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A1 and A2 Bovine Milk, the Risk of Beta-casomorphin-7 and Its Possible Effects on Human Health: (I) *A1 and A2 Milk and the Risk of Beta-casomorphin-7*

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ABSTRACT

Milk proteins are generally composed of proteins such as casein, lactalbumin, lactoglobulin, blood serum albumin, and immunoglobulin. One of them, casein from milk proteins consists of fractions such as alpha, beta, kappa and gamma. Beta casein gene in cattle consists of 209 amino acids and contains proline amino acid (CCT) at 67th position of the amino acid sequence. The milk obtained from animals with this genotype is defined as the original A2 milk. Proline/Histidine amino acid substitution (C/A) at 67th position of the amino acid sequence results in the beta-casomorphin-7 (BCM-7) which is a naturally occurring opioid peptide from cow's milk. By this substitution (CAT), the milk obtained from animals with genotypes (A1A1 or A1A2) is defined as the A1 milk. BCM-7 is a polypeptide consisting of 7 amino acids and is produced from A1 milk during digestion of beta-casein protein. As a result, BCM-7 has harmful effects on human health.

The aim of this study is to raise awareness in the field of animal science as well as to contribute to the taking of professional measures by using the literature on A1 / A2 milk and beta-casomorphin-7.

1. Introduction[1]

In the 1980s, researchers in the field of medicine tried to explain some peptides (such as casein) that occur during digestion that affect human health negatively or positively. In 1990s, the relationship between A1 or A2 milk and some chronic diseases and their relation to A1 milk were investigated. Polypeptide consisting of seven amino acids called beta-casomorphin-7 is produced from A1 milk during the digestion of the beta casein protein. Beta casein, a fraction of milk protein, is composed of 209 amino acids and is the 67th amino acid proline in the original cow's milk, generally in domestic (non-intervened) breeds and in some animals in the cross breeds. This milk containing the proline amino acid at 67th position is defined as the original A2 milk. In the 67th position of the same protein, instead of proline, histidine amino acid comes and causes to form beta-casomorphin 7, an opioid, and this milk is called milk A1. While the genotypes of A1 milk producing cattle are A1A1 and A1A2, the genotype of A2 producing cattle is A2A2. Nowadays, these can be detected by molecular genetic methods.

The purpose of this study is; to describe the A1 and A2 milk, the structure of beta- casomorphin-7 and how it is formed, A2 milk production resources and demonstrate the ways to be followed in the creation of A2 herd and to create awareness in Turkey.

2. *β*-Casein Polymorphism

Milk protein *β*-casein consists of a total of 209 amino acids (Farrell et al 2004). *β* casein constitutes 25-35 % of total milk protein (Eigel et al. 1984; Roginski, 2003). Alleles encoding the *β* casein protein were localized on chromosome 6 in the cattle genome (Rijnkels 2002; Jaiswal et al 2014). Different types of *β* casein gene localization is given in Table 1.

β casein has 13 genetic variants; A1, A2, A3, B, C, D, E, F, H1, H2, I, G and A4 variant found in Korean domestic cattle (base change has not yet been defined) (Kamiński et al 2007). *β* casein variants are shown in Table 2. The most common *β* casein variants in dairy cattle breeds are A1 and A2 (Kamiński et al 2007; Caroli et al 2009), B is less common, and variants A3 and C are rare (Farrell et al 2004).

The difference between *β* casein variants A1 and A2 variants is that only one amino acid changes at 67th position of *β* casein amino acid peptide chain in exon 7. The A1 variant at 67th position of *β* casein contains the histidine amino acid and the A2 variant contains the

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proline amino acid. The codon encoding the A2 variant in the 67th amino acid of the β casein gene is CCT and the codon encoding the A1 variant is CAT (Groves 1969; Roginski 2003). That is, the cytosine nucleotide in the CCT codon is replaced by the adenine nucleotide. As a result, a single nucleotide polymorphism occurs. The β casein gene (CSN2) can be accessed in the Genbank (AC-000163).

