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Peptidomic study on in vitro and in vivo phosphopeptide release during the chewing of gum fortified with a commercial casein hydrolysate

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ABSTRACT

The kinetics of phosphopeptide release from gum fortified with a commercial casein hydrolysate in artificial and human saliva was studied. Using mass spectrometry techniques, the caseinophosphopeptide (CPP) composition of the commercial casein hydrolysate compared with a standard tryptic casein digest from whole raw cow milk was determined. In in vitro trials, the amount of CPPs released was always the same for each chewing time; in contrast, during in vivo chewing, a decrease of peptide levels was detected, from 10% after 5 min to 0.1% at 20 min, due to physiological swallowing. It is concluded that gums are a useful vehicle for CPP intake, owing to their functional role in bone health. However, their use is less satisfactory for prevention of dental caries, due to the brief contact with the enamel surface. Moreover, more calcium would need to be added to enrich the CPP fraction of the commercial hydrolysate.

1. Introduction

The functional role of caseinophosphopeptides (CPPs) in casein micelles is to stabilise amorphous calcium phosphate [ACP; $Ca_3(PO_4)_23H_2O$] at the pH of milk. A decrease in pH destabilises the micellar structure because of the conversion of ACP into soluble calcium phosphate salts. CPPs can be generated from casein fractions by proteolytic enzyme action during fermented milk and cheese production, as well as by gastric protease digestion in vivo. In this regard, studies carried out on CPPs isolated from ripened cheeses (Ferranti et al., 1997; Pinto et al., 2010, 2012) and in vivo digestion (Boutrou et al., 2013; Caira et al., 2016; Cruz-Huerta, Garcia-Nebot, Miralles, Recio, & Amigo, 2015; Garcia-Nebot, Alegria, Barbera, Contreras, & Recio, 2010; Guilloteau et al., 2009), have shown the Ser(P)-Ser(P)-Ser(P)-Glu-Glu sequence to be very resistant to enzymatic degradation by microbial and digestive proteases, respectively.

This behaviour, exhibited by CPPs only in free form, further attests to the bioactivity of three phosphoryl clusters as carriers of bivalent cations (Fe, Ca, Mg) in human metabolism (FitzGerald, 1998). The CPP-ACP complex may act at different sites in vivo. Following ingestion, their first site of potential bioactivity is in the mouth, where they can prevent disease from dental caries and/or remineralise tooth enamel lesions. CPPs have been found in the stomach, duodenum and distal ileum of human subjects (Chabance et al., 1998; Hartmann & Meisel, 2007; Kitasako et al., 2011; Meisel et al., 2003; Walsh, 2009; Wegehaupt & Attin, 2009).

In the last twenty years, numerous studies have been focused on the anticariogenic effect of CPP-ACP complex at the tooth surface, since it can buffer plaque pH, depress enamel demineralisation and enhance its remineralisation (Reynolds, 1991). An in vitro study by means of scanning electron microscopy (SEM) analysis showed the anticariogenic effect of a commercial CPP preparation that was able to prevent the demineralisation of human enamel dipped in a pH 4.8 lactic solution (Ferrazzano, Cantile, Ingenito, Chianese, & Quarto, 2007). The same authors showed, with the same procedure, the preventive anticariogenic effect of yoghurt soluble extract (Ferrazzano et al., 2008). Moreover, among the tryptic CPPs, those

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having the three-phosphoryl cluster showed better ability to stabilise ACP (Adamson & Reynolds, 1996; Kitts, 2006; Meisel et al., 2003).

Currently, commercially available CPP preparations are obtained from casein using different pancreatic endoproteinases to release the clusters of three-phosphorylated pentapeptides (McDonagh & FitzGerald, 1998). The most prevalent technological process uses sodium caseinate as raw material and trypsin as the proteolytic enzyme. The addition of $CaCl_2$ and Na_2HPO_4, followed by ultrafiltration, gives rise to casein hydrolysates containing a CPP-ACP percentage ranging from 6 to 22% (Ellegard, Gammelgard-Larsen, Sørensen, & Fedosovc, 1999; Reynolds, 2008, 2011).

