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OPIOID PEPTIDES DERIVED FROM MILK PROTEINS

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This paper is devoted to opioid peptides derived from milk proteins. The evolutionary aspect of the biological activity of proteins and peptides, protein precursors, amino acid sequences of opioid peptides, and their biological activity are presented herein. Attention was also paid to the relationship between the conformation of β-casomorphins, their hydrophobicity and their ability to interact with opioid receptors. The biological function of opioid peptides derived from milk proteins was shown from the nutritional and pharmaceutical points of view. In this context, the gastrointestinal, analgesic, tolerance and dependence, respiratory, cardiovascular, immunomodulatory and allergic effects of opioid peptides are discussed. Finally, the perspectives of the application of opioid peptides derived from milk proteins in nutrition and pharmacy are also presented.

EVOLUTIONARY ASPECT OF THE BIOLOGICAL ACTIVITY OF PEPTIDES DERIVED FROM FOOD PROTEINS

Nutrition is an attribute of man's life determining his phylo- and ontogenetic development. However, before life could evolve on Earth as biologically-active forms of organic molecules, compounds with specific physicochemical properties first had to develop. These were amino acids which led to proteins. Hence, amino acids can be considered as one of the primary nutrients for living organisms which were only later to come into existence. It seems logical that in the primary mixture of organic substances there were no proteins, but only various amino acid polymers which did not show any sense of purpose in their intermolecular structure; moreover, they lacked any functional properties. It was only in the course of evolution that amino acid polymers, phase-liberated from the environment, could interact in a manner that directed their structure to perform biological functions. The course of organic-molecule evolution towards biological activity was determined first by the appearance of the crystalline structure and the optical properties in the amorphic and racemic mixture of the organic compounds, a fact which is also confirmed by the structure of the amino acids themselves. The importance of amino acids in the evolution of biologically-active systems is partly supported by their geological supremacy over carbohydrates. Regarding evolution, it is worth mentioning that in the beginning a predominance developed of aliphatic amino acids over aromatic amino acids containing comparatively high quantities of sulphur. This is interesting, because the biological properties of peptides are mainly affected by their hydrophobic character and the presence of aromatic acids. As aliphatic amino acids predominated in the Precambrian era, it can be presumed that the biological activity of peptides occurred at a later stage. This biochemical evolution of peptides towards protein structures has been confirmed in research results from the areas of molecular biology, palaeobiochemistry and bioelectronics, which allow us to presume that the evolution of protein molecules was aimed at elongating the polypeptide chain, expanding the side chains, as well as developing aromatization, including closing the chain into a ring, and a secondary structure. However, not only was the chemical structure of the protein important in the evolution of life, but also the proteins' electrical states, which determine their conformation. The phenomenon of protein amphotericity proves their adaptation for oxidoreductive fluctuations in the environment, while on the other hand, the cyclic structure of protein molecules enables them to save the so-called "delocalized electrons". The cyclization of protein is considered to be another remarkable concept regarding life's appearance, *i.e.* the closing of the polypeptide chain into a ring. The formation of such a protein structure offered the possibility of producing metal complexes, the so-called "chelate compounds". Such a degree of protein molecule configuration could in some cases be sufficient to transfer enzymatic properties, which are the basis of biological activity in living organisms, to a protein. Hence, a question arises of the relationship between the structure of food proteins and their biological functions. It seems barely probable that the food proteins

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only were the source of components for the biosynthesis of other proteins and organic molecules, or served as energetic material. Such an opinion would deny the discussed trends of evolution. Furthermore, the evidence of the multi-functionality of products of food protein hydrolysis in the human digestive tract is irrefutable. Thus, from a theoretical point of view, food proteins should be the precursors of biologically-active peptides, including the opioid peptides [Łukowski, 1987; Reinbothe, 1982; Sedlak, 1973].