Table 1.
Chromosomal localization of the casein gene in different species (adapted from Jaiswal et al 2014)

Species		**Localized Chromosome**	**Number of Chromosomes**
Human	(*Homo sapiens*)	HAS-4	46
Cattle	(*Bos taurus*)	BTA-6	60
Sheep	(*Ovis aries*)	OAR-6	54
Goat	(*Capra hircus*)	CHI-4	60
Buffalo	(*Bubalus bubalis*)	BBU-7	Swamp (48)/ River (50)

2. 1. What is A1 and A2 Milk?

The β casein is located on chromosome 6 of the bovine genome. If β contaizns the CCT codon encoding the proline found at 67th position of the casein chain and when the gene in this locus contains the CAT code encoding the histidine instead of the CCT code from A2 allele, then the A1 allele of the gene is mentioned. Accordingly, individuals with A1A1 genotype produce A1 milk, in other words histidine occurs at 67th position of β casein. Individuals with A1A2 genotypes produce both A1 milk" that is, milk with "histidine" and β casein, as well as A2, in other words, milk with "proline" and β casein. Briefly, the milk produced by A2A2 genotype animals is called A2 milk namely original milk.

Table 2.
Changes in the amino acid sequence of beta-casein variants (Farrell et al 2004; Kamiński et al 2007)

β Casein variants	**Change in amino acid sequence**													
	18	25	35	36	37	67	72	88	93	106	117	122	137	138
A2	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**Pro**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	**Ser**	**Leu**	**Pro**
A1	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**His**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	**Ser**	**Leu**	**Pro**
A3	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**Pro**	**Glu**	**Leu**	**Gln**	***Gln***	**Gln**	**Ser**	**Leu**	**Pro**
B	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**His**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	***Arg***	**Leu**	**Pro**
C	**Ser**	**Arg**	**Ser**	**Glu**	***Lys***	**His**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	**Ser**	**Leu**	**Pro**
D	***Lys***	**Arg**	**Ser**	**Glu**	**Glu**	**Pro**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	**Ser**	**Leu**	**Pro**
E	**Ser**	**Arg**	**Ser**	***Lys***	**Glu**	**Pro**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	**Ser**	**Leu**	**Pro**
F	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**His**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	**Ser**	**Leu**	***Leu***
G	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**His**	**Glu**	**Leu**	**Gln**	**His**	**Gln**	***Leu***	**Leu**	**Pro**
H1	**Ser**	***Cys***	**Ser**	**Glu**	**Glu**	**Pro**	**Glu**	***Ile***	**Gln**	**His**	**Gln**	**Ser**	**Leu**	**Pro**
H2	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**Pro**	**Glu**	**Leu**	**Leu**	**His**	**Gln**	**Ser**	**Leu**	***Glu***
I	**Ser**	**Arg**	**Ser**	**Glu**	**Glu**	**Pro**	**Glu**	**Leu**	***Leu***	**His**	**Gln**	**Ser**	**Leu**	**Pro**

Prior to "histidine" amino acid in the 67th position of the β casein chain of individuals with A1A1 and A1A2 genotype; The seven amino acid chain of Tyr + Pro + Phe + Pro + Gly + Pro + Ile produces a non-digestible harmful morphine-like compound called "beta-casomorphin 7" after drinking A1 milk (Figure 1). This compound does not occur during the digestion of the original cow milk with "proline" found at 67th position. A1, A2 and all other variants were formed by mutations occurring in different parts of the DNA chain encoding β casein found in A2 milk. While the changes formed in the codon encoding amino acids at 67th position in A2 is important, the changes in codons encoding the other amino acids do not cause any harmful effects on human health based on literature information.

