Beta-casomorphin 7 in raw and hydrolyzed milk derived from cows of alternative β-casein genotypes

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In addition to its nutritional value, β-casein is a source of bioactive peptides called β-casomorphins, which are produced during digestion of raw or processed milk. It has been shown that β-casomorphin 7 should originate exclusively from β-casein genetic variants A1 or B and may be a serious risk factor in the etiology of human diseases. This study measured the level of this peptide in milk produced by cows of different β-casein genotypes. Ten Polish Holstein-Friesian cows were included in the experiment, 5 carrying the A1/A1 genotype, 5 carrying the A2/A2 genotype. The genotypes of the β-casein locus were determined on the basis of DNA by PCR-Allele Created Restriction Site (ACRS) method. Caseins were extracted and then hydrolyzed by pepsin for a 24 h incubation followed by HPLC isolation of β-casomorphin 7. In hydrolyzed milk from A1/A1 cows, the content of β-casomorphin 7 was 4 times higher than in milk produced by A2/A2 cows. In fresh (not hydrolyzed) milk, traces of β-casomorphin 7 were found. The results confirm the hypothesis that β-casein A1 milk during hydrolysis produces significant amounts of β-casomorphin 7 and, therefore, A1 milk may be considered an undesirable factor influencing human health.

β-Casomorphin 7 in roher und hydrolysierter Milch von Kühen mit unterschiedlichen β-Casein-Genotypen

Außer seinem Nährwert ist β-Casein Quelle für die bioaktiven Peptide β-Casomorphine, die während der Digestion von roher oder verarbeiteter Milch produziert werden. Es wurde gezeigt, dass β-Casomorphin 7 ausschließlich aus den genetischen Varianten des A1 oder B des β-Caseins stammt und ein ernster Risikofaktor bei der Ätiologie menschlicher Krankheiten darstellen kann. In dieser Studie wurde der Level dieses Peptids in Milch von Kühen mit unterschiedlichen β-Casein-Genotypen bestimmt. Von den 10 Polnischen Holstein-Friesian-Kühe dieses Versuchs gehörten 5 zum A1/A1- und 5 zum A2/A2-Genotyp. Die Genotypen des β-Casein-Locus wurden auf der Grundlage der DNA durch die PCR-Allel-created Restriction Site (ACRS)-Methodik bestimmt. Die Caseine wurden extrahiert und durch Pepsin über eine 24 stündige Inkubation hydrolysiert. Anschließend wurde β-Casomorphin 7 durch HPLC isoliert. In der hydrolysierten Milch der A1/A1-Kühe war der β-Casomrophin 7-Gehalt 4mal höher als in der von A2/A2-Kühen. In frischer (nicht hydrolysierter Milch) wurden Spuren von β-Casomorphin 7 gefunden. Die Ergebnisse bestätigen die Hypothese, dass β-Casein A1-Milch während der Hydrolyse signifikante Mengen an β-Casomarophin 7 bildet. Daher A1-Milch ist als unerwünschter Faktor für die menschliche Gesundheit zu betrachten.

18 Casomorphins (β-casein genotypes) **18 Casomorphine** (β-Casein-Genotypen)

1. Introduction

The nutritive value of caseins is not only determined by its amino acid content but also by the peptides released during digestion in the gastrointestinal tract. Beta-casomorphins – the peptides originated from β-casein are currently a well-studied group with a chain length of 4-11 amino acids, all starting with tyrosine residue in position 60 (1). There are 12 genetic variants of CSN2, but only 5 occur in Holstein cattle, A1, A2, A3, B and C, the first two being the most common. In position 67 of the β-casein chain, Prolin in variant A2 and A3 is exchanged by Histidin in variant A1, B and C (2). It was shown that β-casomorphin 7 – a peptide of opioid-like activity, originates only from beta-casein A1 or B (3, 4) and may be serious risk factor for human ischemic heart disease, arteriosclerosis, type 1 diabetes and sudden infant death syndrome (5, 6, 7, 8, 9, 10). There are almost no papers reporting the range of differences in beta-casomorphin 7 quantity in A1 and A2 milk.

In the process of verifying the hypothesis that beta-casomorphin 7 may play a role in the etiology of human diseases, the measurement of the level of this peptide is necessary.