MILK AND MILK PRODUCTS AS A SOURCE OF BIOACTIVE PROTEINS AND PEPTIDES

It is not an exaggeration to state that human milk is the most valuable food in the natural nutritional ecosystem. Human milk is the source of all nutritional components necessary for the proper physical and mental growth of a healthy neonate. The protein portion of milk is one of the major components responsible for its nutritive value [Zinn, 1997]. Human milk is distinguished from other milks by the quality and quantity of proteins. For example, the protein content reaches approximately 1, 3.5, 5.5 and 14 in human, bovine, porcine and rabbit milk, respectively [Jenness, 1985]. Bovine and ovine milks each contain similar quantities of α-lactoalbumin, β-lactoglobulin, α_{S1}-, α_{S2}- and κ-caseins, although ovine milk contains approximately 2.8 times more β-casein than bovine milk [Martin *et al.*, 1993]. However, minimal quantities of κ-casein, 30% of β-casein and the virtual absence of β-lactoglobulin, α_{S1}- and α_{S2}-caseins distinguish human milk from bovine or ovine milks [Martin *et al.*, 1993]. Milk proteins, together with other components, may influence the health of a neonate and the health and level of the milk production of a lactating female. In addition, lactating women demonstrate a reduced incidence of breast cancer [Freudenheim *et al.*, 1994]. For the neonate, there is an increase in healthfulness, especially in terms of an increased immune function and reduced enteric disorders in neonates fed with colostrum. Apart from major milk proteins, milk contains many biologically-active proteins [Donovan *et al.*, 1994; Compana *et al.*, 1995; Maubois, 1989; Tirelli *et al.*, 1977; Ramabadran, 1984]. The following proteins and peptides are classified in this group: hormones from the anterior pituitary gland (*e.g.* prolactin and somatotropin), the hypothalamus (*e.g.* somatotropin-releasing hormone and somatostin), and the gut (*e.g.* vasoactive intestinal peptide, gastrin and substance P). Milk also contains a number of growth factors and a variety of other bioactive peptides, including insulin-like growth factor I and II (IGF), IGF binding proteins, epidermal growth factor (EGF), transforming growth factors, prostaglandin $F_{2\alpha}$ and E, lactofferin and transfferin [Donovan *et al.*, 1994; Compana *et al.*, 1995]. A spectacular example of the biological activity of these protein compounds is the fact that milk prolactin takes part in the neurological development of neonates [Grosvenor *et al.*, 1992; Ellis *et al.*, 1996]. A specific group of biologically-active milk peptides are the opioid peptides, which are discussed in this paper. Opioid peptides are generated *in vivo*, *in vitro* and during food processing. They are largely found in milk, fermented milk and cheeses. Proteolytic enzymes, naturally-occurring in milk and enzymes from lactic acid bacteria or from exogenous sources contribute to the generation of opioid peptides. Dairy processing conditions, such as cheese ripening, are also relevant [Smacchi *et al.*, 2000]. The protein precursors, amino sequences and bioactivity of opioid peptides derived from milk proteins are presented in Table 1.

TABLE 1. Opioid peptides derived from milk proteins.

Opioid peptide	Protein precursor	Amino acid sequence	Bioactivity	Ref.
[His^8]-β-Casomorphin-21	bovine β-casein [60-81]	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-[His]-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr	agonist	[Jinsmaa & Yoshikawa, 1999]
[Pro^8]-β-Casomorphin-21	bovine β-casein [60-81]	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-[Pro]-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr	agonist	[Jinsmaa & Yoshikawa, 1999]
[His^8]-β-casomorphin-13	bovine β- casein [60-72]	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-[His]-Asn-Ser-Leu-Pro-Gln	agonist	[Jinsmaa & Yoshikawa, 1999]
β-casomorphin 11	bovine β-casein [60-70]	Tyr-Pro-Phe-Pro-Ile-Pro-Asn-Ser-Leu	agonist	[Meisel, 1989]
[His^8]-β-casomorphin-9	bovine β-casein [60-68]	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-[His]-Asn	agonist	[Jinsmaa & Yoshikawa, 1999]
pro-[His^8]-β-casomorphin-9	bovine β-casein [59-68]	Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-[His]-Asn	agonist	[Yoshikawa *et al.*, 1994]
pro-[Pro^8]-β-casomorphin-9	bovine β-casein [59-68]	Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-[Pro]-Asn	agonist	[Yoshikawa *et al.*, 1994]
β-Casomorphin-8	bovine β-casein [60-67]	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-[Pro]	agonist	[Ramabadran & Bansinath, 1984]
β -Casomorphin-8	human β-casein	Tyr-Pro-Phe-[Val]-[Glu]-Pro-Ile-Pro	agonist	[Yoshikawa *et al.*, 1994]
pro-β-casomorphin-7	bovine β-casein [59-66]	Val-Tyr-Pro-Phe-Pro-Gly-[Pro]-Ile	agonist	[Brantl *et al.*, 1979]