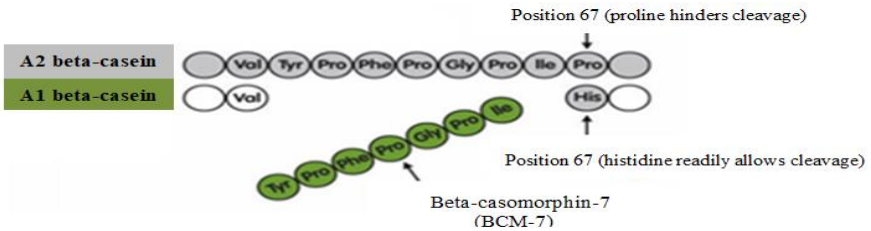


Figure 1.
Formation of β-casomorphin-7 (Woodford 2008; Pal et al 2015)

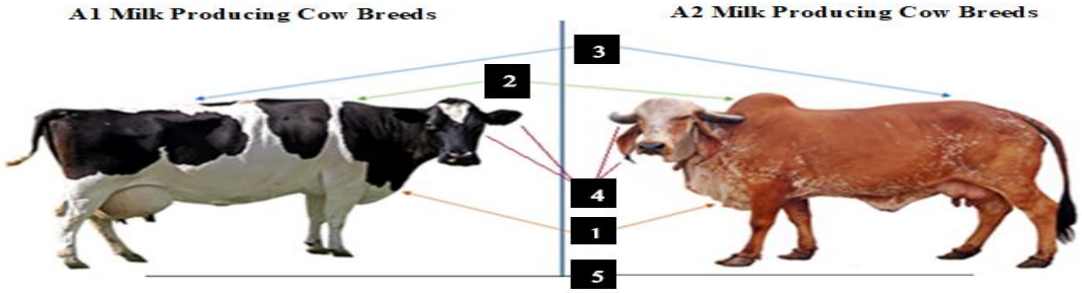
2. 2. Phylogenetics of A2 Milk?

Original cow's milk is A2 milk. That is to say, there is a "prolin" amino acid at 67th position of β casein. Probably 5000 to 10.000 years ago, in the European herds, the CAT codon coding for the histidine was formed by changing the nucleotide base in the codon CCT encoding prolin at the 67th position of the β casein in the European herds as a result of point mutation in *Bos-taurus* species (Ho et al 2014; Jaiswal et al. 2014). In the 1990s, the hypothesis that A1 variant beta-casein was a risk factor was investigated by Robert Elliott on suspicion that it may cause various diseases (De et al 2015). One of the β casein variants, A1 variant appeared in European cows.

The frequency of A1 β casein gene is higher in black and white cows than yellow and brown breeds. There is no A1 β casein variant in the milk of pure Asian and African cattle (Ng-Kwai-Hang & Grosclaude 2003). Clemens (2011) reported that the β casein A1 variant has higher frequencies in Holstein, Holstein - Friesian, Hereford, Ayrshire and Brahman breeds.

2. 3. Morphological Differences between Cattle Types Producing A1 and A2 Milk

Black-white (Holstein - Friesian), yellow (Brown Swiss), brown (Jersey) and brown - white (Simmental) color culture breeds generally produce A1 milk. Some other morphological differences between cattle types producing A1 and A2 milk are given in Figure 2.



1. This type of cows do not have flap.
2. They do not have hump.
3. Their back is straight throughout the body.
4. The ears are small and the forehead is narrow and the neck is short.
5. The rear legs have a straight line

1. Indian breed of cows have flap of skin beneath their neck.
2. They have hump.
3. Their back is curved, especially near the tail.
4. The ears are large, drooping, with a wide forehead and longer length.
5. The rear legs are round and convex.

Figure 2.
Morphological differences between cattle types producing A1 and A2 milk (Mallepalli et al 2017; Anonymous 2018)

2. 4. Heredity of A1 and A2 Milk

Cattle are diploid species. The alleles of the β casein gene are A1 and A2; A1A1, A1A2 and A2A2 genotypes are formed from these alleles. A1 and A2 alleles show codominant inheritance. Therefore, the cow with A1A1 genotype will produce "A1 milk" and the A2A2 genotype will produce "A2 milk". The heterozygous cow with A1A2 genotype will produce milk with a mixture of A1 and A2 beta casein because it is the codominant. In brief, the production capability of a cow in A1 or A2 milk depends entirely on its genetic structure. The genotype of an animal can only be determined by molecular genetic testing (Beavers & van Doormaal 2016). Table 6 shows the possible offspring combinations of beta-casein genotypes of both parents.

