

Peptic digestion of β -casein

Time course and fate of possible bioactive peptides

Christian E.H. Schmelzer^{a,*}, Regina Schöps^b, Lucy Reynell^a,
Renate Ulbrich-Hofmann^b, Reinhard H.H. Neubert^a, Klaus Raith^a

^a Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Straße 4, 06120 Halle (Saale), Germany

^b Institute of Biochemistry/Biotechnology, Martin Luther University Halle-Wittenberg, Kurt-Mothes-Straße 3, 06120 Halle (Saale), Germany

Received 26 June 2007; received in revised form 1 August 2007; accepted 2 August 2007

Available online 9 August 2007

Abstract

Numerous peptides obtained by enzymatic digestion of food proteins have been reported to exhibit biological activities. In this study, the focus was placed on peptides of β -casein from bovine milk after a gastro-analogous *in vitro* digestion with pepsin, a protease with broad specificity. In order to study the time course of the digestion, the process was stopped after specific times and the samples were subjected to HPLC separation followed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) and nanoelectrospray (nanoESI) quadrupole time-of-flight (qTOF) mass spectrometry. A combined sequencing approach using *de novo* interpretation and databases was employed. Overall, 100% of the β -casein sequence was covered by identifying 125 peptides of 4–84 residues in length, including 3 phosphorylated species. The results show that the peptic hydrolysis starts at the C-terminus of the protein. The release of known bioactive peptides from β -casein following the peptic digestion under simulated gastric conditions is unlikely with a few exceptions. Furthermore, an amino acid variation was found, providing evidence for the existence of an additional genetic variant of β -casein.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Pepsin; Genetic variant; Dairy products; Gastrointestinal proteolysis; *De novo* sequencing; LC/MALDI; Nanoelectrospray; β -Casein I

1. Introduction

The major proteins in bovine milk are the four different caseins (CNs) α_{S1} -, α_{S2} -, β -, and κ -CN. β -CN is the second most abundant among them and known to occur in at least 10 genetic variants (A^1 – A^3 and B–H) from which the A^2 , A^1 , and B forms are the most prevalent. It is composed of 209 amino acids (AA) and the molecular mass for variant A^2 is 23,983 u. β -CN contains five serine-phosphate residues located in the N-terminal region.

Numerous peptides derived from β -CN are known to exhibit a variety of bioactivities. These peptides may be released *in vivo* during gastrointestinal digestion. A huge body of literature discussing the hypothesis that the proportion of β -CN A^1 in food intake may have an influence on the incidence of a number of diseases has recently been published. In particular, for type 1

diabetes mellitus, a strong correlation was found in ecological studies [1,2]. The correlation between diseases and the genetic variant A^1 of β -CN has been attributed to the potential release of β -casomorphin 7 (β -CN 60–66), a very strong opioid [3]. In A^1 β -CN the adjacent C-terminal amino acid is His, which does not hinder the formation of this peptide, whereas Pro in the case of A^2 β -CN does. While some dietary products based on A^2 β -CN have already entered the market, the rationale is still subject of heated discussion [4,5].

Other bioactive peptides acting as mineral carriers originating from β -CN were found encoded in the sequence region β -CN 1–28 (β -CN phosphopeptides) [6,7]. β -CN 41–44 and β -CN 60–70 are known to contain opioid agonists (β -casomorphins) [7,8], and peptides derived from the sequences β -CN 6–14, β -CN 47–52, β -CN 73–90, β -CN 140–143, β -CN 169–183, and β -CN 191–202 (β -casokinins) have been found to inhibit angiotensin-I-converting enzyme (ACE) activity [9–14]. Peptides encrypted in the sequences β -CN 60–68 and β -CN 191–202 also possess immunostimulating activity [7], and those in β -CN 98–105 and β -CN 169–183 have been

* Corresponding author. Tel.: +49 345 5525215; fax: +49 345 5527292.
E-mail address: schmelzer@pharmazie.uni-halle.de (C.E.H. Schmelzer).

reported to inhibit lipoxygenase (LOX) [15]. These bioactive peptides were mostly results from the degradation of β -CN with trypsin, proteinase K, or bacterial proteases, or they were synthesized like β -casokinin 10 (β -CN 193–202) [16] and the immunostimulating peptide β -CN 63–68 [6]. The probability of production of these peptides by *in vivo* digestion remains unclear. Before dietary proteins can be cleaved by pancreatic proteases in the intestine, they pass through the stomach, in which food can remain for up to several hours depending on its composition and the degree of comminution during mastication. In the gastric juice, the proteins undergo degradation by pepsin, an endopeptidase with broad specificity. While the peptide products resulting from β -CN digestion with site-specific pancreatic proteases, such as trypsin or chymotrypsin are well investigated [17,18], there are only few papers regarding this primary step of human digestion of milk proteins [19,20].