β-Casomorphin-7	bovine β-casein [60-66]	Tyr-Pro-Phe-Pro-Gly-[Pro]-Ile	agonist	[Teschemacher, 1985]
β-Casomorphin-7	human β-casein	Tyr-Pro-Phe-[Val]-[Glu]-Pro-Ile	agonist	[Lottspeich *et al.*, 1980]
β-Casomorphin-6	bovine β-casein [60-65]	Tyr-Pro-Phe-Pro-Gly-[Pro]	agonist	[Jinsmaa & Yoshikawa, 1999]
β-Neocasomorphin-6	bovine β-casein [114-119]	Tyr-Pro-Val-Gln-Pro-Phe	agonist	[Brantl *et al.*, 1979]
β-Casomorphin-5	bovine β-casein [60-64]	Tyr-Pro-Phe-Pro-Gly	agonist	[Chiba & Yoshikawa, 1986]
β-Casomorphin-4	bovine β-casein [60-63]	Tyr-Pro-Phe-Pro	agonist	[Chang *et al.*, 1985]
β-Casorphin-4-amide (morphiceptin)	bovine β-casein [60-63]	Tyr-Pro-Phe-ProNH_2	agonist	[Loukas *et al.*, 1983]
α-Casein exorphin	bovine α-casein [90-96]	Arg-Tyr-Leu-Gly-Tyr-Leu-Glu	agonist	[Loukas *et al.*, 1983]
α-Casein exorphin	bovine α-casein [90-95]	Arg-Tyr-Leu-Gly-Tyr-Leu	agonist	[Yoshikawa *et al.*, 1986]
α-Lactorphin	human α-lactalbumin [50-53]	Tyr-Gly-Leu-Phe-NH_2	agonist	[Yoshikawa *et al.*, 1986]
β-Lactorphin	bovine β-lactoglobulin [102-105]	Tyr-Leu-Leu-Phe-NH_2	agonist	[Tani *et al.*, 1990]
Serorphin	bovine serum albumin [399-404]	Tyr-Gly-Phe-Gln-Asn-Ala-	agonist	[Yoshikawa *et al.*, 1994]
Casoxin A	bovine χ-casein [35-41]	Tyr-Pro-Ser-Tyr-Gly-Leu-Asn-Tyr	antagonist	[Yoshikawa *et al.*, 1994]
Casoxin C	bovine χ-casein [25-34]	Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg	antagonist	[Yoshikawa *et al.*, 1994]
Casoxin D	human α_{S1}-casein	Tyr-Val-Pro-Phe-Pro-Pro-Phe	antagonist	[Yoshikawa *et al.*, 1994]
Albutensin A	bovine serum albumin	Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg	antagonist	[Yoshikawa *et al.*, 1994]
β-Lactotensin	β-lactoglobulin [146-149]	His-Ile-Arg-Leu	antagonist	[Tani *et al.*, 1990]
Lactoferroxin A	human lactoferrin [318-323]	Tyr-Leu-Gly-Ser-Gly-TyrOCH_3*	antagonist	[Tani *et al.*, 1990]
Lactoferroxin B	human lactoferrin [536-540]	Arg-Tyr-Tyr-Gly-Tyr-OCH_3*	antagonist	[Tani *et al.*, 1990]
Lactoferroxin C	human lactoferrin [673-679]	Lys-Tyr-Leu-Gly-Pro-Gln-Tyr-OCH_3*	antagonist	[Tani *et al.*, 1990]

· Agonist – peptide that binds to opioid receptor and thereby alters its proportion in an active form, resulting in a biological response.
· Antagonist – peptide that reduces the action of the agonist.
·* By the isolation procedure all three peptides were methoxylated at the C-terminus.
· [ ] Boxed amino acid residues represent positional mutations which differentiate variants A, C and D caseins from B one.