Table 6.
Possible combinations when mating animals of various beta casein genotypes (Beavers & van Doormaal, 2016)

Parent (Dam)	Parent (Sire) A1	Parent (Sire) A2
A1	A1A1	A1A2
A1	A1A1	A1A2
50% A1A1 50% A1A2		

Parent (Dam)	Parent (Sire) A1	Parent (Sire) A2
A2	A1A2	A2A2
A2	A1A2	A2A2
50% A1A2 50% A2A2		

Parent (Dam)	Parent (Sire) A1	Parent (Sire) A2
A1	A1A1	A1A2
A2	A1A2	A2A2
25% A1A1 25% A2A2 50% A1A2		

Parent (Dam)	Parent (Sire) A2	Parent (Sire) A2
A1	A1A2	A1A2
A1	A1A2	A1A2
100% A1A2		

Phylogenetic studies have shown differences in the formation of A1 or A2 beta casein between species, breeds and geographic locations (Jaiswal et al 2014; Parashar & Saini 2015). A1 beta casein variant is common in Northern European indigenous dairy cattle such as Friesian, Ayrshire, British Shorthorn and Holstein, but A2 beta casein variant is common in Zebu cattle with African origin, Southern French

breeds such as Charolais and Limousine, and also Channel Island breeds such as Guernsey and Jersey. However, the frequency of A2 allele has been reported to be quite high in most Indian breeds (Truswell, 2005; Mishra et al., 2009).

Mishra et al (2009) reported that A1 allele had never been encountered in the eight buffalo breeds raised in India. In other words, A1 beta casein variant is generally found in European originated pure breeds or crossbreeds, and A2 beta casein variant is found in the native cattle and buffalo breeds of India and African domestic cattle (Ng-Kwai-Hang & Grosclaude, 2003; Boro et al 2016).

The allele frequencies of β-casein gene obtained from studies in different countries are given in Table 7a, the allele and genotype frequencies of β-casein gene in some cattle breeds reared in Indian are shown in Table 7b.

