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Ex vivo degradation of β-Casomorphin-7 by human plasma peptidases: Potential implications for peptide systemic effects

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ABSTRACT

Susceptibility to proteolytic activity is a critical limitation for food-derived peptides possibly influencing human physiological processes. This study explores the *ex vivo* stability and degradation kinetics of the milk-derived opioid peptide β-casomorphin-7 (BCM7) in human blood. Blood specimens collected from three healthy volunteers were individually spiked with synthetic BCM7 and sampled at seven time points over 2 h. Liquid chromatography-electrospray-high resolution tandem mass spectrometry was used to monitor the stability of BCM7 and the formation of its hydrolytic fragments. Human plasma peptidases rapidly hydrolyzed BCM7 generating inactive peptides with similar sharp degradation kinetics across the blood of different individuals. The estimated plasma half-life ($t_{1/2}$) value of BCM7 ranged from 35 to 40 min. The peptide degradation pattern pointed to prolyl oligopeptidase, prolidase, and dipeptidyl peptidase as the primary enzyme candidates responsible for BCM7 hydrolysis. Overall, the findings of this study suggest that BCM7 cannot exert systemic effects in humans.

1. Introduction

The inventory of food-bioactive peptides has seen a significant expansion in recent decades. These functional peptides from a wide range of food protein sources offer the potential for a diverse array of biological functions. However, their claimed health benefits in humans remain elusive and controversial, as they often rely on sporadic intervention studies (Foltz et al., 2010, Nongonierma & FitzGerald, 2015). The disparity between *in vitro* bioactivity and *in vivo* efficacy of food-derived bioactive peptides often arises from the oversight of their post-ingestion behavior, which includes factors as stability against gastrointestinal proteases/peptidases, absorption, persistence during potential transepithelial translocation, plasmatic half-life, hepatic/renal metabolism, and the capacity to access and interact with target organs (Amigo & Hernandez-Ledesma, 2020; Caira et al., 2022a).

Among the food-derived bioactive peptides, β-casomorphins (BCMs) have been extensively studied, primarily due to their alleged associations with adverse effects on human health, including type-1 diabetes, metabolic syndrome, cardiovascular diseases, and autism spectrum disorders. Initially, the link between dietary BCMs and the etiopathology of type-1 diabetes was posited based on epidemiological evidence (Woodford, 2009; Woodford, 2021). According to the mechanisms underpinning this hypothesis, β-casomorphin-7 (BCM7), specifically the fragment 60–66 (YPFPGPI) of bovine β-casein (β-CN), would be released

Abbreviations: BCM7, β-casomorphin-7; BCM6, β-casomorphin-6; BCM5, β-casomorphin-5; BCMs, β-casomorphins; BBM, brush border membrane; LC-ESI-MS/MS, liquid chromatography-electrospray-high resolution tandem mass spectrometry; MALDI-TOF-TOF-MS, matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; RT, retention time; POP, prolyl oligopeptidase; DPPs, dipeptidyl peptidases; ACE, angiotensin-I-converting enzyme.

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during gastrointestinal digestion from the A1 genetic protein variant to a much greater extent than from the A2 counterpart. This distinction arises from the single amino acid substitution Pro^{67} (A2) → His^{67} (A1), which renders the His^{67}-Ile^{68} of β-CN variant A1 a scissile peptide bond. Consequently, BCM7 would be produced through carboxypeptidase processing of peptides mainly derived from variant A1-containing milk (Jinsmaa & Yoshikawa, 1999; Kaminski et al., 2007). However, recent studies have demonstrated that human digestive enzymes can generate BCMs from both β-CN variants (Asledottir et al., 2017; Cattaneo et al., 2023; Lambers et al., 2021). The putative deleterious effects of BCMs were substantiated by elevated antibodies against the A1 variant of β-CN in the plasma of individuals with type-1 diabetes (Padberg et al., 1999). Furthermore, the presence of BCM7 was immunochemically detected in the urine of autistic children (Sokolov et al., 2014) and in the serum of infants experiencing life-threatening apnea (Wasilewska et al., 2011). Opioid-like actions of BCM7 influencing high-fat consumption and neuronal development were demonstrated in rats through intraperitoneal or intracerebellar administration (Dubynin et al., 2008; Lin et al., 1998). In a broader context, BCM7 and various BCM precursors were detected in human small intestinal effluents following milk ingestion (Boutrou et al., 2013; Sanchon et al., 2018), with the observed quantities compatible with a potential local μ-opioid agonist activity, as indicated by the half-maximal inhibitory concentration determined *in vitro* (Yoshikawa et al., 1994). Although at limited amounts, BCM7 and its opioid-active derived peptides BCM6 and BCM5, *i.e.*, β-CN f(60–65) and f(60–64), were identified in milk protein digests resulting from *in vitro* simulated gastrointestinal digestion integrated with jejunal brush border membrane (BBM) enzymes (Picariello et al., 2015) as well as in the blood serum of human infants whose mothers consumed bovine milk (Wasilewska et al., 2011). Despite having a proline-rich motif, BCM7 (YPFPGPI) was shown to undergo cleavage during *in vitro* simulated gastrointestinal digestion yielding at least three proteolytic fragments, namely β-CN f(62–66) (FPGPI), β-CN f(60–65) (YPFPGP), and β-CN f (61–66) (PFPGPI) (Picariello et al., 2015). Furthermore, BCM7 was demonstrated to be susceptible to the action of jejunal BBM peptidases that, under physiologically relevant conditions, degraded 42% and 79% of the initial peptide after 2 and 4 h of digestion, respectively. In comparison, only 5% survived after 24 h (Asledottir et al., 2019). Despite the studies reported above, several crucial questions regarding the ability of BCMs to (i) actively/passively translocate across the intestinal membrane of healthy adults, (ii) endure the action of membrane/cytoplasmic peptidases within enterocytes, and (iii) enter the bloodstream, remain substantially unproven, due to the lack of dedicated rigorous quantitative investigations based on advanced analytical techniques.