STRUCTURAL DETERMINANTS OF THE OPIOID ACTIVITY OF β-CASOMORPHINS

Morphine and β-casomorphins belong to different classes of the chemical components, alkaloids and peptides respectively. Consequently, why do they interact with the same opioid receptors? There is only one explanation to this phenomenon. The communication between biologically-active compounds and the receptors in living organisms is based on their structural similarity. In this case, the biological activity must correlate not only with the steric, but also with the electronic properties of the molecules. This means that energetically stable conformations may exist in all active compounds that possess the potential for similar interaction behavior with the structurally-unknown receptor [Brandt *et al.*, 1994]. This phenomenon can be illustrated by

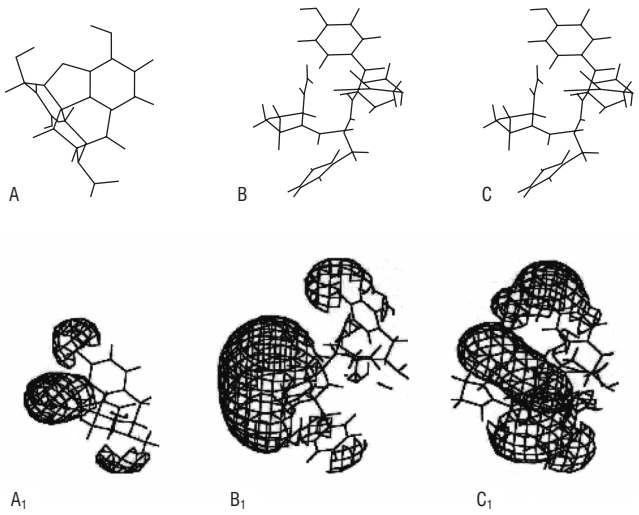

FIGURE 1. Structures of morphine (A), morphiceptin (B) and β-casomorphin-5 (C) and their view of the molecular electrostatic potentials (A_1, B_1,) [Brandt *et al*., 1994, permission].

the comparison of the structures and opioid activities of morphine, morphiceptin and β-casomorphin-5. In this case, the opioid activity means the ability of the opioid compounds to bind with opioid receptors. The opioid receptors are classified in three subtypes: $M_{\mu 1}$, $M_{\mu 2}$ and delta. The highly selective receptor for morphine is $M_{\mu 2}$, although it can also bind with $M_{\mu 1}$. β-Casomorphin-5 is the most active opioid peptide among β-casomorphins. It has a relatively low affinity to the μ-receptor ($K_i \geq 1\mu M$) and a very low affinity to delta receptors ($K_i \geq 10\mu M$) [Liebmann *et al.*, 1991]. The affinity and activity of δ-casomorphins at kappa-receptors seem to be negligible [Liebmann *et al.*, 1994], whereas morphiceptin demonstrates higher μ-selective opioid activity and a lower degree of conformational freedoms than the bovine β-casomorphin-5. Brandt *et al.* [1994] used the method of molecular graphics (MEPS) to explain the relationship between the molecular structure of morphine, morphiceptin and β-casomorphin-5 and their opioid activities. Graphic results of their investigations are presented in Figure 1, and it is worth remembering that the MEPS represents the electrostatic interactions between molecules and a positive unit charge (proton). The analysis of the potential allows certain conclusions to be drawn regarding the interactions of different parts of molecules with nucleophilic or electrophilic receptor binding sites. Thus, for example, a negative molecular electrostatic potential indicates an area of attraction for positively-charged interaction partners [Brandt *et al.*, 1994].

Moreover, these authors ascertained that the occurrence of a nitrogen atom in morphine in a defined spatial position with respect to an aromatic ring system (A-ring) is one of the structural elements essential for opioid activity. The presence of a phenolic hydroxyl group on this ring seems to strengthen the receptor affinity, but it is not necessary for the occurrence of opioid activity. The second benzene ring (F-ring) also seems to be of importance [Brandt *et al.*, 1994]. It is worth noting that all of the essential fragments mentioned above are present in β-casomorphins. The problem of the binding of the opioid peptides to the receptors can also be considered from the perspective of the hydrophobic and hydrophilic interactions. To this end, the aromatic and aliphatic hydrophobicities were calculated using the Tandford method [Tandford, 1962].