Table 7a.
The allele frequencies of β-casein gene in bovine species

Breeds/Cross breeds	Country	Number of Animal (N)	Beta- Casein Allele Frequencies			References
			B	A1	A2	
Guernsey*	USA	3861	0.010-0.020	0.010-0.060	0.880-0.970	Enennam et al. 1991
Jersey*	Germany	43	0.186	0.093	0.721	Ehrmann et al.1997
	Denmark	157	0.350	0.070	0.580-0.650	Bech et al. 1990
	New Zealand	1328		0.123	0.591	Winkelman and Wickham 1997
	USA	387	0.290-0.370	0.090-0.220	0.490-0.540	Eenennam et al.1991
Brown Swedish*	Germany	232	0.170	0.108	0.705	Ehrmann et al. 1997
	USA	282	0.100-0.180	0.140-0.150	0.660-0.720	Swaissgood 1992
	USA	259	0.100-0.180	0.140-0.180	0.660-0.720	Eenennam et al.1991
Simmental*	Croatia	621	0.150	0.190	0.630	Curik et al. 1997
	Germany	229	–	0.343	0.566	Ehrmann et al. 1997
HF*	USA	526	0.010-0.060	0.310-0.660	0.240-0.620	Swaissgood 1992
	USA	6000	0.010-0.040	0.310-0.490	0.490-0.620	Eenennam et al.1991
	Hungary	768	0.107	0.418	0.470	Baranyi et al. 1997
	Germany	229	0.026	0.472	0.496	Ehrmann et al. 1997
	Poland	143	–	0.402	0.598	Kamiñski et al. 2006a
	New Zealand	3761	–	0.465	0.510	Winkelman et al.1997
	Norway	306	0.010	0.400	0.490	Lien et al. 1993
Black-and-White*	Denmark	223	0.030-0.080	0.550	0.390	Bech et al. 1990
Red-and-White*	Sweden	394	0.008	0.460	0.531	Lunden et al. 1997
	Germany	179	0.020	0.573	0.366	Ehrmann et al.1997
Ayrshire*	New Zealand	37	–	0.432	0.527	Winkelman & Wickham 1997
	Finland	686	0.001	0.509	0.490	Ikonen 1997
	UK	29	0-0.003	0.600	0.400	Swaissgood 1992
	USA	45	0	0.720	0.280	Swaissgood 1992
Red*	Denmark	169	0.044-0.060	0.710	0.230	Bech et al.1990
Turkish Grey	Turkey	34	0.029	0.426	0.544	Dinc et al. (2013)
Eastern Anatolian Red		34	0.059	0.118	0.824	
Anatolian Black		34	0.103	0.132	0.765	
Southern Anatolian Red		30	0.117	0.117	0.766	
Turkish Holstein		34	0.029	0.485	0.456	
Holstein Candidate Bulls		18	0	0.278	0.722	
Slovak Simmental	Slovakia	111	-	0.2928	0.7072	Miluchová et al. (2014)
Pinzgau		89	-	0.5618	0.4382	
Holstein		87	-	0.3678	0.6322	
Holstein/cows		92	-	0.54	0.46	Hanusova et al. (2010)
Holstein / bulls		5	-	0.60	0.40	
Shibpur Upazila/ Noakhali Sadar Indigenous and cross-breed	Bangladesh	35		0.17	0.83	Rahman et al. (2016)
Shibpur Upazila/ Noakhali Sadar Indigenous and cross-breed		25		0.10	0.90	

*: Obtained from Kamiński et al. (2007); HF: Holstein - Friesian

Table 7b.
The allele and genotype frequencies of β-casein gene in some cattle breeds reared in Indian

Cattle breed/ Utility type	Country	Number of Animals (N)	β Casein					References
			Allele Frequencies		Genotype Frequencies			
			A1	A2	A1A1	A1A2	A2A2	
Kangayam/Draught	India	48	0	1	0	0	1	Mishra et al. (2009)
Nimari / Draught		45	0	1	0	0	1	
Red Kandhari/ Draught		39	0	1	0	0	1	
Malnad Gidda/ Draught		47	0.096	0.904	0	0.191	0.809	
Kherigarh/ Draught		23	0.109	0.891	0	0.218	0.783	
Malvi/ Draught		44	0	1	0	0	1	
Amritmahal/ Draught		37	0	1	0	0	1	
Kankrej/ Milch		32	0	1	0	0	1	
Gir/ Milch		45	0	1	0	0	1	
Sahiwal / Milch		47	0	1	0	0	1	
Hariana/ Dual		48	0	1	0	0	1	
Tharparkar/ Dual		44	0	1	0	0	1	
Rathi/ Milch		46	0	1	0	0	1	
Mewati/ Dual		40	0	1	0	0	1	
Red Sindhi/ Milch		33	0	1	0	0	1	
Holstein	India	51	0.441	0.559	0.216	0.451	0.333	Sodhi et al. (2012)
Jersey		40	0.325	0.675	0.025	0.600	0.375	
Crossbreed		89	0.298	0.702	0.101	0.393	0.506	
Ongole	India	38	0.06	0.94	0	0.11	0.89	Ganguly et al. (2013)
Frieswal (HF^{+} x Sahiwal) Heifers		124	0.32	0.68	0.12	0.40	0.48	
Frieswal (HFxSahiwal) Bulls		48	0.44	0.56	0.23	0.42	0.35	

$^{+}$HF: Holstein - Friesian

3. The structure of *β*-casomorphins and *β*-casomorphin-7

Beta-casomorphins (BCMs), opioid peptides derived from milk proteins, have amino acid groups starting from tyrosine at 60th position of *β* casein in length ranging from 4 to 11 (Kostyra et al., 2004). The amino acid compositions of *β*-casomorphins in human and cattle milks are given in Table 8.