2. Materials and methods

Ten Polish Holstein-Friesian cows held in one herd and milked in the same season were included in the experiment. The genotype of beta-casein locus was determined using the method described by LIEN et al. (11) with minor modifications. Briefly, 350 µl of blood was drawn to isolate DNA using the MasterPure Purification Kit (Epicentre). The primers had the following sequences:

CASB122L–5'GAGTCGACTGCAGATTTTCAA CATCA GTGAGAGTCAGGCCCTG 3'
CASB67R-5'CCTGCAGAATTCTAGTCTATCC CTTCCCTGGGCCCATCG 3'

To produce 261 bp fragment of CSN2 gene, the following PCR mix was composed: 0.4 µl of the primers 122 L and 67 R, each in concentrations of 50 pmol/µl, 0,7 U of Tfl polymerase (Epicentre), 1, 25 µl MasterAmp 20X PCR Buffer (Epicentre), 1,5 µl magnesium chloride (15 mM), 2,0 µl Enhancer (Epicentre), ca. 150 ng of genomic DNA and H_20 ad 25µl. The samples were amplified in MJ Research thermocycler under following conditions: 3min/ 94°C and 35 cycles of 94°C/25s, 62°C/25s, 72°C/25s.

The yield and specificity of PCR products were evaluated after electrophoresis in 1,5% agarose gel (Promega) and staining with ethidium bromide. The results were observed, analysed and documented with a

Fluor-S Multilmager (Bio-Rad). The PCR product were then digested by Taq I enzyme to generate restriction fragments and electrophoresed in a 2,5% agarose gel (AmpliSize, Bio-Rad).

Peptides were extracted from milk according to HALWARKER and ELLIOT with modification by authors (12). 200 ml of fresh milk, combined with 200 ml of a 1:1 v/v chloroform/methanol mixture, was shaken for 1 h. The extract was made biphasic by the addition of a 0.2 volume of distilled water. After 48 h, the lower layer was discarded and the upper layer, containing peptides and amino acids, was saturated with methanol (3:4 v/v). After 48 h the mixture was centrifuged (3000 g 10 min.) and the supernatant was evaporated (40°C) and lyophilized. The peptide extracts were purified with the SPE method using the column STRATA C-18T, 140Å, 50 µ Phenomenex.

200 mg of the purified peptide extract was injected onto the counterbalance column by 0.1 % TFA and washed with a mixture of 10% acetonitrile and 0.1 % TFA. Peptides were eluted from the column by a mixture of 50% acetonitrile and 0.1 % TFA. The eluate was lyophilized.

Reverse phase HPLC separations were performed directly on the sample aliquots diluted into 1 mg/ml TFA. The column C-12 Phenomenex Jupiter Proteo (250x4.6 mm, 4 µ, 90 Å), fitted to a Shimazu Class VP chromatograph equipped with SPD M-10. A dual wavelength detector in 0.1% TFA was used and filtered through 0.45 µm cellulose acetate filters (Sartorious AG, Germany). [J1]Peptides extracts were first eluted with 100% A (0.1%, v/v TFA in deionized water) for 5 min, then with a gradient from 0 to 30 % B (0.1 %, v/v TFA in acetonitrile) over 45 min. Purified peptides were hydrolized by pepsin (0,0005 g pepsin in 1 ml 0,5 M HCl, 37°C within 24 h). ß-casomorphins were identified in non-hydrolized and hydrolyzed peptides by comparison with standards (Sigma).

3. Results

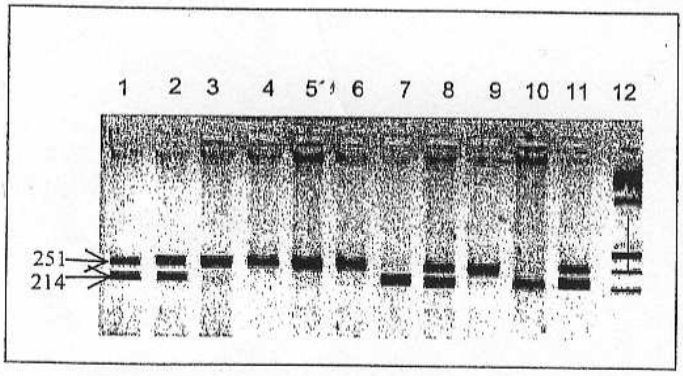

Fig. 1 A typical agarose gel electrophoresis of β-casein genotypes identified in Polish Holstein-Friesian cows. Lanes 1, 2, 8, 11 – A1/A2 genotype. Lanes 3, 5, 9 – A2/A2 genotype. Lanes 7, 10 – A1/A1 genotype. Lane 12 – molecular weight marker PhiX 174/Hae III. Restriction fragment 37 bp is not visible on the gel.