However, a sound understanding of this process is of utmost importance in order to find out how likely these bioactive peptides are to occur. Therefore, in this systematic study, the focus was placed on peptides formed during a gastro-analogous digestion of β -CN. The present paper is a continuation of a former work [21] and differs in terms of the analytical methods applied and particularly in the issues addressed. In the former study, high-performance liquid chromatography (HPLC) with online-coupled electrospray mass spectrometry (MS) and MALDI-TOF-MS without preliminary HPLC fractionation were employed. In the current work, however, the peptide mixtures were analyzed using HPLC followed by MALDI-TOF and nanoESI-qTOF MS. Static nanoESI as opposed to online-coupled HPLC with nanoESI was preferred because of the virtually unlimited time available for tandem MS experiments and thus the possibility of improving the signal-to-noise ratio. Hence, it is possible to investigate even very low abundant peptides, peptides of high molecular masses, and those showing low ionization efficiencies, such as phosphorylated species. Although suppression effects are much less dramatic in the case of nanoESI as compared to classical electrospray [22], a chromatographic separation is still useful for reducing the complexity of the spectra and to avoid overlapping isotopic distributions. Furthermore, fractions obtained by HPLC can be subjected to MALDI experiments without further treatment; this offline coupling is designated as LC/MALDI. The results of MALDI measurements compared to those of nanoESI are usually remarkably different, so that only a combination of both ionization techniques allows a comprehensive analysis of the samples under investigation [23]. Regarding MALDI, complementarity also applies for the use of different suitable matrices [24].

The sequences of peptides can be determined from the fragment ion spectra of post-source decay (PSD) and/or tandem MS (MS/MS) by searching sequence databases and/or *de novo* sequencing. Thus, by checking which residues of the sequence are still intact after peptic digestion under gastric conditions, one can estimate how likely the occurrence of the encrypted bioactive peptides under *in vivo* conditions will be. Furthermore, an investigation of the time course of the proteolysis can provide

additional mechanistic information, e.g. which cleavage sites are attacked by the enzyme first and which later.

2. Materials and methods

2.1. Materials

β -CN derived from bovine milk for human consumption (product number, C6905; lot number, 082K7405; country of origin, New Zealand; purity, $\geq 90\%$) and pepsin from porcine gastric mucosa (product number, P7000; lot number, 12K0100; activity, 871 U mg⁻¹ protein) were obtained from Sigma–Aldrich (Taufkirchen, Germany). According to the supplier's information, the β -CN was derived from dissolved total CN by pH adjustment from pH 7.5 to 4.6. The β -CN containing supernatant was chromatographed using DEAE-sepharose and the peak fractions were then pooled and precipitated in approximately 25% ammonium sulfate. Finally, the product was dialyzed and lactose as extender was added prior to lyophilization.

Acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany), trifluoroacetic acid (TFA) of analytical grade from Fluka (Buchs, Switzerland), and the water used was doubly distilled. The matrices α -cyano-4-hydroxycinnamic acid (CHCA) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) were purchased from Sigma–Aldrich. All substances were used without further treatment.

2.2. Gastro-analogous digestion of β -CN

The *in vitro* hydrolysis was performed simulating gastric conditions of human adults in terms of temperature, pH value, digestion time, as well as protein and protease concentrations. For this purpose, bovine β -CN was dissolved in water at a concentration of 25 mg mL⁻¹, adjusted to pH 2 with 1 M HCl, and digested with pepsin (1 mg mL⁻¹) at 37 °C under agitation. Samples of 0.1 mL of the digest were collected at different times between 1 and 180 min (see Fig. 1), the digestion immediately stopped by neutralization with 1 M NaOH, aliquoted and stored at –20 °C for further analyses.

2.3. HPLC characterization and fractionation

The digests were initially characterized by reversed-phase HPLC (RP-HPLC) fingerprints using a Merck-Hitachi system with a Eurospher 100 C₁₈ column (250 mm $\times$ 4 mm I.D.; 5 μ m particle size; 100 Å pore size; Knauer, Berlin, Germany). A quantity of 20 μ L of each sample solution was loaded on the column, and peptides were eluted using a linear binary gradient: 5–50% acetonitrile in 0.05% TFA from 5 to 50 min. The column was maintained at room temperature, and the flow rate was 1 mL min⁻¹. Ultraviolet detection was performed at a wavelength of 220 nm.

Samples of the 10-, 30-, and 60-min digest showing distinct qualitative changes in the peak pattern were separated by manual peak fractionation and subjected to mass spectrometric analyses.