Some milk proteins have been identified as active peptide-opioid sources (Brantl et al 1979; Chang et al 1985; Kostyra et al 2004). Opioids bind to opioid μ-receptors in the central nervous system and gastrointestinal tract (Teschemacher 2003). However, in the base form such substances are not active, but they become active. When they are digested during stomach/intestinal proteolytic digestion or product processing and so on. On the other hand, it causes the formation of various bioactive peptides after the consumption of such dairy products that are proteolyzed in the gastrointestinal proteolytic digestion (Kostyra et al 2002). Opioid peptides play a role in various biological processes such as respiration, analgesic, constipation and behavior in humans (Ng-Kwai-Hang & Grosclaude 2003).

Table 8
β-casomorphins and amino acid compositions from human and bovine milk (Kamiński et al 2007; Nguyen et al 2015)

***β*-Casomorphin**	**Amino acid composition**
Bovine BCM-4	Tyr-Pro-Phe-Pro
Bovine BCM-5	Tyr-Pro-Phe-Pro-Gly
Bovine BCM-6	Tyr-Pro-Phe-Pro-Gly-Pro
Bovine BCM-7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile
Bovine BCM-8	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro
Bovine BCM-9	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn
Bovine BCM-10	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser
Bovine BCM-11	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro--Asn-Ser-Leu
Human BCM-7	Tyr-Pro-Phe-Val-Glu-Pro-Ile
HumanBCM-8	Tyr-Pro-Phe- Val-Glu -Pro-Ile-Pro

While histidine found in the 67th position during A1 *β*-casein milk digestion releases a bioactive peptide with 7 amino acids called beta-casomorphin 7 (BCM-7) in the small intestine, proline in the 67th position of the *β* casein amino acid peptide chain in A2 *β*-casein milk prevents this degradation (Roginski 2003; Kostya et al 2004). BCM-7 was first isolated in 1979 as a peptide with morphine-like activity (Brantl et al 1979). As the peptide bind between isoleucine and histidine in A1 *β* casein milk is broken down with elastase, the linkage between isoleucine and proline in A2 *β* casein milk cannot be broken. The product digested in A1 milk, ie *β* casein, constitutes a seven amino acid long polypeptide having the sequence Tyr60-Pro61-Phe62-Pro63-Gly64Pro65-Ile66. This polypeptide is called beta-casemorphin-7 (BCM-7). This bioactive peptide exhibits a strong opoid activity (Kürek et al 1992). Other polypeptide products such as BCM-9, BCM-13 and BCM-21 may also be formed together with BCM-7 (Kostya et al 2004; Parashar & Saini, 2015). BCM-7 is degraded as far as the BCM-5 and BCM-3 by the dipeptide peptidose IV (DPP IV) found on the surface of the absorptive cells and in the blood (Elliot et al 1999; McLachlan 2001). Figure 3 shows enzymes that are effective in the formation of *β*-casomorphin-7.

As can be seen from Figure 3; pepsin elastase enzyme causes the formation of BCM-21 by breaking the peptide bond between tyrosine and proline in the 80th position with the peptide bond between the leuricine and valine found in the 59th position of the *β*-casein molecule. The leucine amino peptidase (LAP) breaks the link between the valine and tyrosine at the 60th position, and the elastase enzyme simultaneously breaking the link between isocyanine and histidine between 66-67, causes the formation of BCM-7 *β*-casomorphin-7. The LAP also breaks the link between the valine and tyrosine amino acids at 60th position, as well as the chymotrypsin trypsin elastase enzyme breaks the link between 68-69 and BCM-9 is formed.