Notably, the exposure time of teeth to CPP-ACP appears to be a crucial parameter for the remineralising effect (Nongonierma & FitzGerald, 2012). To date, CPPs included in vehicles such as toothpaste, lozenges and chewing gum are available in the nutraceutical market. Chewing gum, in particular, presents the advantage of a longer residence time in the mouth compared with toothpaste or mouthwash (Cai et al., 2009; Sanares, King, Itthagarun, & Wong, 2009). However, the remineralising effect of CPPs, alone or included in a vehicle, has been evaluated in both in vitro and in situ studies by assessing hydroxyapatite or calcium ion occlusion into enamel lesions (Ferrazzano et al., 2007; Reynolds, 1997). For this reason, in the present study, gum fortified with 10% of a commercial casein hydrolysate, previously analysed for qualitative-quantitative CPP composition, has been produced and chewed with artificial and human saliva. During each chewing process, salivary samples were collected at different times and analysed by high performance liquid chromatography (HPLC) coupled with matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry. The objective was to define the kinetics of CPP release to evaluate the preventive use of CPPs against caries disease.

2. Materials and methods

2.1. Samples

Two samples of bovine casein hydrolysate were analysed: a commercial hydrolysate (CH) product (LACPRODAN DI-2021, purchased from Arla Foods company) and a standard tryptic hydrolysate (SH) of bovine isoelectric casein obtained from raw bulk milk (Aschaffenburg & Drewry, 1959).

2.2. Trypsin enzymatic digestion of casein

The enzyme/substrate ratio was 1:50 (w/w) in 50 mM Tris–HCl buffer, pH 7.8 containing 0.2 M KCl, 4.5 M urea, and 10 mM DTT. The reaction was carried out at 37 °C for 16 h and stopped by adding 2 mL of 4 M trifluoroacetic acid (TFA). The hydrolysate was freeze-dried and stored at −20 °C before use. The enzymatic digestion was performed twice.

2.3. Chemical analysis

2.3.1. Determination of CPP content

The CPP content in the commercial hydrolysate (CH) was assessed by gravimetric analysis (Ferranti et al., 1997). Briefly, 1 mL 10% barium nitrate solution [$Ba(NO_3)_2$] and 50/50 water/ethyl alcohol (30 mL^{-1}) was added to a 10% aqueous solution of CH sample (5 g per 50 mL^{-1}). The suspension was centrifuged (4000×*g*, 10 min), the pellet dissolved in deionised water, acetone was added (10 mL^{-1}) and pH adjusted to 4.6 with hydrochloric acid. The new pellet was recovered by centrifugation (4000×*g*, 10 min), freeze-dried and weighed. The determination was performed in triplicate, and the powdered sample was stored at −20 °C before use.

2.3.2. Determination of calcium content

Total calcium determination was carried out according a modified version of the procedure of Kindstedt and Kosikowski (1985). Briefly, 3 g of dry ash sample was dissolved in water, then titrated with ethylenediamine tetra-acetate, as described by Ntailianas and Whitney (1965).

2.3.3. Determination of the different nitrogen fraction content in casein hydrolysate

Different nitrogen fractions of CH, i.e., total nitrogen (TN), soluble nitrogen fraction in pH 4.6 acetate buffer (SN), 12% trichloroacetic acid (TCA) soluble nitrogen fraction (NPN, non-protein nitrogen) and degree of hydrolysis (DH, %SN/TN), were prepared and determined according to the method of IDF (1993), using a nitrogen protein conversion factor of 6.38.

2.4. In vitro and in vivo chewing study

2.4.1. Chewing gum preparation

Gum preparation, in vivo mastication and in vitro mastication were carried out following the procedure of Di Stasio, Nazzaro, and Volpe (2013) as summarised below. The components of the chewing gum with relative percentages were: gum-base (69%), CH (10%), flavouring agents and sweeteners (20%), co-adjuvant excipients (talc, magnesium stearate; 1.0%). These were blended in a laboratory mixer (HulaMixer™ Sample Mixer) and produced by a single-punch tableting machine (Matrix 2.2 A, Ataena Srl, Ancona, Italy) at room temperature.

2.4.2. Mechanical chewing

The device for mechanical chewing, well described in Di Stasio et al. (2013), consisted of a thermostatic cell in which a vertically oriented piston holding an upper chewing plate was mounted, with a second chewing plate on the lower cell surface. The cell, filled with 50 mL of artificial saliva and the chewing gum, was loaded onto the lower chewing surface. The chewing procedure consisted of up and down strokes of the upper surface, providing mastication of the chewing gum. The temperature of the test medium was 37 °C and the chew frequency was 50 ± 2 strokes per min. At predetermined time intervals, 500 μL of supernatant was recovered. The dissolution medium was replaced with fresh artificial saliva after each sampling. Three basic tests were carried out, differing from each other in the chewing duration (5, 10 and 20 min). Each sample was analysed in triplicate. In each experiment, saliva was collected, filtered, freeze-dried and stored at −20 °C before use.