Recently, our research group implemented liquid chromatography-electrospray-high resolution tandem mass spectrometry (LC-ESI-MS/MS) to identify inactive BCM precursors and possible bioactive compounds in the plasma of human volunteers upon milk consumption. Notably, BCM-containing domains originating from A1 and A2 variants of β-CN were detected, showcasing a remarkable inter-individual pattern variability (Caira et al., 2022b). These precursors exhibited a relatively brief persistence in the plasma, indicating a transient presence in the bloodstream. None of BCM7, BCM6, and BCM5 were detected, consistent with findings from other dedicated intervention studies (Osborne et al., 2014; Teschemacher et al., 1986) and in parallel with other randomized controlled human trials showing no conclusive link between BCMs and adverse effects (Daniloski et al., 2021). Crucial kinetic parameters of BCMs, including half-life, stability, and degradation mechanisms mediated by plasma peptidases, remained unexplored.

The balance between stability and degradation of bioactive peptides circulating within the human bloodstream bears critical physiological importance, as exemplified by the fine modulation of endogenous insulin and glucagon hormones. This aspect, relevant for successfully using peptide-based drugs or biomarkers for diagnosis and prognosis, is often assessed through peptide monitoring in *ex vivo* specimens, such as blood and cerebrospinal fluid (Yi et al., 2015). The present study delved into the *ex vivo* stability and degradation kinetics of synthetic BCM7 treated with blood samples from healthy volunteers. The key aim was to shed light on BCM7's susceptibility to hydrolysis by human plasma peptidases and its potential implications for peptide systemic effects.

2. Material and methods

2.1. Reagents

Synthetic BCM7 (YPFPGPI) was synthesized using solid-phase with Fmoc chemistry (Bio-Fab Research, Rome, Italy). The peptide identity, with a monoisotopic $[M + H]^+$ ion at m/z 790.4134, was confirmed by matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry (MALDI-TOF-TOF-MS) analysis, using an UltrafleXtreme mass spectrometer (Bruker Daltonics, Bremen, Germany) and α-cyano-4-hydroxycinnamic acid (4 g/L in 60 % v/v aqueous acetonitrile containing 0.1 % v/v trifluoroacetic acid) as the matrix (Loebel et al., 2000). The peptide purity (>99 %) was assessed via reversed-phase HPLC-UV, monitored at 214 and 280 nm, using an HP1100 modular system (Agilent, Santa Clara, CA) equipped with a reversed-phase column (Aeris Widepore XB-C18, 250 x 2.1 mm, 3.6 μm, Phenomenex, Torrance, CA) running at a flow rate of 0.2 mL/min. Solvent A consisted of 0.1 % v/v trifluoroacetic acid (TFA) in water, while solvent B was 0.1 % v/v TFA in acetonitrile. After 5 min of isocratic elution at 4 % solvent B, a 4–50 % gradient of solvent B over 45 min was used for peptide separation (Allegrini et al., 2001). Chromatograms were processed using Chemstation software, v. 10.7 (Agilent). The above-reported peptide purity data were confirmed by independent LC-ESI-MS/MS experiments on the commercial product, which demonstrated the total absence of BCM7 fragments (Supporting Information Table S1). Acetonitrile, methanol, water, and formic acid were LC − MS grades from Merck (Darmstadt, Germany). Other reagents were of analytical grade and were purchased from Merck.

2.2. Blood specimens

Blood samples were collected from a cohort of healthy volunteers (N = 3) consisting of one male and two females, aged between 35 and 55. Before blood sampling, these volunteers provided informed consent and refrained from consuming milk and dairy products for at least seven days. No additional dietary restrictions were imposed. Blood was drawn by a skilled operator from the volunteers' brachial vein after 12 h of fasting and collected into multiple 3 mL vacutainer tubes containing EDTA. The collected blood specimens were gently mixed through tube inversion and immediately used for the subsequent BCM7 spiking procedures. The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committee of the University of Naples Federico II (protocol number 266/17); written informed consent was obtained from participants before starting the experimental protocol.

2.3. Ex vivo blood hydrolysis and peptide purification

To determine the optimal ratio of plasmatic enzymes to peptide substrate, various concentrations of BCM7 were spiked into the human plasma, and the corresponding experimental results were evaluated. The selection of the final BCM7 concentration used for exhaustive experiments was based on a realistic evaluation of the peptide levels detected in the human plasma, considering previous studies (Padberg et al., 1999; Sokolov et al., 2014; Wasilewska et al., 2011) and the subsequent LC-ESI-MS/MS responses of the parent compound and its proteolytic fragments (Bottger et al., 2017). The experiments were conducted in technical triplicate in sterile glass tubes. In each tube, 200 μL of EDTA-treated blood specimens were spiked with 200 μL of a BCM7 solution (10 μg/mL) in water. For an adult individual, this concentration is equivalent to the intake of about 1.5 g of β-CN, which roughly corresponds to a 200 mL serving of milk, assuming that this protein yields an

equal molar amount of BCM7 and that the peptide is fully absorbed into the bloodstream. Plasma samples without BCM7 or plasma with inactivated peptidases were also prepared as control samples. In the case of peptidase-inactivated plasma, EDTA-treated blood was acidified to a pH value of 2 with formic acid (5 μL), vigorously mixed, and then added to the BCM7 solution. In all cases, the hydrolysis process was carried out at 37 °C under gentle agitation. Aliquots (60 μL) of incubation mixtures were sampled at 0, 1, 10, 30, 60, and 120 min. The incubation mixtures and control samples were diluted ten times with 0.2 % v/v formic acid and thoroughly mixed. Subsequently, all samples were transferred to 3 kDa Amicon centrifugal filter units (Merck-Sigma) in parallel, then centrifuged at 12,000 × *g* for 30 min at 4 °C. The resulting permeate was vacuum-dried and reconstituted in 50 μL of 0.1 % v/v formic acid.