The LAP breaks the link between the valine and tyrosine at 60th position, in addition, it breaks the link between chymotrypsin 72-73 and constitutes BCM-13. Breaking the link between the valine at the 60th position and the tyrosine link of pepsin between and 80-81, LAP allows the formation of the BCM-21.

Traces of BCM-7 were identified in fresh milk (Cieœliñska et al. 2007; Cieślińska ve ark., 2012), some cheeses and commercial infant formulas, but not found in commercial yoğurt (De Noni ve Cattaneo, 2010). Therefore, Nguyen et al. (2015) stated that whether BCMs do form and the amount of BCM forming at different processing steps needs further investigation and possibly will depend on the heat treatment and fermentation process but remains an intriguing unknown.

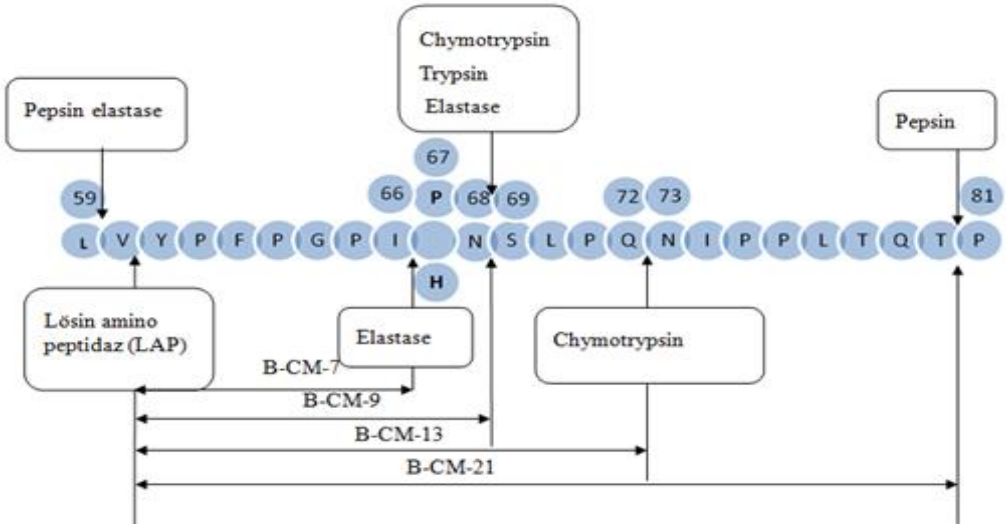


Figure 3
Cleavage sites in β-casein for gastrointestinal proteases leading to the release of BMCs (Jinsmaa & Yoshikawa 1999)

4. Conclusion and Recommendations

It can be concluded from the above that A1 milk is a variant of A2 milk. More specifically, A1 milk is a variant that causes the formation of β-casomorphin-7. Some other variants have also emerged with single base changes from different positions of codons encoding the β casein protein in A2 milk. However, the most prominent effect is that in the codon encoding the amino acid at 67th position of the β casein, the base A is replaced by the base C. When the CCT codon encodes the CAT proline 'amino acid at the 67th position of the β casein chain in A2 milk, the CAT codon is formed and this encodes histidine amino acid. Briefly, the following recommendations should be taken into consideration.

- Breeding studies together with DNA studies to remove the A1 gene from population should be carried out by determining A1 or A2 cows. The animals of A1 and A2 should be genetically established and the cattle population should be formed from animals with A2A2 genotype.
- The position of β-casomorphin-7 in milk and dairy products should be researched. Studies should be carried out to determine which process inactivates β-casomorphin-7.
- The bulls used in artificial insemination programs must be the ones with A2A2 genotypes.
- Animals that are imported for breeding or production should be cared for whether they are with A2A2 genotype in terms of the beta casein gene.
- By supporting the farmers producing A2 milk, we should contribute to the national economy by reducing the additional costs of beta-casomorphin -7 induced diseases.

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