2.4.3. Preparation of artificial saliva

The artificial saliva consisted of an aqueous solution of 14 mM sodium chloride, 16.1 mM potassium chloride, 1.7 mM calcium chloride dihydrate, 0.54 mM magnesium chloride hexahydrate, and 1.49 mM potassium phosphate dibasic, pH 5.7.

2.4.4. Human saliva collection

The in vivo chewing experiment was performed by three volunteers. Each volunteer chewed 1 g of gum for 5, 10 and 20 min. At each time point, the saliva was collected, lyophilised and stored at −20 °C before use.

2.5. Reversed-phase high-performance liquid chromatography analysis

Samples (casein hydrolysate, artificial and human saliva) were dissolved in 0.1% TFA at a 2 mg mL^{-1} concentration and loaded onto a Vydac reversed-phase (RP) C_{18} column (250 mm, 2.1 mm i.d.). Samples of 100 μL were injected onto a high-performance liquid

chromatography (HPLC) system, and peptide mixtures were separated by applying a linear gradient from 5 to 60% of solvent B (0.1% TFA in acetonitrile, v/v) over 60 min at a constant flow rate of 0.2 mL min^{-1}; solvent A was 0.1% TFA in water (v/v). The column effluent was monitored by UV detection at 220 nm.

2.6. Matrix-assisted laser desorption/ionisation-time-of-flight-mass spectrometry analysis

Mass spectrometry experiments were carried out by a Voyager DE-PRO TOF mass spectrometer (PerSeptiveBiosystems, Framingham, MA, USA) equipped with N_2 laser (337 nm, 3 ns pulse width). Dihydroxybenzoic acid was used as a matrix. The sample (1 μL from a solution in water) was loaded on the target and dried. Afterwards, 1 μL of a mixture of 10 g L^{-1} dihydroxybenzoic acid containing 0.1% phosphoric acid in H_2O/acetonitrile (1/1, v/v) was added. For each sample, mass spectrum acquisition was performed in positive reflector mode accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed using the signal of the matrix dimer together with the monoisotopic masses of peptide standards, including renin substrate (MW 1761), bovine β-casein f(33–48) 1P (MW 2061.83) and bovine β-casein f(1–25) 4P (MW 3122.27) peptides. The mass resolution in the working *m/z* range was >10,000. Post-source decay (PSD) fragment ion spectra were obtained after isolation of the appropriate peptide precursor using timed ion selection. Fragment ions were refocused onto the final detector by stepping the ratio of the voltage applied to the reflector to 1.0000 (precursor ion segment), 0.9126, 0.6049, 0.4125, 0.2738 and 0.1975 (fragment ion segments) and recording data at the digitisation rate of 20 MHz. All precursor ion segments were acquired at low laser power to avoid saturating the detector; the laser power was increased for all of the remaining segments of the PSD acquisitions. Typically, 200 laser shots were acquired for each fragment ion segment. The individual segments were finally combined using the software developed by PerSeptiveBiosystems for data handling. Raw data were processed using the software program Data Explorer version 4.0 (Applied Biosystems).

3. Results and discussion

3.1. Chemical analysis

The nutraceutical functionality of casein hydrolysate for remineralisation purposes is strictly dependent on the quantity and bioavailability of soluble CPPs-ACP complex, which assures, in this form, calcium transport across the distal small intestine. For this aim, nitrogen fractions, CPPs and calcium content were assessed and DH (degree of hydrolysis) was calculated (Table 1). Enzymatic digestion carried out on the raw material, namely casein or caseinate, gave rise to a high amount of SN, which allowed the determination of a 72.2% DH and a 12% TCA-NPN fraction containing oligopeptides, CPPs included, accounting for 38.3% of TN. Moreover, the calculated percentage of CN indicates that 26.1% of TN consists of native casein and/or very large peptides while the remaining percentage (6%) did not contain nitrogen (Table 1).

Table 1
Protein composition, degree of hydrolysis, calcium and casein phosphopeptide content in a commercial casein hydrolysate (CH).[a]

Parameter	CH
Protein (%, w/w)	
TN	94.0 ± 2.1
SN	67.9 ± 1.8
NPN	38.3 ± 1.1
CN	26.1 ± 0.9
DH (%, w/w)	72.2
Calcium (%, w/w)	0.30 ± 0.0
CPP (%, w/w)	17.0 ± 0.8

[a] Abbreviations are: TN, total nitrogen; SN, soluble nitrogen; NPN, non-protein nitrogen; CN, casein nitrogen; DH, degree of hydrolysis (SN/TN); CPP, casein phosphopeptide. Values are the means of 3 replicates ± standard deviations.