The calculation of hydrophobicity of opioid peptides derived from milk proteins is presented below:

$$Q = \Sigma \Delta f/n \qquad Q_1 = \Sigma \Delta f/n_1$$

where: Q – total average hydrophobicity of peptide; Q_1 – aromatic average hydrophobicity of peptide; Q_2 – aliphatic average hydrophobicity of peptide ($Q_2 = Q - Q_1$); Δf – value of amino acid residues (Table 2); n – number of all amino acid residues; and n_1 – number of aromatic amino acid residues.

TABLE 2. Δf values of the side chains of amino acids, representing their hydrophobicity, according to Tanford [1962].

Amino acid	Δf – values (cal/mol)
Gly	0
Ser	40
Thr	440
His	500
Asp	540
Asn	-10
Glu	550
Gln	-100
Arg	730
Ala	730
Met	1330
Lys	1500
Val	1690
Leu	2420
Pro	2620
Phe	2650
Tyr	2870
Ile	2970
Trp	3000

It is necessary to emphasise that the casual relationship of the pattern and the ability of exiorphins to specifically bind with receptors is still unclear. Bakalkin *et al.* [1992] looked at this problem from the perspective of the presence of specific hydrophobic and hydrophilic segments in an opioid peptide. They classified the amino acid residues into four groups: hydrophobic residues Pro, Tyr, Ile, Val, Trp and Cys (group P); Phe, Leu, Ala, Met (group F); hydrophilic residues Gly, Asn, (G); and Asp, Glu, His, Gln (B). The P group more often precedes rather than follows an adjacent Gly or Asn. The G residues more frequently precede rather than follow any other group of hydrophobic residues (Phe, Leu, Ala and Met-group F). The F group more frequently precedes Arg, Ser, Thr and Lys (group R of hydrophilic residues) rather than follows them; and, finally, the R group more frequently precedes rather than follows the P group. In conclusion, the researchers stated that there was a correlation between certain characteristics of the primary structure and biological activity in the opioid peptide family. The characteristics of the primary structure are the contents of certain segment pairs as well as the density of their arrangement in a peptide [Bakalkin, 1992]. These results seem to coincide with our simulated evaluation of the hydrophobicity of the opioid peptide derived from milk (Table 3). The majority of opioid peptides are characterized by a total

TABLE 3. Hydrophobicity of opioid peptides derived from milk proteins.

Opioid peptide	Total hydrophobicity	Aromatic hydrophobicity	Aliphatic hydrophobicity
[His^8]-β-casomorphin-21	1582	263	1319
[Pro^8]-β-casomorphin-21	1683	263	1420
[His^8]-β-casomorphin-13	1709	263	1446
[His^8]-β-casomorphin-9	1871	613	1258
pro-[His^8]-β-casomorphin-9	2294	613	1681
pro-[Pro^8]-β-casomorphin-9	2530	613	1917
β-Casomorphin-8 (bovine)	2371	613	1758
β-Casomorphin-8 (human)	2323	613	1710
pro-β-casomorphin-7 (bovine)	2255	690	1565
β-Casomorphin-7 (bovine)	2335	789	1546
β-Casomorphin-7 (human)	2203	789	1414
β-Casomorphin-6	2230	920	1310
β-Neocasomorphin-6	2058	920	1138
β-Casomorphin-5	2152	1104	1048
β-Casomorphin-4	2690	1380	1310
β-Casomorphin-4-amide	?> 2690	?> 1380	?> 1310
α-Casein exorphin (90-96)	1694	957	737
α-Casein exorphin (90-95)	1885	1380	505
α-Lactorphin	1985	1380	605
β-Lactorphin	2590	1380	1210
Serorphin	1023	920	103
Casoxin A	1710	358	1352
Casoxin C	1908	574	1334
Casoxin D	2531	1167	1364
Albutensin A	1286	333	953
β-Lactotensin	1655	000	1655
Lactoferroxin A	1367	957	410
Lactoferroxin B	2282	1722	560
Lactoferroxin C	1740	820	920