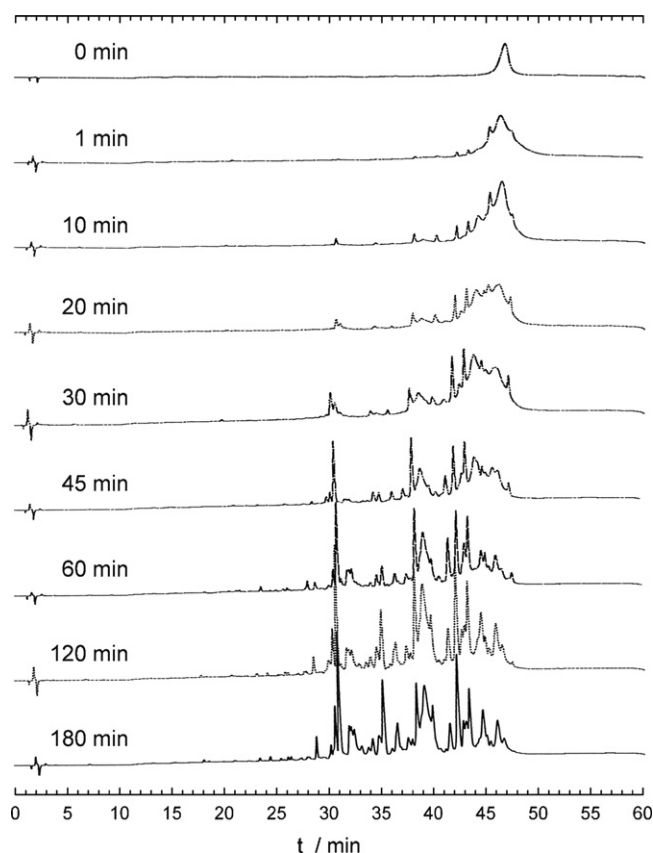


Fig. 1. Reversed-phase HPLC-UV chromatograms ($\lambda = 220$ nm) of bovine β -CN after different times of peptic digestion. The chromatograms are offset for clarity; absorbance in arbitrary units.

2.4. MALDI-TOF-MS

MALDI experiments were carried out on a delayed extraction TOF mass spectrometer Voyager-DE PRO (Applied Biosystems, Foster City, CA, USA) equipped with a pulsed nitrogen laser ($\lambda = 337$ nm, 3 ns pulse width, 20 Hz repetition rate). Two standard matrix systems were used: a solution of 10 mg mL^{-1} of α -cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/0.1% TFA (1:1, v/v) and 20 mg mL^{-1} of sinapinic acid in acetonitrile/0.1% TFA (1:1, v/v). The sample solution was mixed with the matrix solution 1:1 (v/v). $0.5 \mu\text{L}$ of the resulting mixture was applied to each spot of the sample plate. The samples were then dried in a gentle stream of air ($\sim 35^\circ\text{C}$). Measurements were performed operating in the positive-ion reflector mode at an acceleration voltage of 20 kV, grid voltage set to 75%, 0.005% guide wire voltage, and an extraction delay of between 100 and 400 ns depending on experimental conditions. A low mass gate was set to m/z (mass-to-charge ratio) 600 to prevent detector saturation from matrix peaks. Survey spectra of the peptide mixtures were obtained by accumulating data from 500 to 2000 laser shots.

2.5. MALDI-PSD/CID analysis

Post-source decay experiments were carried out by performing 10–15 steps of the reflection voltage, and for each individual

step the voltage was reduced to $\sim 75\%$ of the previous step. Using the timed ion selector (TIS), different precursor ions were selected from the peptide mixtures and subjected to fragmentation experiments. The TIS resolution was specified with a resolution of 80 (full width at half maximum). After acquisition, all spectra were stitched together by the data system Data Explorer (v. 4.0.0, Applied Biosystems). Each segment spectrum obtained was the mean of at least 500 laser shots. For some peptides, PSD was aided by collision-induced dissociation (CID). For this purpose the collision cell was filled with air so that the pressure in the source chamber reached $\sim 800 \times 10^{-6}$ Pa.

2.6. NanoESI-qTOF-MS

Static nanoESI experiments were conducted on a qTOF mass spectrometer Q-TOF-2 (Waters/Micromass, Manchester, UK) equipped with a nanoESI Z-Spray source. The nanoESI glass capillaries were obtained precoated from DNU-MS (Berlin, Germany). The TOF analyzer was calibrated externally using a mixture of sodium iodide and caesium iodide or internally using sample peptides of known sequence. Several HPLC fractions were concentrated using ZipTip C_{18} units (Millipore, Schwalbach, Germany) according to the manufacturer's instructions prior to elution with acetonitrile/water (1:1, v/v) containing 0.05% TFA. Two microliters of the sample solution were loaded into the nanoESI glass capillaries using Microloader pipette tips (Eppendorf, Hamburg, Germany).

The typical operating conditions for the qTOF mass spectrometer were as follows: capillary voltage, 800–1000 V; sample cone voltage, 30–100 V; source temperature, 80°C . The instrument was operated in the positive ion mode. Full scans were performed over the m/z range of 50–2500. Peptides of interest were selected manually for tandem MS experiments using CID. The quadrupole mass filter, followed by the TOF analyzer, was adjusted with low mass (LM) and high mass (HM) resolution settings of between 10 and 16 (arbitrary units) and the CID collision energy was varied depending on charge state and mass-to-charge ratio between 18 and 80 eV.

