specifically, narrowing the mass range to only allow transfer of precursor ions of $m/z$ 50–600, minimising collision cell RF to 140 Vpp and transfer time to 70 μs resulted in the efficient transfer and optimal fragmentation of short peptides and these conditions were therefore subsequently used for all experiments. Fragmentation spectra of representative synthetic di- (Val-Tyr), tri- (Lys-Tyr-Pro) and tetra-peptides (Val-Leu-Gly-Pro) are shown

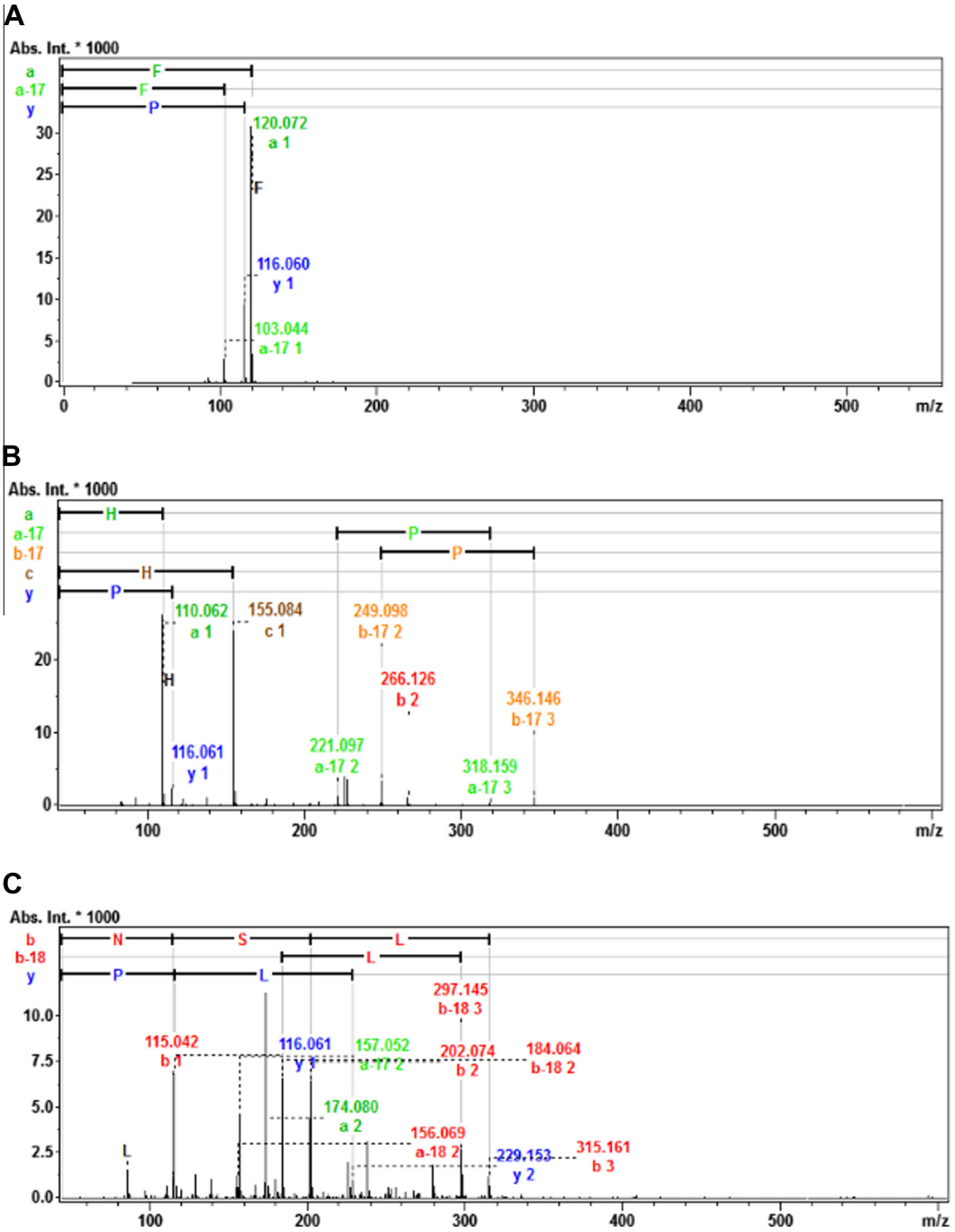


**Fig. 2.** Mass spectrometry fragmentation spectra for (A) Phe-Pro, (B) His-Gln-Pro and (C) Asn-Ser-Leu-Pro detected within an *Aspergillus niger* prolyl endoproteinase hydrolysate of bovine β-casein. The *x*-axis represents the $m/z$ of the fragment ions while the *y*-axis represents the intensity of the detected ions.