hydrophobicity of over 2000 cal/mol and by an aromatic hydrophobicity of over 600 cal/mol. The total and aromatic hydrophobicities seem to correlate with the molecular weight of opioid peptides. The highest hydrophobicity is demonstrated by the opioid peptides with the lowest molecular weight. This fact agrees with Bakalkin's data, which proved the presence of specific hydrophobic domains in exorphins and endogenous peptides [Bakalkin *et al.*, 1992]. Similarly, a correlation is also observed between aromatic hydrophobicity and the affinity to opioid receptors. For example, to characterize the ability of opioid peptides to interact with an opioid activity receptor, Bakalkin *et al.* [1992] used IC_{50} and K_i values obtained in pharmacological assays on isolated electrically-stimulated tissues (guinea pig ileum, GpI) of mice or rats vas deferens (M/RVD), and in biochemical binding assays, respectively. The $-\log[IC_{50}](M)$ were 6.2, 5.6, 4.2 for morphiceptin, β-casomorphin-5 and α-casein (90–95), respectively. The binding assays correlate well with the aromatic hydrophobicity of these peptides, which reaches 1380, 1104 and 1380 cal/mol, respectively. Summing up the above-mentioned results, the following view on the structure of an opioid peptide-opioid receptor complex could be postulated thus: the effective binding of opioid peptide to opioid receptors requires the triple functioning of the donor-acceptor system and hydrophobic site. The C and side terminal residues of amino acids could function in a donor-acceptor system. Furthermore, the hydrophobic-hydrophilic balance in a peptide molecule seems to be an additionally important factor.

BIOLOGICAL ACTIVITY OF β-CASOMORPHINS

The biological activity of β-casomorphins has also been investigated from the physiological and pharmacological perspectives as a result of the fact that β-casomorphins are the natural components of food and, additionally, they have properties similar to those of morphine and exhibit naloxone-inhibiting activities [Smacchi, 2000; Krawczuk *et al.*, 2000; Chang *et al.*, 1985]. α- and β-casomorphins and lactorphins act as opioid agonists, whereas casoxins behave as an opioid antagonist [Meisel *et al.*, 1989; Meisel, 1990].

OPIOID EFFECT ON GASTROINTESTINAL MOTILITY AND FOOD INTAKE

Opioids can influence gastrointestinal functions by interacting with opioid receptors located in the central nervous system and in the myenteric plexus. The administration of opioids, including β-casomorphins, delays gastric emptying and inhibits the intestinal transit of the digesta in various species [Shook, 1986; Daniel *et al.*, 1990]. This means that β-casomorphins can influence satiety. β-Casomorphin-5 and morphiceptin induce a naloxone-reversible reduction in a short-circuit current when applied to the mucosal side in the isolated rabbit ileum. Therefore, it appears that β-casomorphins can alter the intestinal electrolyte transport [Hautefeuille *et al.*, 1986]. Schusdziarra *et al.* [1983] investigated the influence of bovine casein peptone and β-casomorphins on the release of insulin, somatostatin and pancreatic polypeptide. They proved that the mixture of casein peptone and β-casomorphins stimulated insulin, somatostatin and pancreatic polypeptide release in dogs [Schusdziara *et al.*, 1983 a,b,c]. β-Casomorphin-7 and β-casomorphin-5 have also been reported to stimulate the intake of a high-fat diet when given either intraperitoneally or centrally ICV to satiated rats [Lin *et al.*, 1998]. On the other hand, Christy *et al.* [2000] found that enterostatin injected intragastrically into rats fasted overnight caused a U-shaped dose-dependent reduction in the intake of the high-fat diet for the first two hours after infusion, but had no effect on the low-fat diet intake. β-Casomorphin-7 stimulated the intake of the high-fat diet. Furthermore, β-casomorphin-7 blocked the inhibitory effect of enterostatin on high-fat diet intake in fasted rats [Christy *et al.*, 2000].

ANALGESIC ACTIVITY

When β-casomorphins are injected in the blood stream, they induce an analgesic and sedative effect due to their action on the nervous system [Paroli, 1988]. β-Casomorphin-4, β-casomorphin-5, and casomorphin-6 cause analgesia for up to 45 minutes, whereas casomorphin-7 causes analgesia for more than 90 minutes [Mathies *et al.*, 1984]. Furthermore, early neonatal exposure of rats to morphiceptin results in a hyperalgesic state in later life (4.5 months) [Zadina *et al.*, 1987]. These results indicate that the extrapolation of these data to humans requires further study, especially due to the wide differences in the developmental processes of human neonates [Fitzgerald, 1987].