The low assessed calcium content (0.3%) can be explained by taking into account the demineralisation of caseins taking place during the acidification of milk carried out in the industrial preparation.

Furthermore, the content of CPPs represents 17% of the CH composition. In this regard, a very high CPP/Ca (w/w) ratio (56.6) was calculated. In studies carried out on the remineralising effects of CPPs on tooth enamel (Ferrazzano et al., 2007; Reynolds, 1998; Reynolds, Riley, & Adamson, 1994), this positive effect was reached using 1% CPP stabilising 60 mM $CaCl_2$ at pH 7 corresponding to a calculated CPP/Ca ratio of 1:2.4. These data suggest the need for calcium supplementation of such hydrolysates before use.

3.2. Mass spectrometry analysis of standard casein tryptic digest

The technological parameters (pH of hydrolysis, heat treatment) involved in casein hydrolysate production can result in a loss of phosphate groups as a consequence of β-elimination reactions (Calabrese, Mamone, Caira, Ferranti, & Addeo, 2009). For this reason, a standard tryptic digest of whole casein obtained from raw whole cow milk was prepared and determined, on analysis with MALDI-TOF-MS (data not shown), to have an available standard CPP composition from unheated milk. Among the peptides identified (results not shown) thirteen were CPPs, six of which were derived from α_{S1}-casein (α_{S1}-CN), four from α_{S2}-CN and three from β-CN (Table 2). In particular, two forms of α_{S1}-CN(f59–79)5P differing from each other in the modification of N-terminal Gln_{59} to pyroglutamic acid (pyroGln) were found, as were two phosphorylated forms of α_{S2}-CN(f46–70) peptide, differing from each other in one phosphate (4P and 5P). This occurrence, as reported above, is due to the different kinase specificity on Ser or Thr phosphorylation in the triplet code [Ser/Thr-X-Glu]. As expected on the basis of related primary structure and the specificity of action of trypsin, nine of the identified CPPs (3 α_{S1}-, 4 α_{S2}- and 2 β-CN) contain the three-phosphorylated cluster enabling ACP stabilisation in the CPP-ACP complex (Reynolds, 1998).

3.3. Mass spectrometry analysis of commercial casein hydrolysate

To evaluate the peptide composition of the CH sample, MALDI-TOF analysis was used, the relative mass spectrum was registered (Fig. 1) and the peptides were identified (Table 3). The results show that this product contains a mixture of eight non-phosphorylated peptides and five CPPs, two mono-phosphorylated [α_{S1}-CN(f104–119)1P, α_{S2}-CN(f22–41)1P], and three containing the phosphoseryl cluster [α_{S2}-CN(f2–21)4P, β-CN(f1–25)3P, β-CN(f1–25)4P]. This CPP composition, lacking α_{S1}-CN derived peptides containing the three-phosphorylated pentapeptide clusters, seems poorer than that of SH (Table 3) as well as that of similar commercial products (Cross et al., 2007; Reynolds, 2008) having, among others, the CPP α_{S1}-CN(f59–79)5P. At the same time, it is known that these peptides have low ionisation power, in particular of their phosphate group, when analysed in mixture with other non-phosphorylated peptides by MALDI-TOF-MS in positive mode (Mamone, Picariello, Ferranti, & Addeo, 2010). For this reason, an enriched CPP fraction (EF) from CH hydrolysate was prepared by addition of barium nitrate $Ba(NO_3)_2$, then analysed by MALDI-TOF analysis (Fig. 1B), and the identified peptides are reported in Table 3.

Table 2
Phosphorylated peptides identified by MALDI-TOF MS analysis in the standard tryptic casein digest from raw whole cows' milk.