**Table 2**
Short (di-, tri-, tetra- and penta-) peptides identified in the 5 kDa permeate of a Corolase PP hydrolysate of bovine sodium caseinate.

| Peptide sequence[a] | Parent protein | Experimental mass (*m*/*z*) | Theoretical mass (*m*/*z*) | Error (Da) | RT (min) |
|---|---|---|---|---|---|
| KH | Several | 284.1699 | 284.1717 | 0.0018 | 0.8 |
| RPK | $\alpha_{s1}$-CN | 400.2557 | 400.2667 | 0.0011 | 1.1 |
| HIQ | $\alpha_{s1}$-CN | 397.2178 | 397.2194 | 0.0016 | 2.0 |
| LSH | BSA | 365.1905 | 365.1928 | 0.0023 | 2.0 |
| LEK | β-CN | 389.2354 | 389.2395 | 0.0041 | 2.0 |
| YQ | Several | 310.1345 | 310.1397 | 0.0052 | 2.1 |
| MAPK | β-CN | 446.2277 | 446.2432 | 0.0155 | 3.1 |
| LQS | β-CN | 347.1958 | 347.1925 | −0.0033 | 4.6 |
| SI/L | Several | 219.1255 | 219.1339 | 0.0084 | 4.9 |
| HPI | $\alpha_{s1}$-CN | 366.2040 | 366.2136 | 0.0096 | 5.0 |
| QQKPV | κ-CN | 300.1742 (2+) | 300.1792 | 0.0050 | 7.6 |
| VLS | κ- and β-CN | 318.1989 | 318.2023 | 0.0034 | 7.9 |
| EVV | $\alpha_{s2}$-CN | 346.1951 | 346.1973 | 0.0022 | 9.2 |
| FPQ | $\alpha_{s2}$-CN | 391.1899 | 391.1976 | 0.0077 | 9.9 |
| PPK | κ-CN | 341.2122 | 341.2183 | −0.0061 | 10.1 |
| IPIQ | κ-CN | 470.2882 | 470.2973 | 0.0091 | 10.9 |
| IHPF | β-CN | 513.2853 | 513.2820 | −0.0033 | 11.5 |

CN: casein.
BSA: bovine serum albumin.
[a] One letter amino acid code.

in Fig. 1, confirming the suitability of the MS/MS parameters employed herein to effectively fragment short peptides. Fragmentation resulted in the production of *a*, *b* and *y*, as well as immonium ions arising from internal fragmentation (e.g. Fig. 1C shows a strong signal at *m*/*z* 86 representing the immonium ion for Leu).

### 3.2. Identification of short peptides of known mass within a complex mixture

A purified individual protein (β-CN) was hydrolysed with *An*-PEP in order to analyse the suitability of the UPLC–MS/MS conditions employed herein for the efficient fragmentation of short peptides of known (predicted) mass within a defined mixture. Theoretically, *An*-PEP digestion of β-CN should result in the generation of a set of peptides of known mass. In a previous study of this hydrolysate, not all peptides predicted *in silico* to be present were detected under the general MS/MS conditions employed (Norris et al., 2014). The mass range employed therein was 100–2500 *m*/*z*, allowing for transfer of a wide range of precursor ion sizes through to the collision cell. The collision cell RF and transfer time were 600 Vpp and 120 μs, respectively, resulting in the efficient fragmentation of a wide range of peptide masses. However, these conditions resulted in the over-fragmentation of short peptides, negating their accurate identification. In the current study, the masses of the predicted peptides (generated when β-CN was incubated with *An*-PEP) were entered on an 'include' list in the MS/MS protocol such that they were preferentially fragmented. Furthermore, the MS/MS conditions were also set as outlined for short peptide fragmentation. A list of the 21 theoretically expected di-, tri- and tetra-peptides generated by *An*-PEP hydrolysis of β-CN can be seen in Table 1, as well as information on their detection in the 24 h hydrolysate using the MS/MS conditions employed herein. Nine peptides that had remained undetected in a previous analysis of this sample (which used a method for the detection of peptides having a wide range of masses, Norris et al., 2014) have now been identified using the conditions described above, resulting in a total of 15 short peptides being identified within this sample. However, five peptides predicted to be present using the *in silico* approach remain undetected within this sample. This may be due to missed cleavages or perhaps these peptides are not well ionised within the mixture and thus remain undetected. Two of the five undetected, theoretically expected, peptides have Ala at their C-teminus (Phe-Ala and Gln-Lys-Ala). As *An*-PEP has a strong preference towards cleavage post Pro residues (Edens et al., 2005) it is possible that these two peptides were not generated in the hydrolysate. Alternatively, it may be that the undetected peptides were further digested to free amino acids. Examples of representative fragmentation spectra for di- (Phe-Pro), tri- (His-Gln-Pro) and tetra-peptides (Asn-Ser-Leu-Pro) identified within the *An*-PEP β-CN hydrolysate are shown in Fig. 2. High mass accuracy was achieved for all peptides identified (Tables 1 and 2). The use of strict tolerance levels (0.1 Da for MS/MS) when confirming the identity of these peptides helps to ensure the reliability of the data.