2.4. Liquid chromatography-electrospray-high resolution tandem mass spectrometry

LC-ESI-MS/MS analyses were accomplished using an Ultimate 3000 RS liquid chromatographic system coupled to an LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, Waltham, MA) operating in the positive ion untargeted data-dependent scanning mode. Without further dilution, 5 μL of the final peptide solution was injected using a refrigerated (4 °C) RS 3000 autosampler (ThermoFisher Scientific). Peptide separation was achieved on a reversed-phase column equipped with a positively charged surface (Luna Omega PS C18, 100 x 2.1 mm, 2.6 μm, Phenomenex), thermostated at 35 °C, and running at a flow rate of 0.2 mL/min. The mobile phases consisted of 0.1 % v/v formic acid in water (solvent A) and 0.1 % v/v formic acid in methanol (solvent B). The following solvent B gradient was used (minutes/%B): (0/10), (2/10), (14/95), (16/95). The electrospray interface parameters were spray voltage 4.8 kV, capillary voltage 25.0 V, and capillary temperature 300 °C. Sheath gas flow and auxiliary gas flow were maintained at 30 and 5 arbitrary units, respectively. Full scan Fourier transformed profile data were acquired within the mass scanning range of 75–1200 *m/z*. The current ion of each singly charged peptide was scanned with a mass tolerance of ± 3 ppm. In data-dependent scanning mode, MS/MS normalized collision energy was set at 35 %, activation Q at 0.25, and activation time at 25 ms. An isolation window of 1 amu was used, and the dynamic exclusion interval was 3 s. Additionally, a reject mass list was generated through blank sample injections. Data responses were monitored using Xcalibur 2.1 software (ThermoFisher Scientific).

2.5. Statistical analysis

Sample were injected in technical triplicate for each blood sample specimen, resulting in 3 observations for each time point per individual. Numerical data are reported as mean ± standard deviation. Hydrolysis results were graphically depicted, illustrating the percentage of remaining peptide (signal intensity %) versus sampling time. All statistical analyses were conducted using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA). BCM7 half-life in blood was estimated by the MS ion intensity *vs.* time plots. Experimental points were best fitted with a linear decay rate over time (min^{-1}), consistent with a zero-order kinetic decay. Statistical analysis was performed using Student's *t*-test, with a significance level set at $P < 0.05$.

2.6. Peptide identification and visualization

Peptide fragments were characterized using the Andromeda search engine within the MaxQuant suite (Tyanova et al., 2016), selecting the non-enzymatic cleavage-specificity option. Mass tolerance values were set at 5 and 10 ppm for precursor and MS/MS fragment ions, respectively. MS/MS were individually validated through manual inspection. The distribution of oligopeptides was visually represented by uploading the MaxQuant output file to the Peptigram open-source tool (*https://bioware.ucd.ie/peptigram/new*). In this representation, the height