Peptide	Peptide sequence	Theoretical molecular mass (Da)	Determined molecular mass (*m/z*)	Phosphate residues per mole
α_{S1}-CN(f106–119)	VPQLEIVPNSAEER	1659.8	1660.8	1
α_{S1}-CN(f43–58)	DIGSESTEDQAMEDIK	1927.7	1927.7	2
β-CN(f33–48)	FQSEEQQQTEDELQDK	2060.8	2061.8	1
α_{S2}-CN(f2–21)	NTMEHVSSSEESIISQETYK	2619.0	2618.3	4
α_{S1}-CN(f37–58)	VNELSKDIGSESTEDQAMEDIK	2678.1	2678.0	3
α_{S1}-CN(f59–79)	pyro-QMEAESISSSEEIVPNSVEAQK	2702.9	2703.9	5
α_{S1}-CN(f59–79)	QMEAESISSSEEIVPNSVEAQK	2721.1	2720.9	5
α_{S2}-CN(f1–21)	KNTMEHVSSSEESIISQETYK	2746.0	2747.0	5
α_{S1}-CN(f35–58)	EKVNELSKDIGSESTEDQAMEDIK	2934.2	2935.1	3
α_{S2}-CN(f46–70)	NANEEEYSIGSSSEESAEVATEEVK	3007.1	3008.0	4
α_{S2}-CN(f46–70)	NANEEEYSIGSSSEESAEVATEEVK	3087.0	3087.9	5
β-CN(f1–25)	RELEELNVPGEIVESLSSSEESITR	3042.4	3042.4	3
β-CN(f1–25)	RELEELNVPGEIVESLSSSEESITR	3122.4	3123.3	4

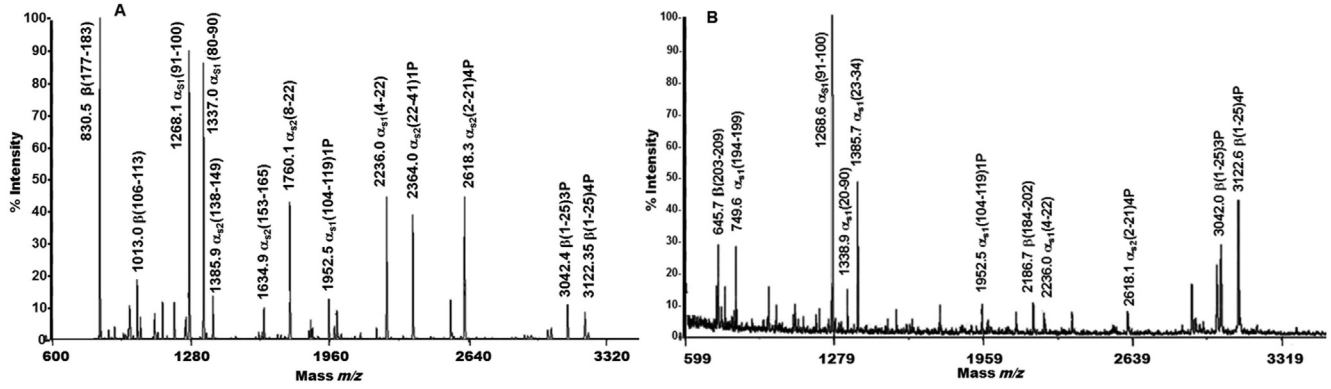

Fig. 1. MALDI-TOF mass spectrum of peptides contained in commercial casein hydrolysate (A) compared with its enriched CPP fraction (B).

Table 3
Peptides identified by MALDI-TOF MS analysis in commercial hydrolysate, its enriched caseinphosphopeptide fraction, in artificial saliva by mechanically chewing and in human saliva after in vivo chewing.[a]