2.7. Peptide sequencing

The fragment ion spectra obtained from the MALDI-PSD measurements were processed using Mascot Distiller (Matrix Science, London, UK); a software program that reduces MS raw data to peak lists for database searching. In the case of nanoESI, the mass spectra and tandem mass spectra were analyzed by MassLynx (version 3.4, Waters/Micromass). Then, the MS/MS data for each peptide was processed by the MassLynx add-on Maximum Entropy 3 (MaxEnt3), and converted into SEQUEST files, which were suitable for further analyses. Both the PSD and nanoESI fragment ion peak lists, generated as described, were analyzed by searching sequence databases with Mascot (Matrix Science, London, UK) [25]. For these purposes, mass spectrometry protein sequence database (MSDB), Swiss-Prot, and an in-house database containing the different milk proteins and their variants were used. The searches were taxonomically restricted to "other mammalia" and the enzyme was set to "none"

because of the broad specificity of pepsin at pH 2. The mass error tolerances for precursor and fragment ions were typically set to 100 and 500 ppm for PSD data and to 0.15 and 0.20 u for qTOF-MS/MS data, respectively. The following variable modifications were considered: phosphorylation of Ser, hydroxylation of Pro, oxidation of Met, dehydration of Ser and Thr, and deamidation of Asn and Gln.

Additionally, automated *de novo* sequencing combined with database searching was performed on the qTOF-MS/MS data using the software PEAKS Studio (version 3.1, Bioinformatics Solutions, Waterloo, Canada) [26] with precursor and fragment mass error tolerances of 0.10 u. The enzyme entry was set to “unknown” and the same post-translational modifications as used for the Mascot search were considered. Besides MSDB, the same in-house protein database as implemented in Mascot was also used with PEAKS.

3. Results and discussion

The samples obtained from peptic digestion of β -CN and HPLC fractionation were subjected to mass spectrometry in order to characterize their peptide patterns as comprehensively as possible. In total, 125 different peptides of up to 84 amino acid residues in length were identified in the 10-, 30-, and 60-min samples. Among them, five peptides from κ -CN and one from α_{S1} -CN were identified (data not shown). Since the substrate was investigated using UV-HPLC and MALDI-MS prior to digestion and no peptide impurities were found, it can be assumed that these peptides are the result of the proteolysis. Three of the remaining 119 β -CN peptides were phosphorylated species, one of them, β -CN 1–58, contains all five phosphoserines. The largest peptides, whose sequences could be determined with the respective fragmentation technique, had remarkable monoisotopic masses of $m = 5499.88$ u (β -CN 94–142) in the case of MALDI-PSD and $m = 9303.92$ u (β -CN 59–142) for nanoESI-MS/MS. For the identification of the latter, its $[M+6H]^{6+}$ ions were fragmented over 35 min, processed with MaxEnt 3, and sequenced using Mascot.

It is notable that several peptides with partial deamidation of Gln and Asn or partial dehydration of Ser (S69, S142, S168) or Thr (T55, T80, T154) were identified. Heat treatment is known to initiate deamidation or dehydration by β -elimination [27,28]. It therefore can be assumed that these modifications already existed in the commercial β -CN used, which was prepared from pasteurized milk.

The β -CN used in acidic solution at pH 2 only shows a single peak in the chromatogram without any detectable peptide impurities (Fig. 1). Degradation starts very fast after treatment with pepsin. After 1 min of digestion, peptide peaks at retention times of 47.5, 45.5, 43, and 42 min containing the C-terminal peptides β -CN 190–209, β -CN 191–209, β -CN 192–209, and β -CN 193–209 are clearly present (see Fig. 2A; peptides were identified from the respective peak-fractions of the 10 min digested sample).

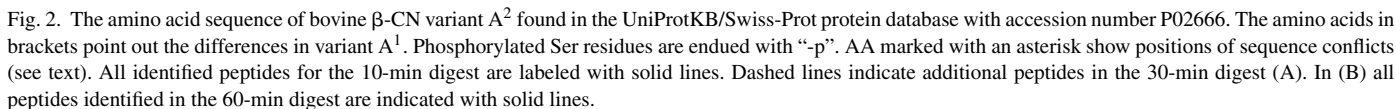
The fractions of additional chromatographic peaks after 10 min of digestion, at retention times of 41, 39, 34.5, and 31 min, mainly contain the less hydrophobic complementary

peptides up to AA 164 (β -CN 164–192, β -CN 164–191, β -CN 164–190, β -CN 164–189, and β -CN 164–188) as well as at 31 min the entire residue β -CN 164–209. β -CN 128–163, the next part of the sequence in N-terminal direction, and its fragments β -CN 126–142 and β -CN 143–163 were identified from the 10-min digest (Fig. 2A). In samples digested for 30 min only the additional peptide β -CN 69–93 and some further degraded C-terminal peptides (Fig. 2A, dashed lines) were found. The complete degradation scheme resulting from analysis after 60 min of gastro-analogous hydrolysis of β -CN is shown in Fig. 2B. Liquid chromatograms of longer digested samples up to 180 min show that there is no more substantial change in the peptide pattern as compared to 60 min. Thus, it can be assumed that the digest is widely finished after 60 min.