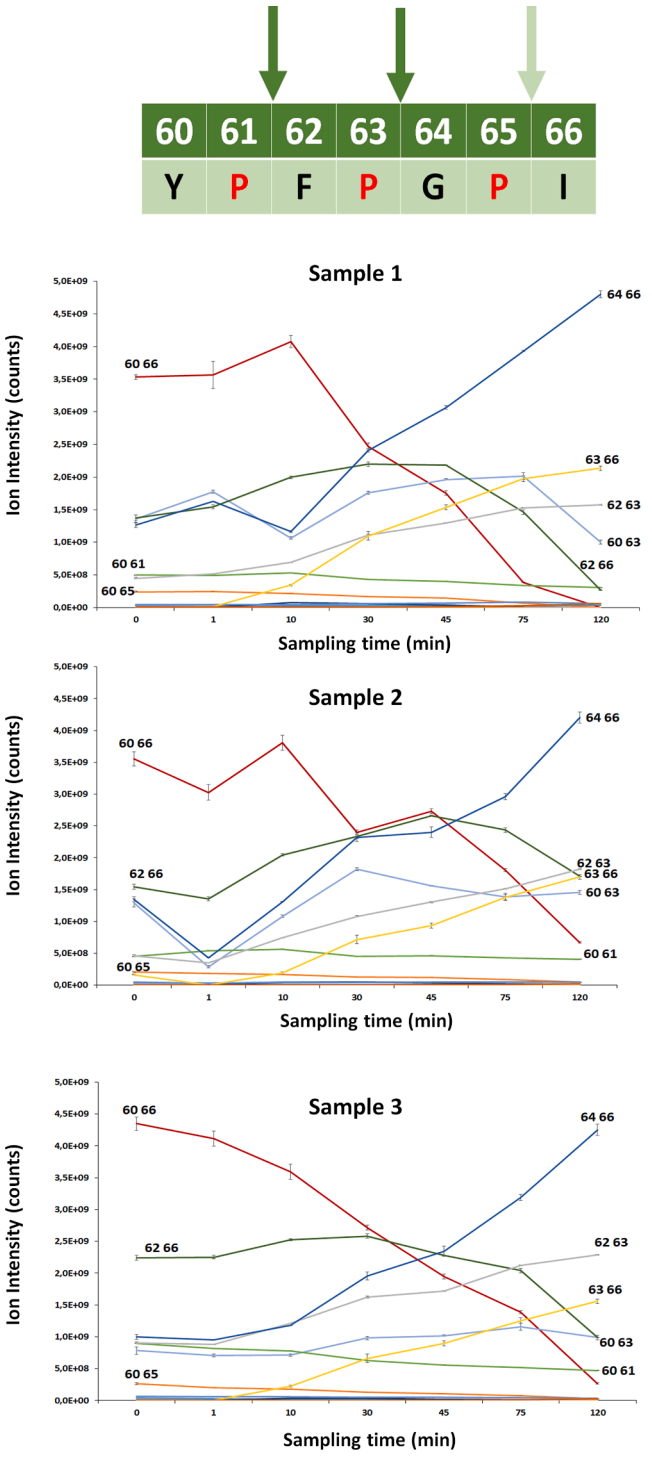

Fig. 1. Time-dependent degradation of BCM7 and evolution of derived hydrolytic fragments in blood specimens from three human individuals. Ion intensity reports signal mass spectral count values. Error bars are also reported. The time values reported in the figure do not consider the initial temporal range (about 30 s) necessary to add synthetic BCM7 to blood specimens, gently shake the resulting mixture, and then take the samples for their subsequent 10-fold dilution in 0.2 % v/v formic acid and final analysis through LC-ESI-MS/MS. The absence of BCM7 hydrolytic fragments in the synthetic peptide was confirmed by LC-ESI-MS/MS experiments on the commercial peptide product (Supporting Information Table S1).

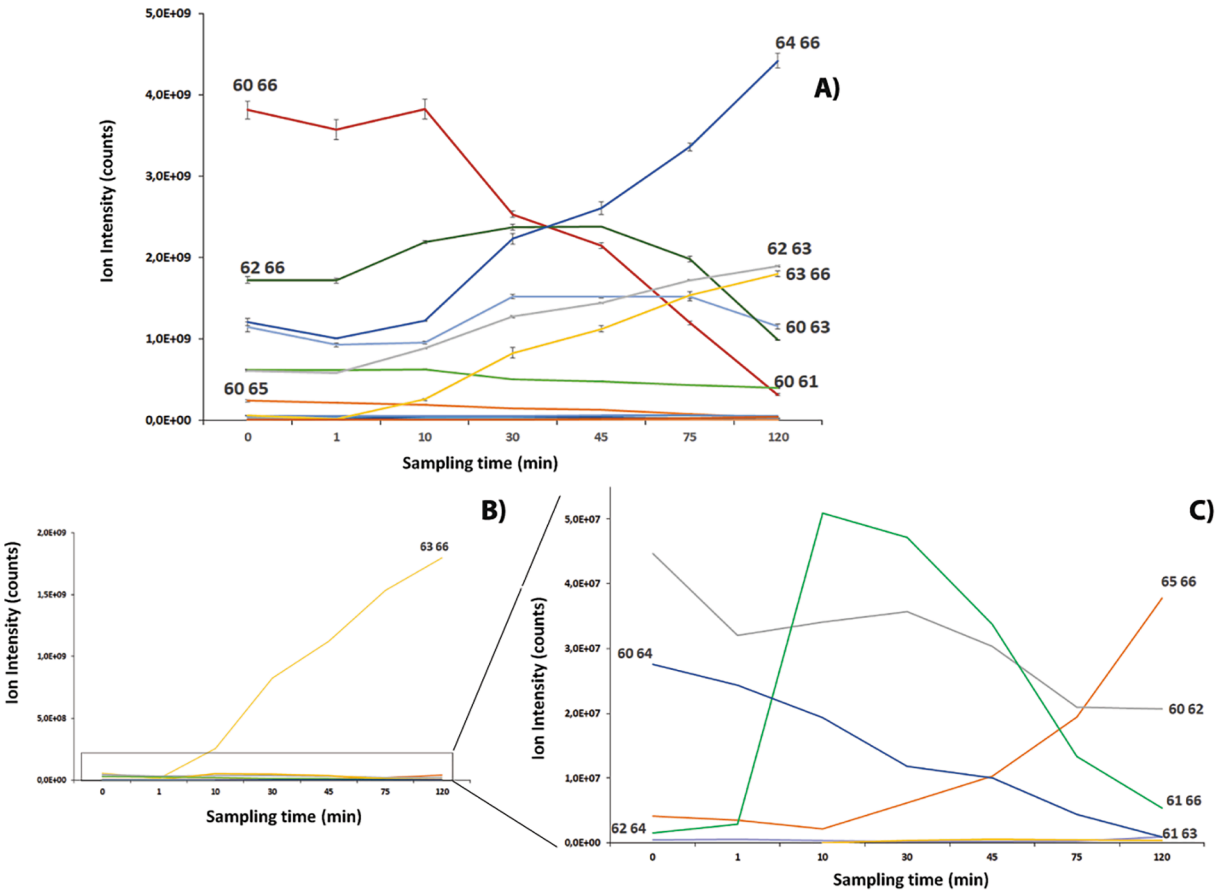

Fig. 2. Detailed evolution of the hydrolytic fragments of BCM7 was reported as average values of the three analyzed blood specimens. Panel A illustrates the formation of peptides likely generated from cleavage at internal Pro residues of BCM7. Panel B depicts the progression of peptide fragments likely resulting from the activity of exopeptidases. The inset (panel C) provides a magnified view of the low-intensity ion signals from Panel B. The time values reported in the figure do not consider the initial temporal range (about 30 s) necessary to add synthetic BCM7 to blood specimens, gently shake the resulting mixture, and then take the samples for their subsequent 10-fold dilution in 0.2 % v/v formic acid and final analysis through LC-ESI-MS/MS. The absence of BCM7 hydrolytic fragments in the synthetic peptide was confirmed by LC-ESI-MS/MS experiments on the commercial peptide product (Supporting Information Table S1).

of the bars and the green color intensity were proportional to the MS peptide ion count. This visualization provided insights into cumulative peptide intensities corresponding to their respective positions (Manguy et al., 2017).