Molecular mass (*m/z*)		Peptide	Peptide sequences	CH	EF	MC	VC_5	VC_{10}	VC_{20}
Theoretical	Measured								
645.4	645.7	β-CN(f203–209)	GPFIIV		EF				
679.5	679.6	α_{S1}-CN(f101–105)	LKKYK				VC	VC	VC
746.5	745.3	α_{S2}-CN(f200–205)	VIPYVR			MC			
748.4	749.3	α_{S1}-CN(f194–199)	TTMPLW		EF	MC	VC	VC	VC
780.5	780.5	β-CN(f170–176)	VLPVPQK			MC	VC	VC	
830.4	830.5	β-CN(f177–183)	AVPYPQR	CH		MC	VC	VC	VC
903.5	903.4	α_{S2}-CN(f182–188)	TVYQHQK			MC			
1013.5	1013.0	β-CN(f106–113)	HKEMPFPK	CH		MC	VC	VC	
1098.6	1098.6	α_{S2}-CN(f189–197)	AMKPWIQPK			MC	VC	VC	VC
1267.7	1268.1	α_{S1}-CN(f91–100)	YLGYLEQLLR	CH	EF	MC	VC	VC	VC
1337.7	1337.8	α_{S1}-CN(f80–90)	HIQKEDVPSER	CH	EF	MC	VC	VC	VC
1384.7	1384.9	α_{S1}-CN(f23–34)	FFVAPFPEVFGK		EF	MC	VC	VC	VC
1386.6	1385.9	α_{S2}-CN(f138–149)	TVDMESTEVFTK	CH					
1633.9	1634.9	α_{S2}-CN(f153–165)	LTEEEKNRLNFLK	CH		MC			
1759.9	1760.1	α_{S1}-CN(f8–22)	HQGLPQEVLNENLLR	CH		MC	VC	VC	
1951.9	1952.5	α_{S1}-CN(f104–119)1P	YKVPQLEIVPNSAEER	CH	EF	MC			
2186.2	2187.0	β-CN(f184–202)	DMPIQAFLLYQEPVLGPVR		EF	MC	VC		
2235.2	2236.1	α_{S1}-CN(f4–22)	HPIKHQGLPQEVLNENLLR	CH	EF	MC	VC	VC	
2363.0	2364.6	α_{S2}-CN(f22–41)1P	QEKNMAINPSKENLCSTFK	CH		MC			
2553.2	2553.9	α_{S2}-CN(f138–158)1P	TVDMESTEVFTKKTKLTEEEK			MC	VC		
2619.0	2618.3	α_{S2}-CN(f2–21)4P	NTMEHVSSSEESIISQETYK	CH	EF	MC	VC	VC	VC
3042.4	3042.4	β-CN(f1–25)3P	RELEELNVPGEIVESLSSSEESIT	CH	EF				
3122.4	3123.3	β-CN(f1–25)4P	RELEELNVPGEIVESLSSSEESIT	CH	EF	MC	VC		
Total				13	11	19	15	12	8

[a] Abbreviations are: CH, commercial hydrolysate; EF, enriched CPP fraction; MC, artificial saliva by mechanically chewing; VC_5, VC_{10}, VC_{20}, human saliva after in vivo chewing at 5, 10, 20 min, respectively.

However, the enrichment procedure, even though it intensified the signals of the two β-CN(f1–25) phosphorylated peptides, resulted in a decrease in the α_{S2}-CN(f46–70)4P signal and the loss of the α_{S1}-CN(f22–41)1P fragment (Fig. 1B). This behaviour shows that, as in the case of ferric ion (Kibangou et al., 2005), the barium-binding capacity of CPPs depends on their structural and/or conformational features. Moreover, the presence of non-phosphorylated peptides in the EF sample suggested that another group, such as carboxyl, may also be involved in calcium binding, enabling an increase in the free calcium content of the hydrolysate (Meisel, 1998).

3.4. *In vitro and in vivo release of CPPs from gum fortified with 10% casein hydrolysate*

3.4.1. *High performance liquid chromatography analysis*

To study the kinetics of CPP release in vitro, gum fortified with 10% CH was mechanically chewed in artificial saliva (MC) for 5, 10 and 20 min. At the end of each chewing time, the three collected saliva samples were analysed by RP-HPLC giving rise to three very similar elution profiles, of which a representative one is shown in Fig. 2A. These preliminary results indicate the inability of the gum to gradually release peptides, since the release occurred in the first 5 min of chewing. From a qualitative point of view, the highest heterogeneity of peptide composition can be observed in the hydrophobic zone of the elution gradient (Fig. 2A).

The RP-HPLC profiles of human saliva collected after 5 (VC_5), 10 (VC_{10}) and 20 min (VC_{20}) of human gum chewing are shown in Fig. 2B. Unlike the in vitro counterpart, a quantitative decrease of extracted peptides during the chewing time was observed in RP-HPLC profiles, obtained by operating at the same full scale value [Fig. 2B(i)]. In contrast, a very similar peptide composition can be observed in the hydrophobic zone of the elution gradient, through the zooming of chromatogram profiles [Fig. 2B(ii)]. These quantitative differences, compared with the respective MC results (Fig. 2A), depend not only on the saliva composition but also on the characteristics of the reservoir where the mastication occurs. As reported in the Materials and Methods section, for each extraction time, both saliva volume and composition remained unchanged during the mechanical chewing; in the in vivo test, however, the chewing of gum increased salivation, with consequent dilution of the peptides until their disappearance with physiological swallowing.

3.4.2. *Mass spectrometry analysis*

MALDI-TOF-MS analysis carried out on the same samples, according to the above HPLC results, gave rise to the representative mass spectrum shown in Fig. 3A, and the identified peptides are reported in Table 3. By comparison with the peptide composition of CH, the highest number of peptides was released in artificial saliva (19 versus13), of which five are CPPs as in the CH sample (Table 3). In particular, the β-CN(f1–25)3P CPP was not extracted by artificial saliva; instead, α_{S1}-CN(f138–158)1P appears.