Multiple pepsin cleavages were observed at the C-terminal region, whereas no degradation characteristic for pepsin was found in the N-terminal region from AA 12–44 and in the central region from AA 94–123. Several preferred cleavage sites of pepsin are located between the AA 124 and 130, AA 140–144, AA 155–159, and AA 187–200. Some rather unexpected cleavage sites were found in these regions after at least 60 min of hydrolysis. However, it is worth mentioning that the pepsin was investigated using MALDI-TOF-MS and no masses of other proteases were detected. Although impurities by other enzymes cannot be fully excluded, to the authors' best knowledge other proteases originating from the gastric mucosa that may be considered as contaminants are either inactive at pH 2 or do not show specificity which would explain those cleavage sites.

Pepsin is an aspartic protease with broad specificity. It exhibits preferential cleavage for hydrophobic, preferably aromatic, residues in P1 and P1' positions [29]. It can be seen from Fig. 2 that the degradation of β -CN starts at the hydrophobic C-terminal region of this amphiphilic protein with “soap-like” properties. It is known from recent studies, that removing the C-terminal part from AA 192 to 209 has not only an effect on aggregation behavior but also a strong destabilizing effect on the whole molecule [30]. From the results of the analyses of the 10 min digested sample (Fig. 2A, solid lines) one can assume that the next preferred cleavage sites of pepsin are at AA 163/164 and AA 125/126, or AA 127/128, each C-terminal to Leu and N-terminal to Ser or Thr. The N-terminal region up to AA 125 turns out to be more stable.

With regard to the suggested three-dimensional model of the β -CN structure by Kumosinski et al. [31], no correlation of preferred pepsin cleavage sites to predicted secondary structure elements was found. The course of degradation seems to be determined predominantly by the primary structure. Although standard literature for pepsin only notes the preference for cleavages between nonpolar AA with large hydrophobic residues, such as Phe and Leu [29], additional cleavage sites are reported, including peptide bonds between other nonpolar AA, acidic AA (nonpolar at pH 2) as well as Cys, Thr, and Ser [21,32–34]. Our results show that peptide bonds between Leu and Ser, Leu and Thr, Ser and Trp are cleaved relatively fast (Fig. 2A and B), while several X-Pro cleavages, Trp–Met, Val–Met, and other cleavages between nonpolar AA were detected only after 60 min of digestion.



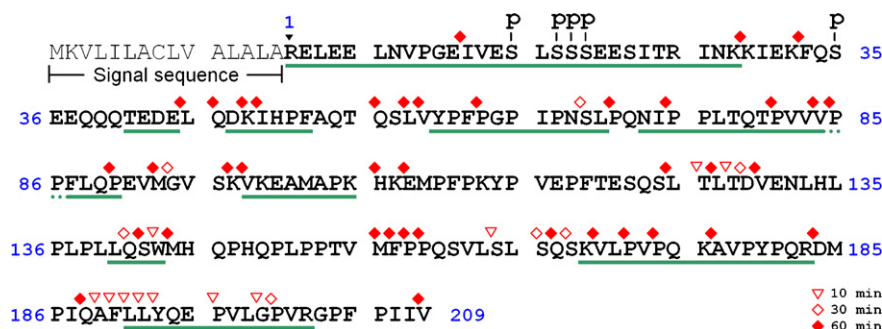


Fig. 3. The sequence of bovine β -CN variant A². Regions containing known bioactive peptides (see Section 1) are underlined and all cleavage sites for pepsin first identified in the 10-, 30-, or 60-min digest are indicated.

Furthermore, it can be seen from Fig. 3 that pepsin cleavage sites under *in vitro* but gastro-analogous conditions can be found throughout the β -CN sequence. Although the situation *in vivo* is much more complex, it is likely that most of the peptides reported to show bioactivity will not survive this process as part of an intact sequence. The probability of their release decreases with increasing digestion times. For example, within regions containing the ACE inhibiting peptides β -CN 140–143, β -CN 169–174, and β -CN 169–175 [10] as well as the β -casokinin β -CN 80–90 [9] numerous cleavage sites were found. Also the part of the sequence in which the strong opioid agonist β -casomorphin 7 is encrypted shows two cleavage sites. The peptide itself was not detected in the digests. In contrast, no cleavages were found within regions containing the opioid agonist β -CN 41–44 [8], the synthesized immunomodulating peptide β -CN 63–68 [7], and the LOX-inhibiting peptides β -CN 98–105 and β -casokinin 7 (β -CN 177–183) [15,35]. Moreover, the release of three peptides with sequences identical or almost identical to known bioactive peptides was found (Table 1). It is worth highlighting that all these cleavage sites are partial (see Fig. 2) and therefore, conclusions concerning the survival of sequence regions containing bioactive peptides can only be drawn to a limited extent.

Another issue is the deliberate administration of externally produced β -CN-derived peptides for nutraceutical or pharmaceutical purposes. Since pepsin is a protease with eight substrate side-chain binding sites [29], shorter peptides will not be preferred substrates and may survive the gastric digestion after oral application. Larger peptides, however, with several cleavage sites like β -casokinin 10 [16], β -casokinin β -CN 80–90 [9], or β -casomorphin 11 (β -CN 60–70) [7], are assumed to be degraded in the stomach.

In the past, contrary results concerning the nature of two amino acids in the β -CN sequence have been published.