3. Results and discussion

The assessment of peptide stability to human plasma peptidases *ex vivo* encompasses inherent complexities. Peptides navigating the bloodstream encounter diverse peptidases, each one characterized by a peculiar cleavage specificity. Numerous factors, such as the peptide/serum (plasma) ratio, the potential use of anticoagulants, preanalytical treatment(s), buffer composition, and pH value, may exert influence on the peptidase activity. Consequently, these factors can affect the kinetics of peptide degradation and the resulting hydrolytic end-products (Cavaco et al., 2021). In this study, we designed experiments to monitor the stability of BCM7 against plasma peptidases under pseudo-physiological conditions, simulating its exposure to the bloodstream. We used human EDTA-treated blood specimens without separating blood cells, enhancing the physiological relevance of our approach (Bottger et al., 2017). The primary aim was to investigate the mechanisms of BCM7 degradation, focusing on the formation of shorter fragments that could potentially retain or even enhance the bioactive properties of the parent peptide (Sánchez-Rivera et al., 2016).

3.1. *Ex vivo degradation of BCM7 in blood*

To track the *ex vivo* degradation profile of BCM7, blood specimens were collected from three healthy volunteers at seven distinct time points spanning from 0 to 120 min. Alongside the degradation profile of synthetic BCM7, we monitored the quantitative evolution of 14 peptide degradation products by LC-ESI-MS/MS. Fig. 1 illustrates the time-dependent MS signal intensities of BCM7 and its most abundant hydrolytic products; detailed quantitative information is provided in Supporting Information Table S1. Despite minor differences attributable to interindividual variability, shared trends in peptide quantities were observed in the BCM7-spiked blood specimens. Control samples exhibited no quantitative changes in BCM7 over time and the absence of peptide hydrolytic products. In particular, the recorded MS ion signal intensity values of BCM7 in peptidase-inactivated blood specimens remained constant throughout the investigated time interval, and no LC-ESI-MS/MS peaks associated with β-CN fragments f(60–65) (YPFPGP), f(60–64) (YPFPG), f(60–63) (YPFP), f(60–62) (YPF), f(60–61) (YP), f(61–66) (PFPGPI), f(61–63) (PFP), f(62–66) (FPGPI), f(62–65) (FPGP), f(62–64) (FPG), f(62–63) (FP), f(63–66) (PGPI), f(64–66) (GPI) and f(65–66) (PI) were detected (Supporting Information Table S2).

3.2. *BCM7 half-life*

BCM7 was introduced into the human blood at a concentration of 10 μg/mL (12.6 nmol/mL), which is approximately 2.5 times lower than

the value used in similar studies evaluating the stability of other synthetic peptides (Bottger et al., 2017). This chosen peptide level closely aligns with a physiologically realistic concentration range associated with milk protein consumption. Analysis of the corresponding degradation curves revealed that the estimated *ex vivo* half-life ($t_{1/2}$) value of BCM7 ranged from 35 to 40 min for all blood samples (Supporting Information Figure S1). This value is lower than those generally measured for other food-derived peptides in this body fluid (Caira et al., 2022a). Notably, consensus regarding the practical significance of $t_{1/2}$ in assessing peptide stability within the blood is yet to be universally established (Cavaco et al., 2021). Although $t_{1/2}$ is generally reported for *in vivo* studies and generally reflects a multitude of physiological and biochemical processes ongoing in the human body, whose interpretation generally requires multi-response modeling instruments able integrating different physical events, reaction pathways, mechanistic insights, and kinetic variables, even in an *ex vivo* context, as in the case reported here, it should be considered as an indicative parameter arising from a complex concomitance of diverse processes and constraints (among those reported above), rather than representing a single decay event with a precisely defined kinetics. Despite these considerations, the results of this study highlight the susceptibility of BCM7 to enzymatic degradation by plasma peptidases, as also evidenced by the progressive formation of the corresponding fragments. This observation parallels with *in vivo* studies on other bioactive peptides (Pollaro & Heinis, 2010), in which the time-dependent drop of molecular concentration observed in blood specimens was attributed to enzymatic hydrolysis rather than renal clearance. In conclusion, our findings demonstrate the high susceptibility of BCM7 to the action of plasma peptidases, suggesting that the relatively low levels of the peptide possibly entering the bloodstream undergo a significant further degradation. This declining trend implies that the potential of BCM7 to manifest systemic bioactive effects is attenuated.

3.3. Hydrolytic release of oligopeptides

Time-dependent hydrolysis of BCM7 resulted in the generation of short peptides that marked various stages of the degradation process within the bloodstream. Identifying these short peptides posed a challenge in some instances due to the possibility of generating isobaric peptides in the reaction products, such as β-CN f(61–62) (PF) and β-CN f (62–63) (FP) (with a theoretical *m/z* value of 263.1391, $[M + H]^+$), as well as β-CN f(61–64) (PFPG) and β-CN f(62–65) (FPGP) (with a theoretical *m/z* value of 417.2132, $[M + H]^+$). These isobaric peptides were definitively assigned through MS/MS sequencing following chromatographic separation. For example, Supporting Information Figure S2 shows the MS/MS spectrum of an isobaric peptide reported above, having an experimental *m/z* value of 417.2133 and eluting at a retention time (RT) of 6.48 min. Diagnostic fragment ions observed at *m/z* 302.15 (b_3 ion) and 270.15 (y_3 ion) unequivocally assigned this component to FPGP. Similarly, Supporting Information Figure S3 illustrates the fragmentation spectrum of an isobaric peptide reported above, having an experimental *m/z* value of 263.1401 and eluting with an RT of 5.76 min. Diagnostic signals observed at *m/z* 246.11, 116.07, and 120.08 were associated with the $[M-NH_2]^+$ fragment ion resulting from the loss of the amino moiety, the y_2 fragment ion, and the characteristic fragment ion of phenylalanine, respectively; they definitively assigned this component to FP. Most of these fragment ions cannot be detected in the case of PF.