TOLERANCE AND DEPENDENCE

As β-casomorphins belong to the opioid group, it is logical to expect that with repeated use they can cause the phenomenon of tolerance and physical dependence. The tolerance was observed after continuous intrathecal infusion (0.5 μL h^{-1} for 3.5 to 4.5 days) of morphiceptin in rats, although dependence was not assessed in this study [Russell *et al.*, 1987]. However, Chang *et al.* [1983] found a relationship between the physical dependence and the use of morphiceptin. In their study, the ability of morphiceptin to induce physical dependence was measured after infusion (1–23 μL h^{-1} for 70–72 h) of the peptide into the junction of the aqueduct and the fourth ventricle in rats. The withdrawal was precipitated by naloxone (4 mg kg^{-1}). Generally, it can be concluded that chronic infusion of β-casomorphins produces a physical dependence which is comparable to that of other opiates.

RESPIRATORY EFFECT

Bovine β-casomorphin-5 and 7 and morphiceptin, when administered intracerebroventricularly to pre-term newborn rabbits, cause a dose-related depression of respiratory frequency and tidal volume [Hedner, 1987]. Morphiceptin and β-casomorphin-7 were as potent as morphine, whereas β-casomorphin-5 was 10 times as potent. Respiratory depression could also be elicited by the systematic administration of morphiceptin, but not of β-casomorphin-5 and 7. All ventilatory effects induced by β-casomorphins could be readily reversed by naloxone. This phenomenon is connected with sudden infant death syndrome (SIDS). Sudden infant death syndrome is defined as the sudden death of an apparently healthy infant less than 1 year of age, which remains unexplained after a thorough investigation, including the performance of a complete autopsy, an examination of the scene of death and a review of the clinical history [Sun *et al.*, 2003]. Probably, certain β-casomorphins culpability in SIDS were justified by Peruzzo's [2000] investigations, which reported on a progressive development of the barrier between the median eminence and the caudate nucleus in rats during the first postnatal weeks, suggesting that the brain is not fully developed in the newborn rat. Thus, it could be presumed that the introduction of β-casomorphins into the central nervous system would be expected to occur more readily in infants before the blood-brain-barrier is fully developed. On the other hand, there is no convincing evidence that an increased opioid activity is related to SIDS. However, the similarities between the hyperendorphin syndrome and near-miss SIDS have led to speculation that excessive endogenous opioid activity might be one of many causes of SIDS [Kuich, 1981]. An extension of this premise would be that the excessive exogenous opioid activity of β-casomorphins may induce or add to the exogenous opioid tone to impair breathing in the neonate.

CARDIOVASCULAR EFFECTS

The administration of opioid peptides intracerebrally, intravenously or directly to the brain stem areas produces powerful cardiovascular responses [Holaday, 1983]. For example, the intracerebroventricular or intraveneous injection of morphiceptin into normotensive rats produce bradycardia and hypotension [Widy-Tyszkiewicz *et al.*, 1986; Wei *et al.*, 1980], whereas in hypertensive rats, intraventricular injections of morphiceptin produce a dose-related increase in heart rate and blood pressure. In isolated guinea-pig heart preparations, β-casomorphin-5 exerts a positive inotropic effect at low doses and a cardiodepressive action at higher doses [Liebman *et al.*, 1986].

IMMUNOMODULATORY AND ALLERGIC EFFECTS

Certain findings indicate that β-casomorphins may play an immunomodulatory and allergic role in human and animal organisms [Teschemacher, 1985; Kurek *et al.*, 1990]. Casein-derived immunopeptides, including immunopeptides from α_{S1}-casein (194–199) and β-casein (63–68 and 191-193), stimulate the phogocytosis of sheep red blood cells by mirine peritoneal macrophages, and exert a protective effect against *Klebsiella pneumoniae* infection in mice after intravenous treatment [Parker *et al.*, 1984; Migliore-Saymour, 1989]. Kayser and Meisel [1996] investigated the *in vitro* modulation of the proliferation of human peripheral blood lymphocytes by different peptides derived from milk proteins. They found that lymphocyte proliferation was suppressed at lower concentrations, and stimulated at higher concentrations for β-casomorphin-7 and β-casoxin-10. Nymberg *et al.* [1989] measured plasma and cerebrospinal fluid β-casomorphin-8 by RIA in women during late pregnancy and lactation as well as in non-pregnant, non-puerpeal women. They suggested that fragments of the milk protein β-casein may cross the breast parenchyma-blood barrier into plasma, and subsequently penetrate the blood-brain barrier to reach the central nervous system. The mentioned investigations should also be considered in the context of the postpartum psychosis [Lindstrom *et al.*, 1984]. The postpartum psychosis occurs in 0.1–0.2% of women 6 months after delivery. Lindstrom *et al.* [1984] measured opioid receptor-active components in the cerebrospinal fluid (CSF) of 11 women with postpartum psychosis, 11 healthy lactating women, and 16 healthy women who were not lactating. They concluded that certain cases of postpartum psychosis are associated with the occurrence in plasma CSF of unique opioid peptides probably related to bovine β-casomorphins. There are only scarce data on the allergic properties of opioid peptides derived from milk proteins [Kurek *et al.*, 1990; Jarmołowska *et al.*, 1999]. Nevertheless, their immunogenic properties and their ability to produce an anaphylactic reaction suggest their allergic potential.