Jimenez-Flores et al. cloned and sequenced cDNA of bovine β -CN and found Leu and Gln for the translated amino acids in position 93 and 195 [36], whereas other authors found Met and Glu [37–42], respectively. Apart from these variations, the sequence identified by Jimenez-Flores et al. is identical to the A² form published by Ribadeau-Dumas et al. [37] and the two differences between them are up to now handled as sequence conflicts in the nucleotide and protein databases. In 2002, Jann et al. published a PCR-SSCP-based DNA test for the differentiation of five alleles (A¹–A³, B, and C) using exons 6 and 7 of the β -CN gene (CSN2). The authors identified for several of 318 unrelated animals an A $\rightarrow$ C point mutation in exon 7 resulting in a Met $\rightarrow$ Leu substitution of the amino acid in position 93 [43]. Unfortunately, Jann et al. did not relate their results to the sequence of Jimenez-Flores et al.

However, our commercial sample was derived from various animals and contains different genetic variants of bovine β -CN. Although 100% of the sequence of variant A² is covered, also several peptides with His instead of Pro in position 67 were found, possibly originating from the widespread A¹ form. More interestingly, a peptide (β -CN 59–93) with a C-terminal Leu was identified, providing evidence on the protein level that behind the putative sequence conflict, another not yet classified genetic variant is hidden. Due to the fact that the mass difference between this peptide and the corresponding A² variant is $\Delta m \approx 18$ u, our first assumption was that this is caused by dehydration of a Ser or Thr within the sequence. Although this modification was observed for several other peptides, this possibility has been ruled out for the respective peptide based on *de novo* sequencing data. In Fig. 4, two homologous tandem mass spectra of the peptide β -CN 59–93 are plotted against each other. Both were found in fractions of the 60-min digest, but the peptide from the not classified variant had a 1 min higher retention time compared to the peptide from the A² variant. This is in agreement with the higher hydrophobicity of Leu over Met. The tandem mass spectra only differ in a mass shift of ≈ 18 u for all y fragments, whereas the masses of all b fragments are identical. This fact proves that the difference between both peptides is located at the C-terminus. To confirm the results of the tandem MS experiments, accurate mass determination for the peptide under investigation was employed by electrospray qTOF as well as Fourier transform ion cyclotron resonance (FT-ICR) MS on a Reflex III (Bruker Daltonics, Billerica, MA, USA). An *m/z*

Table 1
Peptides identified in the peptic digest of β -CN with identical or slightly longer sequences as compared to known bioactive peptides (underlined)

Literature		Experiment	
Peptide	Bioactivity	Peptide	Sequence
β -CN 60–70 [6]	Opioid agonist	β -CN 59–70	VYPFGPIPNSL
β -CN 75–82 [14]	ACE inhibition	β -CN 75–83	PPLTQTPVV
β -CN 177–183 [15]	ACE inhibition	β -CN 177–183	AVPYPQR