An additional six peptides, unfound in CH sample and with low molecular mass, determined the highest heterogeneity of MC in the low mass range 746.5–1384.7 kDa of MALDI-TOF spectrum.

Moreover, the use of external standard synthetic peptides [β-CN(f1–25)4P, β-CN(f16–40)1P, α_{S2}-CN(f2–21)4P] in MALDI-TOF analysis allowed the assessment of an 80% recovery yield of hydrolysate components. This decreased recovery rate may depend on the absorption capacity or affinity of the peptides for the gum.

In agreement with the RP-HPLC results [Fig. 2B(i)], the MALDI-TOF spectra of human saliva extracts (Fig. 3B) showed a general decrease in the number and the signal intensity of released peptides as chewing time increased (Table 3). After 5 min chewing, 15 peptides were detected in human saliva of which 14 were common to the MC counterpart. On the basis of relative signal intensity, a gradual release of some non-phosphorylated peptides occurs during the chewing time, such as the increased α_{S1}-CN(f101–105) and decreased α_{S1}-CN(f91–100) peptide signals. Regarding the former,

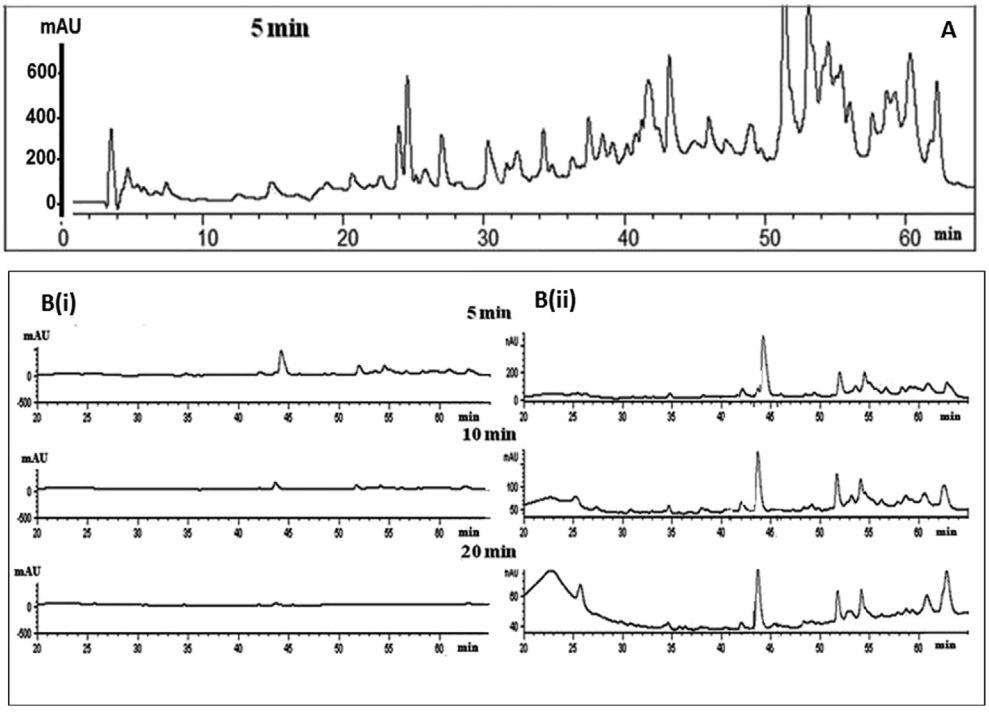

Fig. 2. RP-HPLC profiles of peptides released in artificial saliva by mechanically chewed gum (A), compared with those released in human saliva from chewed gum at 5, 10 and 20 min (B): B(i), chromatographic profiles obtained operating at the same full scale value; B(ii) obtained from B(i) by zooming.