In order to determine if the 15 short peptides detected in this sample (using an 'include' list) could be detected without the 'include' list approach, the sample was re-run under the same MS/MS conditions but without an include list. This should more closely mimic real conditions where a list of predicted peptides would generally not be known (due to the broad range of specificities of many food-grade proteolytic preparations). All 15 peptides that had been identified using the 'include' list were efficiently fragmented in the protocol without the 'include' list leading to their correct identification within this sample (data not shown). This indicates that, in this sample, and under the MS/MS conditions employed, an include list was not essential for short peptide identification.

The β-CN hydrolysate analysed in this study has previously been shown to have highly potent angiotensin converting enzyme (ACE) inhibitory activity (Norris et al., 2014). Some of the newly identified short peptides in this study have also previously been shown to have ACE inhibitory activity, i.e. FP (Dziuba & Darewicz, 2007) and IP (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980).

### 3.3. Identification of short peptides of unknown mass within a complex mixture

The identification of short BAP sequences within complex mixtures of peptides having potent bioactivity remains a challenge. Sodium caseinate was therefore employed as an example of a complex protein mixture. It is often impossible to predict the masses of short peptides within food protein hydrolysates due to the broad range of hydrolytic activities contained within many common food-grade proteolytic/peptideolytic preparations that are used to generate the hydrolysates. Corolase PP is a proteolytic preparation derived from porcine pancreas mainly containing trypsin, chymotrypsin and elastase but it also has exopeptidase activities (Mullally, O'Callaghan, FitzGerald, Donnelly, & Dalton, 1994). Therefore, digestion of sodium caseinate with Corolase PP is expected to result in a hydrolysate with various short peptide sequences of unknown mass. The hydrolysate was processed through an ultrafiltration system with a 5 kDa molecular mass cut-off membrane in order to enrich for short peptides. GPC analysis of this sample showed that ~81% of the material present was <1 kDa (data not shown). Seventeen short peptides were identified within the hydrolysate (Table 2). Representative fragmentation spectra for Tyr-Gln, Leu-Glu-Lys, and Met-Ala-Pro-Lys detected within the Corolase PP hydrolysate of sodium caseinate are shown in Fig. 3. Some of the short peptides identified have been previously shown to have different bioactivities or to have been located within bioactive peptide sequences. For example, Phe-Pro-Gln is found within the peptide Phe-Pro-Gln-Tyr which has previously been shown to have antioxidative properties, i.e. with the ability to scavenge oxygen radicals (Contreras, Sanchez, Sevilla, Recio, & Amigo, 2013). Ser-Leu has previously been shown to have DPP-IV inhibitory activity (Nongonierma, Mooney, Shields,