The progression of all BCM7 degradation products, expressed as average values of the intensities of the three analyzed samples, is illustrated in Fig. 2. Specifically, Fig. 2A reports the formation of peptides likely generated from the cleavage at internal Pro residues of BCM7. Fig. 2B, along with the enlarged view of Fig. 2C, describes the evolution of peptide fragments likely generated by the action of exopeptidases. The appearance of fragment β-CN f(61–66) (PFPGPI) indicated the prompt aminopeptidase-mediated hydrolysis at Tyr^{60}, and the formation of a molecule devoid of a part of the tripeptide moiety essential for the interaction with opioid receptors (Tyagi et al., 2020). This component underwent a fast quantitative decline shortly after reaching its peak, sequentially yielding β-CN f(62–66) (FPGPI), β-CN f(63–66) (PGPI), and β-CN f(64–66) (GPI) as degradation products. Notably, this study did not track these accumulating peptides' subsequent evolution or clearance, which warrants dedicated investigations using ^{13}C and ^{15}N labeled precursors. Opioid-active BCM6 (YPFPGP) and BCM5 (YPFPG) were rapidly generated in small quantities and exhibited a slightly lower persistence than BCM7. In a quantitative context, it is essential to note that the intensity of the MS ion signals may not directly correlate with the quantity of the corresponding peptide fragments, as ionization efficiency can vary depending on the molecular sequence and amino acid composition. Therefore, the intensity of the MS signals was used as a pseudo-quantitative indicator.

3.4. Mechanistic insights of BCM7 degradation in blood

The most recent version of the Human Plasma Peptide Atlas database (*https://peptideatlas.org/*) comprehensively catalogs a panel of enzymatic components in human blood, including 34 aminopeptidases, 25 carboxypeptidases and various established endopeptidases. This extensive enzymatic repertoire highlights the complex biomolecular organization predisposed to degrading exogenous peptides. The degradation of BCM7 progressed through the cleavage of its internal peptide bonds and the removal of N- and C-terminal amino acids, indicating the concerted involvement of a consortium of endo- and exopeptidases operating simultaneously.

As a Pro-rich peptide, BCM7 contains three Pro-Xaa motifs, where Xaa represents any amino acid. The process of BCM7 hydrolysis within the blood specimens resulted in the generation of multiple peptides with a C-terminal proline, including β-CN f(60–61) (YP), β-CN f(62–63) (FP), β-CN f(60–63) (YPFP), and β-CN f(60–65) (YPFPGP), as well as the accumulation of β-CN f(64–66) (GPI), which is also released by the cleavage at the carboxy-side of Pro^{63} (Fig. 2A). Prolyl oligopeptidase (POP; EC 3.4.21.26), a serine protease widely represented in human tissues and body fluids, is the most likely enzyme candidate to mediate BCM7 hydrolysis in the blood (Breen et al., 2004; Garcia-Horsman, 2020). POP was already shown to efficiently cleave other circulating Pro-rich peptides in healthy individuals, while polypeptides larger than 30 residues remained unaffected (Moriyama et al., 1988). POP does not cleave all Pro-Xaa bonds with the same efficiency (Lone et al., 2010). Based on the intensity of the complementary fragments β-CN f(60–63) (YPFP) and β-CN f(64–66) (GPI), it is reasonable to consider Pro^{63}-Gly^{64} as the preferential peptide cleavage site, likely accomplished by POP. On the other hand, the formation of the relatively high-intensity fragment β-CN f(63–66) (PGPI) may be due to the action of a different endopeptidase having a cleavage specificity for aromatic amino acids (*i.e.*, Phe) (Figs. 1 and 2). Although the release of the intense complementary fragments β-CN f(60–61) (YP) and β-CN f(62–66) (FPGPI) could also be, in principle, attributed to POP activity, it more likely involves dipeptidyl peptidases (DPPs, EC 3.4.14.11) or related members of the POP family, which are selective for Xaa-Pro substrates.

3.5. Multiple degradation routes of BCM7 in blood

In principle, β-CN f(62–66) could also be generated by a double sequential aminopeptidase action, as Pro at the N-terminal position of β-CN f(61–66) (PFPGPI) can be released by prolyl aminopeptidases (EC 3.4.11.5) or even generic aminopeptidases, albeit at a slower rate compared to other N-terminal amino acids. The possibility that this degradation route may be dominant appears cumbersome. However, evidence of the simultaneous intervention of aminopeptidases was suggested by the formation of low-intensity β-CN f(61–66) and the stepwise trimming at the N-terminal end of the peptide. Similarly, the relatively intense dipeptide β-CN f(62–63) (FP) could partly result from