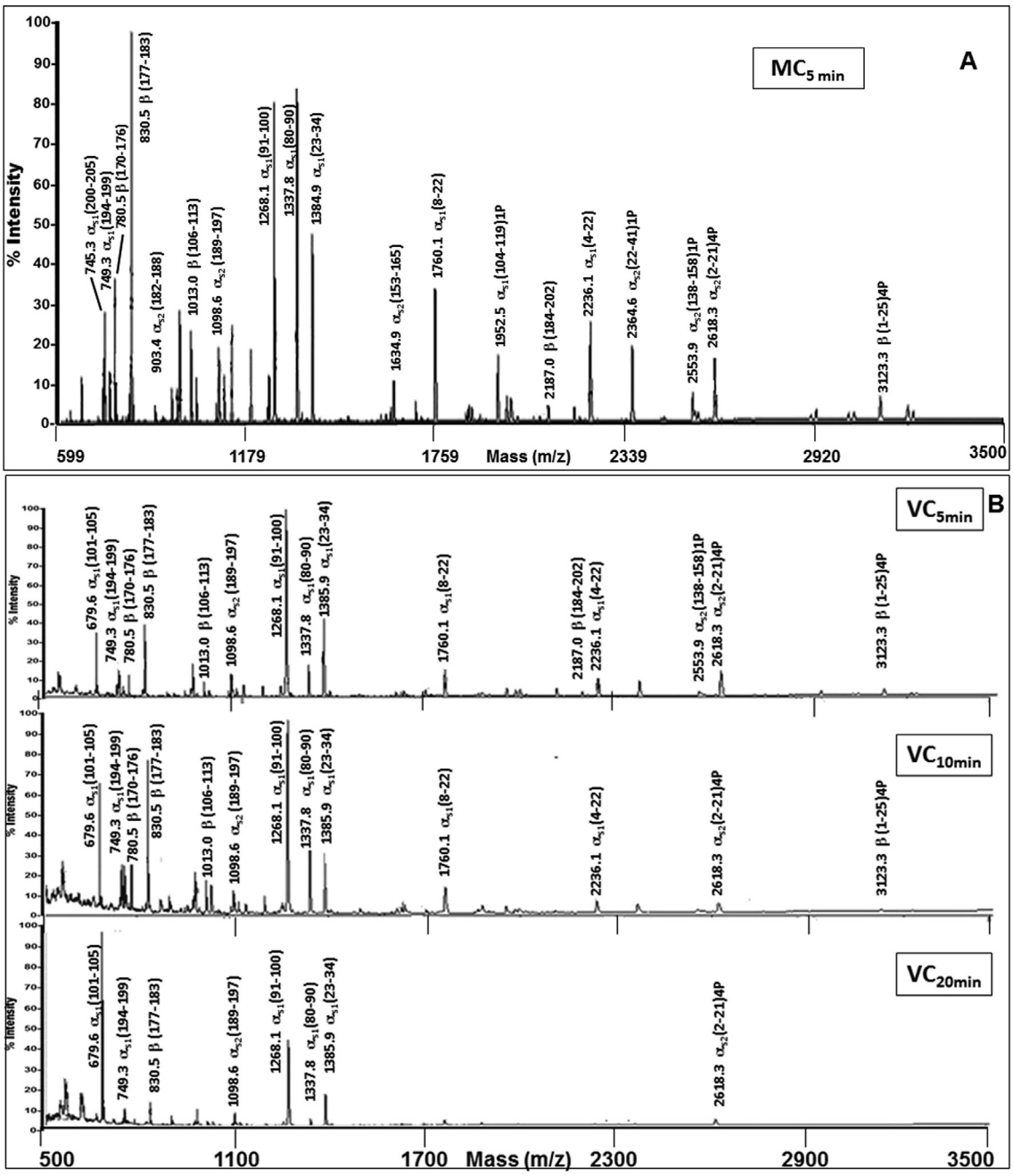

Fig. 3. MALDI-TOF mass spectra of peptides released in artificial saliva by (A) mechanically chewed gum at 5 min ($MC_{5\ min}$) compared with those released in human saliva (B) from chewed gum at 5, 10 and 20 min (VC_5, VC_{10}, VC_{20}, respectively).

since it has only been found in human saliva, further studies are needed. In the same way, a gradual selective decrease of CPPs occurs, three [α_{S2}-CN(f138–158)1P, α_{S2}-CN(f2–21)4P and β-CN(f1–25)4P] after 5 min and just one [α_{S2}-CN(f2–21)4P] after 20 min. These results show a higher affinity for the gum of α_{S2}-CN(f2–21)4P than β-CN(f1–25)4P having the same number of phosphate groups. Finally, by using standard synthetic peptides, the calculated recovery yield of released peptides after 5, 10 and 20 min was calculated as 10, 2 and 0.5%, respectively.

4. Conclusions

Fortified gum including CPPs extracted from commercial casein hydrolysate can be a useful medium for CPP release in the oral cavity during chewing. However a further calcium addition is aimed at enriching the CPP fraction of commercial hydrolysate employed in fortified gum. Finally, an "omic" approach was a powerful tool for underlining the different CPPs released during mechanical and in vivo chewing.

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