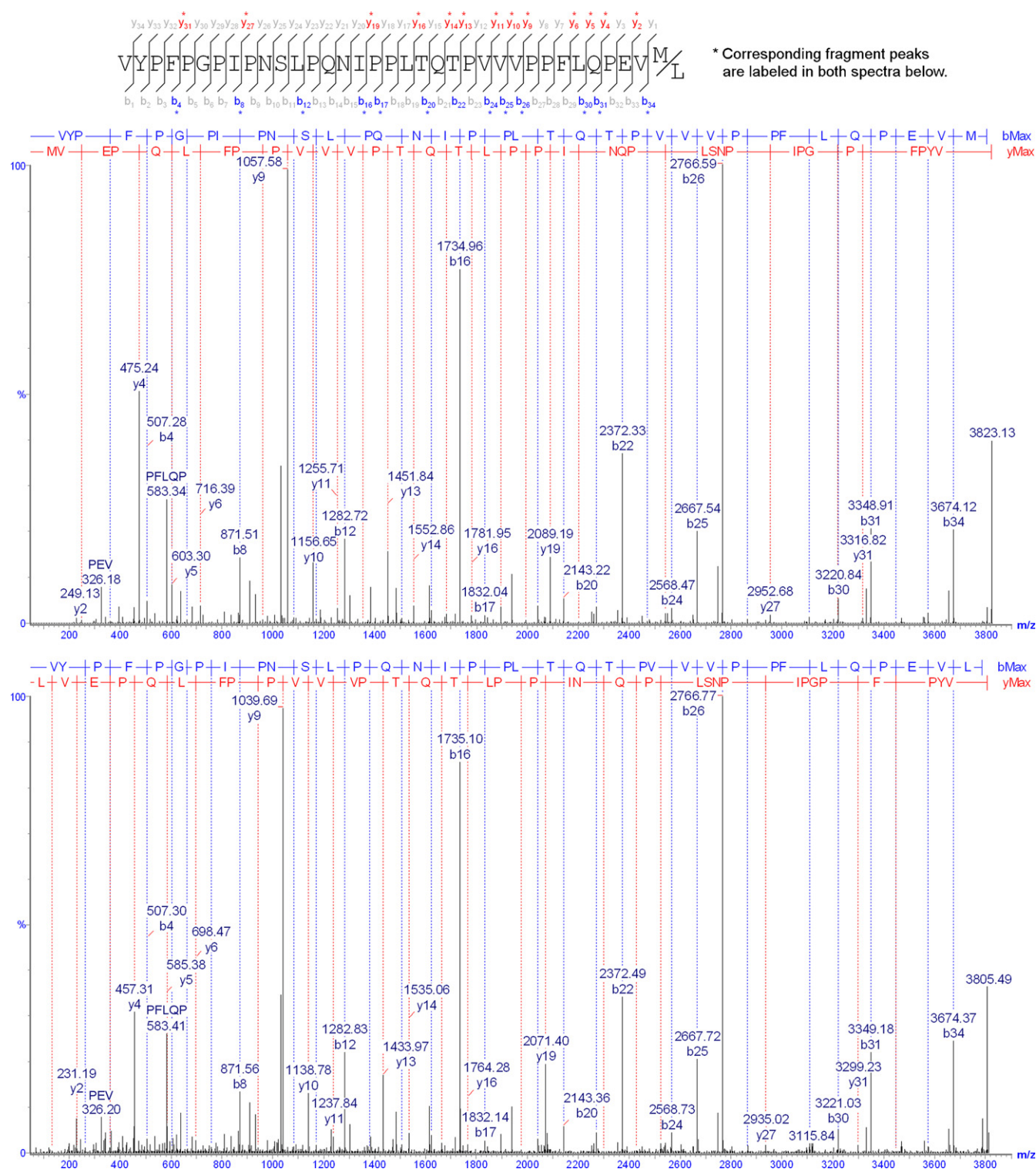


Fig. 4. Deconvoluted nanoESI-qTOF fragment ion spectra of the residue β-CN 59–93 acquired at a collision energy of 30 eV. The upper spectrum shows the peptide with a C-terminal Met (m/z 1275.02), whereas the lower spectrum shows the same peptide but identified with a C-terminal Leu (m/z 1269.04). The b and y series ions are annotated and their respective amino acid residues are indicated.

of 1269.0357 for the $[M+3H]^{3+}$ ion was determined by FT-ICR MS which differs from the theoretical m/z of 1269.0375 by only 1.4 ppm.

This peptide, together with the DNA results of Jann et al. [43], verifies the existence of a further (11th) genetic vari-

ant of β-CN. Although no peptide containing Gln in position 195 was found, this variant is most likely the one already sequenced from cDNA clones by Jimenez-Flores et al. [36]. Hence, this up to now neglected variant should be classified as β-casein I.

4. Conclusion

This in-depth study of peptic digestion of β -CN under gastro-analogous *in vitro* conditions revealed that the enzyme pepsin starts to cleave its substrate in the C-terminal region rich in large, hydrophobic residues. After 60 min, the digestion is practically finished, leaving only small proportions of the sequence completely intact. The results provide details about the cleavage sites and thus the specificity of the enzyme under gastric conditions and, furthermore, evidence for the formation and/or non-formation of known bioactive peptides during a peptic digestion of bovine β -CN. These findings add to the current understanding of bioactive peptides with respect to the probability of their release under physiological conditions. Moreover, a variation observed in position 93 proves the existence of an 11th genetic variant of bovine β -CN, which has not yet been defined.

Acknowledgements

The authors wish to thank the German Federal Ministry of Education and Research (BMBF) for the financial support within the scope of the research project 0312750A. C.E.H.S. would like to thank the Graduiertenförderung des Landes Sachsen-Anhalt for funding. The authors thank Dr. J. Schmidt for performing the FT-ICR experiments and A. Heinz for helpful discussions.