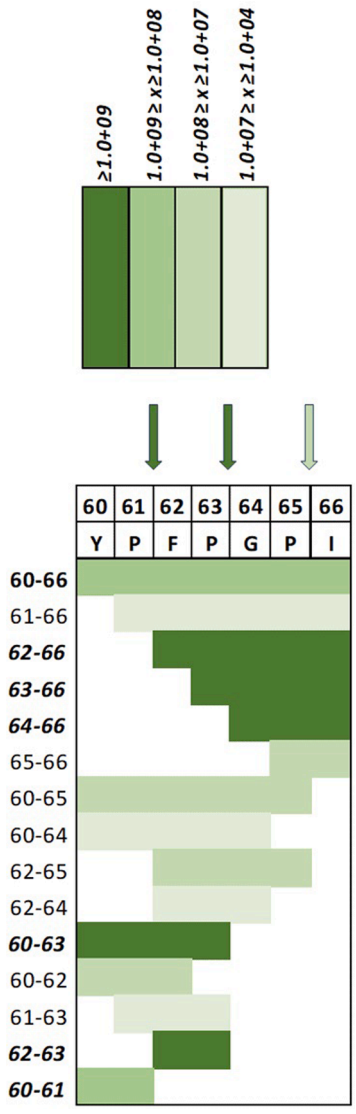

Fig. 3. Identification of *ex vivo* cleavage sites by blood peptidases in BCM7. Prolyl endopeptidase was identified as the primary candidate for hydrolyzing BCM7 into primary fragments (indicated by dark green arrows and bold italic numbers), which can be further degraded by the secondary action of amino- and carboxypeptidases. The gradation from dark to light green shades reflects decreasing MS ion signal intensity. The maximum intensity achieved by individual peptides at 120 min of time-hydrolysis is represented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a double action of a DPP, sequentially removing YP and FP. Consequently, multiple simultaneous degradation routes might contribute to the hydrolysis of BCM7 in the blood, highlighting the intricate cooperation of endo- and exopeptidases. These enzymes collectively cleave peptide bonds with varying specificity, emphasizing the complexity of BCM7 degradation within the bloodstream. An intriguing aspect arises from the ability of specific milk-derived peptides like β-CN f(59–66) (VYPFPGPI) to inhibit POP (Taraszkiewicz et al., 2023). If inhibitor sequences are formed and absorbed *in vivo*, milk consumption could modify the degradation kinetics of BCM7, adding another layer of complexity to the fate of this peptide. This suggests that various dietary factors could potentially influence the metabolism of BCM7 within the human body.

Prolidase (EC 3.4.13.9) may also play a role in truncating BCM7-derived peptides with a C-terminal Pro (Surazynski et al., 2008). This enzyme was already shown to be active on dipeptides containing C-terminal Pro, while its action on longer peptides might be much lower or absent (Eni-Aganga et al., 2021). It is worth noting that no human carboxypeptidases classified by BRENDA (*https://www.brenda-enzymes.org/*) and MEROPS (*https://www.ebi.ac.uk/merops/*) databases exhibit significant activity toward Pro residues. Nevertheless, the presence of β-CN f(60–64) (YPFPG) and β-CN f(62–64) (FPG) suggests the activity of a carboxypeptidase capable of removing C-terminal Pro residues. This enzymatic action would provide a pool of recycled Pro for protein biosynthesis.

Fig. 3 provides a snapshot of the observed cleavage sites of BCM7 in the blood. Each peptide fragment represents the maximum intensity achieved over the examined time range. The detection of BCM6 (YPFPGP) at intermediate intensity implies the presence of carboxypeptidases operating within the blood milieu. However, the progressive accumulation of β-CN f(64–66) (GPI) and β-CN f(63–66) (PGPI) over time, emerging as the most abundant peptides after 2 h-incubation (Figs. 1 and 2), suggests a reduced carboxypeptidase activity compared to the aminopeptidase counterpart. The observed removal of N-terminal Pro residues from peptides containing more than two amino acids does not seem to be attributable to prolinase (EC 3.4.13.8), which is an enzyme primarily acting on dipeptides, but rather to other aminopeptidases.

3.6. Physiological implications of BCM7 degradation

In this study, the occurrence of BMC7 in human blood was strongly associated with its degradation and the significant formation of shorter peptides, suggesting that hydrolysis is the predominant factor contributing to its decay over time. Drawing parallels from a similar study, Walsh and colleagues demonstrated that β-lactoglobulin f(142–148), a lactokinin exhibiting potent angiotensin-I-converting enzyme (ACE)-inhibitor activity *in vitro*, underwent swift degradation by blood peptidases, thereby failing to elicit any antihypertensive activity in humans (Walsh et al., 2004). In an *in vivo* setting, the formation and potential persistence of BCM7 within the bloodstream are subject to numerous factors, including the nature and structure of dairy products, individual parameters of consumers (*e.g.*, age, pharmacological therapies, meal volume, diet), co-administration of non-dairy foods, putative interaction with other food components, and intestinal permeability (van Lieshout et al., 2020). Collectively, these variegate factors underscore the complex interaction between individual characteristics and nutrition elements, ultimately shaping the metabolic trajectory of food-derived peptides, their bioavailability, and their *in vivo* impact on human physiology.

Fig. 4 summarizes the routes of BCM7 degradation. The primary pathway of BCM7 degradation initiates with the endopeptide splitting operated by POP. This is one of at least four simultaneous processes mediated by blood peptidases that can be tracked. The probable action of DPPs can also play a significant role in the primary degradation pathway. Exopeptidases can intervene at various stages of the reported routes, highlighting a tightly coordinated enzymatic interplay within the human blood, aimed at the complete breakdown of exogenous peptides. The rapid disappearance of BCM7 compared to the delayed degradation by brush border membrane enzymes (Asledottir et al., 2019) highlights the physiological necessity of a sophisticated apparatus in the human blood that functions in the post-absorption stage to inactivate potentially harmful peptides. Looking at a broader picture, the entire machinery of protein degradation, including gastroduodenal proteases, intestinal mucosal, and plasmatic peptidases, marks an evolutionary adaptation with the dual purposes of maximizing the reuse of amino acid building blocks and preventing the undue accumulation of foreign peptides in the bloodstream. This orchestrated enzymatic degradation cascade serves as a formidable defensive mechanism against the systemic impact of peptides from dietary sources, in a perspective that