In Fig. 1, a typical result of CSN2 genotyping is shown. Three genotypes can be identified in random population of cows. Among the 10 cows analyzed in this study, two genotypes were identified as A1/A1 and A2/A2. The cows carrying alternative beta-casein genotypes were selected from 400 cows – daughters of bulls of 5 previously screened ones for CSN2 locus (13). To minimize the effect of environmental influences on milk properties, including the quantity of β-casomorphin 7, all cows came from one herd and were milked in the same season and stage of lactation. In raw milk (not hydrolized) a trace amount of casomorphin 7 was found in all samples (data not shown). Hydrolysis of milk proteins extracts by pepsin produced a release of β-casomorphin 7 at two distinct levels: low (av. 2,87±1,48 µg/mg) characteristic of 5 A2/A2 samples, and high (11,59±0,75 µg/mg) characteristic samples of A1/A1 genotypes (Fig. 2, Table 1).

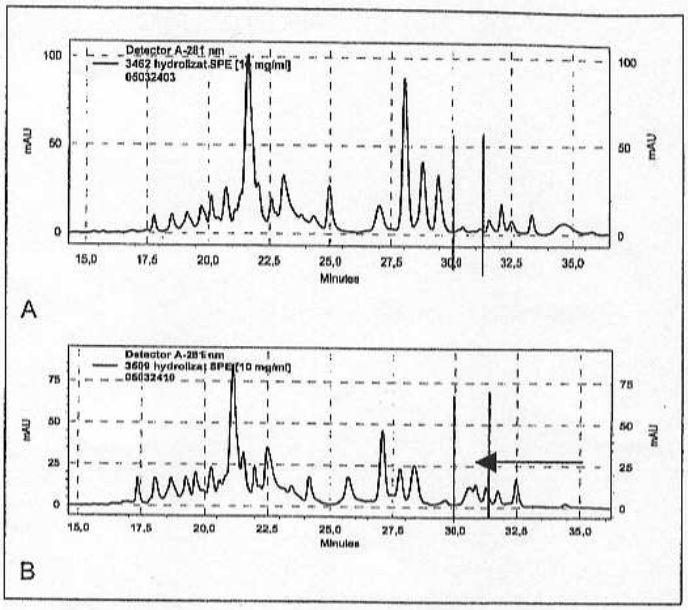

Fig. 2: Result of HPLC for hydrolyzed bovine β-casein cow's milk. A – milk from cow A2/A2; between the 30th and 31st min; there is no peak characteristic for β-casomorphin 7 standard; B – milk from cow A1/A1; characteristic peak for β-casomorphin 7 is depicted by the arrow.

Table 1: Content of β-casomorphin 7 (in µg/mg) of SPE extracts of pepsin hydrolyzates originating from cows of alternative β-casein genotypes

Cow's genotype and ID no	β-casomorphin 7 content	Mean	Standard deviation
A2/A2			
1	0.95		
2	4.48		
3	3.76		
4	1.72		
5	3.45	2.87	1.48
A1/A1			
6	11.73		
7	10.68		
8	12.73		
9	11.46		
10	11.37	11.59	0.75

The difference was ca. 4 fold. These results confirm findings obtained by HARTWIG (3). Although a mix of proteolytic enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase A and B, leucine-aminopeptidase) were used, no casomorphin 7 in digests of β-casein A2 and A3 was found, whereas they were found in digests of β-casein A1, B and C. It is known that hydrolysis of caseins may be conducted in many options, including different incubation time or different concentration of en-

zyme/s. Such experiments are planned, however, the results reported in this paper show very clearly that, even in a single enzyme digestion, the difference in β-casomorphin 7 in A2 and A1 milk is very high and sufficiently supports the hypothesis of beta-casein allele A1 as a source of casomorphin 7. In the near future we are going to investigate the level of β-casomorphin 7 in different types of milk-based products, especially in milk powder, yoghurts and baby formula produced from milk of alternative beta-casein phenotypes to find out whether the technology of milk processing influences the quantity of β-casomorphin 7.

Assuming that the prevalence of allele A1 in the most popular dairy cattle – Holstein-Friesian is relatively high (40%) (2), a large amount of milk produced all over the world is able to release the "undesirable" peptide β-casomorphin 7. Eventual limitation of production of such milk is difficult, long-term, and needs many actions through breeding organizations and dairy industry. A practical strategy preferringto favour the A2 allele in the dairy industry has been established in New Zealand. A commercial company, A2 Corporation (http://www.a2 corporation.com/), offers A2 Milk™, which is obtained exclusively from cows of the A2/A2 genotype.

4. Conclusions

The results sustain the hypothesis that the hydrolysis of β-casein derived from cows carrying the alternative β-casein genotype (A1/A1 *vs.* A2/A2) produced a β-casomorphin 7 at two distinct levels with a 4-fold difference. Although, the clinical implication of A1 milk on human health is still under discussion (14, 15), the authors feel it is necessary to continue research into the role of β-casomorphin 7 for human health. *In vivo* experiments are necessary to verify the presence of β-casomorphin 7 in the blood of individuals fed a diet containing milk of the alternative β-casein genotype.

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