References

- [1] C.N. McLachlan, *Med. Hypotheses* 56 (2001) 262.
- [2] M. Laugesen, R. Elliott, *N. Z. Med. J.* 116 (2003) 295.
- [3] G. Koch, K. Wiedemann, H. Teschemacher, *Naunyn Schmiedeberg's Arch. Pharmacol.* 331 (1985) 351.
- [4] A.S. Truswell, *Eur. J. Clin. Nutr.* 59 (2005) 623.
- [5] K.B. Woodford, *Eur. J. Clin. Nutr.* 60 (2006) 437.
- [6] H. Meisel, H. Frister, E. Schlimme, *Z. Ernährungswiss* 28 (1989) 267.
- [7] E. Smacchi, M. Gobetti, *Food Microbiol.* 17 (2000) 129.
- [8] A.M. Fiat, D. Miglioresamou, P. Jolles, L. Drouet, C.B.D. Sollier, J. Caen, *J. Dairy Sci.* 76 (1993) 301.
- [9] A. Abubakar, T. Saito, H. Kitazawa, Y. Kawai, T. Itoh, *J. Dairy Sci.* 81 (1998) 3131.
- [10] M. Maeno, N. Yamamoto, T. Takano, *J. Dairy Sci.* 79 (1996) 1316.
- [11] R.J. FitzGerald, B.A. Murray, D.J. Walsh, *J. Nutr.* 134 (2004) 980S.
- [12] H. Meisel, W. Bockelmann, *Antonie van Leeuwenhoek* 76 (1999) 207.
- [13] M. Gobetti, L. Stepaniak, M. De Angelis, A. Corsetti, R. Di Cagno, *Crit. Rev. Food Sci. Nutr.* 42 (2002) 223.
- [14] M. Gobetti, P. Ferranti, E. Smacchi, F. Goffredi, F. Addeo, *Appl. Environ. Microbiol.* 66 (2000) 3898.
- [15] S.G. Rival, S. Fornaroli, C.G. Boeriu, H.J. Wichers, *J. Agric. Food Chem.* 49 (2001) 287.
- [16] H. Meisel, E. Schlimme, in: V. Brantl, H. Teschemacher (Eds.), *Beta-Casomorphins and Related Peptides: Recent Developments*, VCH, Weinheim, 1994, p. 27.
- [17] L. Leadbeater, F.B. Ward, *J. Chromatogr.* 397 (1987) 435.
- [18] L. Lemieux, J. Amiot, *J. Chromatogr.* 473 (1989) 189.
- [19] J. Svedberg, J. de Haas, G. Leimenstoll, F. Paul, H. Teschemacher, *Peptides* 6 (1985) 825.
- [20] E. Miquel, J.A. Gomez, A. Alegria, R. Barbera, R. Farre, I. Recio, *Food Sci. Technol. Int.* 12 (2006) 379.
- [21] C.E.H. Schmelzer, R. Schöps, R. Ulbrich-Hofmann, R.H.H. Neubert, K. Raith, *J. Chromatogr. A* 1055 (2004) 87.
- [22] A. Schmidt, M. Karas, T. Dülcks, *J. Am. Soc. Mass Spectrom.* 14 (2003) 492.
- [23] M.D. Stapels, D.F. Barofsky, *Anal. Chem.* 76 (2004) 5423.
- [24] M. Karas, R. Krüger, *Chem. Rev.* 103 (2003) 427.
- [25] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, *Electrophoresis* 20 (1999) 3551.
- [26] B. Ma, K. Zhang, C. Hendrie, C. Liang, M. Li, A. Doherty-Kirby, G. Lajoie, *Rapid Commun. Mass Spectrom.* 17 (2003) 2337.
- [27] J.R. Whitaker, R.E. Feeney, *Crit. Rev. Food Sci. Nutr.* 19 (1983) 173.
- [28] Z.E. Sikorski, in: Z.E. Sikorski (Ed.), *Chemical and Functional Properties of Food Proteins*, Technomic Press, Lancaster, PA, 2001, p. 191.
- [29] J. Tang, *Handbook of Proteolytic Enzymes*, Elsevier, London, 2004.
- [30] P.X. Qi, E.D. Wickham, E.G. Piotrowski, C.K. Fagerquist, H.M. Farrell Jr., *Protein J.* 24 (2005) 431.
- [31] T.F. Kumosinski, E.M. Brown, H.M. Farrell Jr., *J. Dairy Sci.* 76 (1993) 931.
- [32] H. Fujioka, H.A. Scheraga, *Biochemistry (Mosc.)* 4 (1965) 2197.
- [33] J.E. Vance, D.A. LeBlanc, R.E. London, *Biochemistry* 36 (1997) 13232.
- [34] A. Magalhaes, B.C. Da Fonseca, C.R. Diniz, J. Gilroy, M. Richardson, *FEBS Lett.* 329 (1993) 116.
- [35] S. Maruyama, K. Nakagomi, N. Tomizuka, H. Suzuki, *Agric. Biol. Chem.* 49 (1985) 1405.
- [36] R. Jimenez-Flores, Y.C. Kang, T. Richardson, *Biochem. Biophys. Res. Commun.* 142 (1987) 617.
- [37] B. Ribadeau-Dumas, G. Brignon, F. Grosclaude, J.C. Mercier, *Eur. J. Biochem.* 25 (1972) 505.
- [38] A.F. Stewart, J. Bonsing, C.W. Beattie, F. Shah, I.M. Willis, A.G. Mackinlay, *Mol. Biol. Evol.* 4 (1987) 231.
- [39] G. Simons, W. van den Heuvel, T. Reynen, A. Frijters, G. Rutten, C.J. Slangen, M. Groenen, W.M. de Vos, R.J. Siezen, *Protein Eng.* 6 (1993) 763.
- [40] S. Visser, C.J. Slangen, F.M. Lagerwerf, W.D. Van Dongen, J. Haverkamp, *J. Chromatogr. A* 711 (1995) 141.
- [41] C. Dong, K.F. Ng-Kwai-Hang, *Int. Dairy J.* 8 (1999) 967.
- [42] S.K. Han, Y.C. Shin, H.D. Byun, *Anim. Genet.* 31 (2000) 49.
- [43] O. Jann, G. Ceriotti, A. Caroli, G. Erhardt, *J. Anim. Breed. Genet.* 119 (2002) 65.