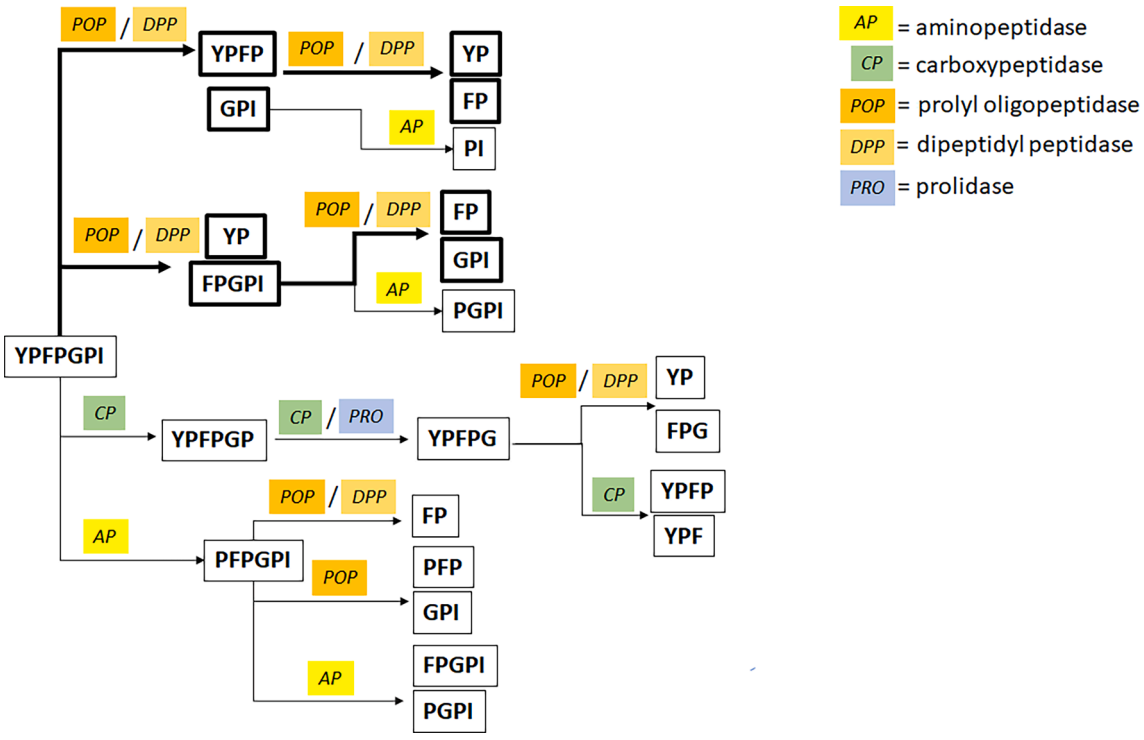

Fig. 4. Schematic reconstruction of the degradation routes of BCM7 in human blood according to the corresponding hydrolytic fragments identified in this study. Based on the intensity of these peptides, two prevailing routes have been outlined, which are represented by thicker lines. The enzymes postulated to be involved in each step are indicated with different colors. This study did not investigate the release of free amino acids; accordingly, their expected formation has been omitted.

substantially challenges the notion that food-derived peptides could exert systemic actions under physiological conditions (Miner-Williams et al., 2014). In this context, the functional deficiency of POP and prolidase as phenotypic traits of rare genetic disorders (Eni-Aganga et al., 2021), or alterations of endopeptidase/exopeptidase plasma concentrations, as occurring in aged individuals and patients with chronic liver and renal diseases, might result in a prolonged persistence of BCMs in the bloodstream.

4. Conclusion and future perspectives

In this study, we sought to contribute a pivotal piece to the complex puzzle surrounding the potential effects of BCMs on human health, exploring the *ex vivo* degradation dynamics of BCM7 within the human blood. The stability of a peptide to plasma peptidases over a suitable period is a fundamental requirement for any conceivable systemic effect. The prevailing notion that Pro-rich peptides, like BCMs, are impervious to digestive hydrolytic enzymes has been recently reevaluated, and specialized endo- and exopeptidases targeting Pro residues have been identified (Gülseren & Vahapoglu, 2022; Picariello et al., 2023). The results of this study unequivocally demonstrate that BCM7 is vulnerable to the attack of human plasma peptidases under physiologically relevant conditions. The estimated *ex vivo* $t_{1/2}$ value of BCM7 in blood was in the range 35–40 min, which is similar to that of other food-derived peptides with fast elimination kinetics or peptide drugs lacking chemical stabilization (Foltz et al., 2010; Gülseren & Vahapoglu, 2022). These results, together with other factors, such as the gastrointestinal release of BCMs in an active form, peptide vulnerability to intestinal brush border membrane peptidases (Asledottir et al., 2019), and unproven ability of these molecules to cross the human small intestinal mucosa and penetrate the blood–brain barrier, collectively lessen the likelihood that BCMs can exert systemic activities within the human body.

The continuous delivery of BCMs over the temporal course of digestion of dairy products cannot exclude harmful effects on human health that depend on chronic exposure to these peptides rather than on acute administrations. Furthermore, potential pathological effects of BCMs would likely manifest during early childhood, when milk is the primary nutritional source, and the intestinal permeability is still developing. Additionally, the intestinal bioaccessibility of BCM7, which has already been established in humans, does not rule out the possibility that BCMs agonize intestinal μ-opioid receptors of the enteric nervous system or interact with immunocompetent cells (Tyagi et al., 2020). In such a complex information landscape, it is essential to acknowledge that our findings by themselves do not provide conclusive outlines but, together with additional investigations, simply emphasize the decreased likelihood that BCMs elicit systemic effects on target receptors of organs other than intestine. Future studies with chemically labeled precursors will precisely monitor the kinetics of BCM7 in human tissues/body fluids and will uncover the true impact of this peptide on human physiology, dispelling the uncertainty that has enshrouded this field for far too long.

CRediT authorship contribution statement

Sabrina De Pascale: . **Gianluca Picariello:** Software, Formal analysis, Data curation. **Antonio Dario Troise:** . **Simonetta Caira:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Gabriella Pinto:** Writing – original draft, Formal analysis, Data curation. **Francesca Marino:** Writing – review & editing. **Andrea Scaloni:** Writing – review & editing, Writing – original draft, Funding acquisition. **Francesco Addeo:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106004.

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