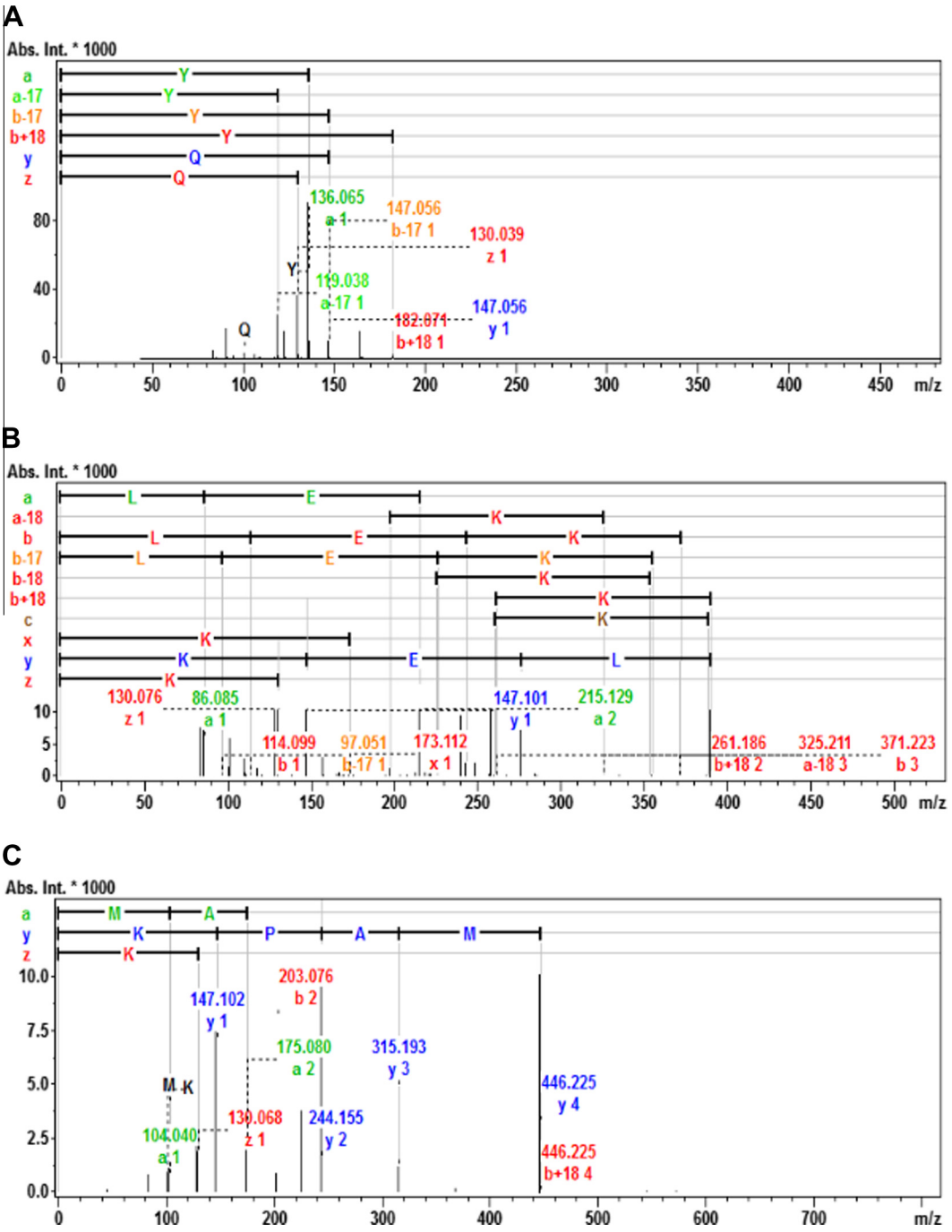


**Fig. 3.** Mass spectrometry fragmentation spectra for (A) Tyr-Gln, (B) Leu-Glu-Lys and (C) Met-Ala-Pro-Lys detected within a Corolase PP hydrolysate of sodium caseinate. The *x*-axis represents the *m*/*z* of the fragment ions while the *y*-axis represents the intensity of the detected ions.

& FitzGerald, 2013). Arg-Pro-Lys, Lys-His and His-Pro-Ile are all part of a nonapeptide, Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln that has previously been shown to have ACE inhibitory activity ($IC_{50}$ 13.4 μM, Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000). Furthermore, Phe-Pro-Gln, is found both within the ACE inhibitory peptide, Phe-Pro-Gln-Tyr-Leu-Gln-Tyr (Tauzin, Miclo, & Gaillard, 2002) and the antihypertensive peptide, Tyr-Gln-Lys-Phe-Pro-Gln-Tyr (Contreras, Carrón, Montero, Ramos, & Recio, 2009).

The method employed herein identifies short peptide sequences from high quality MS/MS fragmentation data without the need for (a) derivitisation of peptides prior to MS/MS analysis, (b) primary sequence knowledge or (c) knowledge of masses of peptides of interest as is the case for MRM experiments. This method has resulted in a detailed analysis of the peptides within a complex milk protein-derived hydrolysate.

The current study focused on the identification of short peptide sequences. However, their quantitation within mixtures is also of interest. Direct quantitative/semi-quantitative analysis of short peptides within complex mixtures may be complicated by the potential of ion suppression by other analytes present in the mixture. However, this may be overcome through the use of appropriate standards as recently published by García-Tejedor, Sánchez-Rivera, Recio, Salom, and Manzanares (2015).

In conclusion, the method presented herein has been shown to efficiently fragment short (di-, tri- and tetra-) synthetic peptides, as well as peptides from a purified protein hydrolysed with an

enzyme having defined activity (*An*-PEP). In addition it could identify short peptides in a complex protein mixture (sodium caseinate) hydrolysed with a food-grade proteolytic preparation having a broad range of specificities (Corolase PP). This direct MS/MS identification of short peptides eliminates the need for additional steps such as derivitisation of peptides prior to MS/MS analysis. The method is also relevant to the identification of peptides where the primary sequence is as yet unknown. The identification of short peptides still requires a significant amount of manual evaluation of the MS/MS spectra; it is expected that ongoing advances in software will help to ameliorate this time-consuming analysis. The MS/MS method described herein provides appropriate conditions for the identification of short peptides; the combination of these short peptide settings with those appropriate for the transfer and fragmentation of longer peptides, leading to one method for the identification of all peptides within a bioactive fraction, is worthy of further research.

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