PERSPECTIVES OF THE APPLICATION OF MILK PROTEIN-DERIVED OPIOID PEPTIDES IN NUTRITION AND PHARMACY

Investigations over the last few years have shown that proteins are no longer only considered nutritional components. They are also claimed to possess other biological properties [Kostyra, 1992; Meisel, 1990; Rokka *et al.*, 1997; Jarmołowska *et al.*, 1999; Muehlenkamp, 1996]. This means that novel food should be evaluated not only from the nutritional but also from the physiological point of view [Bos *et al.*, 2000]. Opioid peptides are generated *in vivo*, *in vitro* and during food processing, and they are in an inactive state in their peptide precursors. It has been shown that these

peptides may be released in the proteolysis induced by digestive and bacterial enzymes [Rokka *et al.*, 1997; Smacchi, 2000; Ferranti *et al.*, 2004]. Milk-derived peptides, including opioid peptides, can be produced on an industrial scale and, as a consequence, they have already been considered for application both as dietary supplements in "functional foods" and as drugs. Hence, they are claimed to be health-enhancing nutraceuticals for food and pharmaceutical preparations [Meisel, 1997]. The production of bioactive peptides can proceed during food processing upon the application of heat, alkali or acid conditions which hydrolyse proteins; the enzymatic hydrolysis of food proteins; and the microbial activity of fermented foods [Pihlanto--Leppälä, 2001; Bitri, 2004]. It should be noted that there is a growing interest in employing genetic engineering in the production of human milk proteins, peptides, growth factors, and other bioactive substances [Korhonen *et al.*, 1998; Rosen *et al.*, 1996; Karatzas, 1997]. Human milk proteins that have been cloned from a mammary gland library are α-lactoalbumin, lactopherrin, lysozyme, collagen, β-casein, and κ-casein [Lönnerdahl, 1996; Colman, 1996]. These facts prove that there is a real perspective for designer milks to be improved through various combinations of genetics and by farm and feed management [Boland *et al.*, 2001].

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FINAL REPORT

Title of the research ordered project:
THE METHODOLOGICAL BASES OF THE EVALUATION OF THE QUALITY AND SAFETY OF THE NEW GENERATION FOOD (PBZ-KBN-020/P06/1999).

Title of the individual project:
Characterization of the opioid activity of the chosen dairy products.

Institution:
Department of Biochemistry, Faculty of Biology, University of Warmia and Mazury in Olsztyn, Poland.

Leader:
Prof. Dr. Elżbieta Kostyra

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Key words: women colostrum and milk, cow milk, acidophilic buttermilk, kefir, yogurt, cheeses, β-casomorphin-4, 5 and 7, opioid activity, HPLC, poly- and monoclonal antibodies, ELISA

SYNTHESIS OF RESULTS

Milk is the only natural food of mammalian newborns. Each mammalian species provides the specific nutrients needed by its newborn by producing milk with a unique composition which mirrors these individual requirements. The milk proteins are the source of not only amino acids but also of biologically-active peptides. Consistently, the dairy products containing milk proteins can be the source of these peptides. An important group of biological peptides are the opioid peptides. Both human and bovine caseins contain amino acid sequences displaying opioid activity. The physiological importance of β-casomorphin peptides has not been fully recognised yet (see previous paper). For example, some authors suggest that the opioid peptides are responsible for such pothological symptoms as: sudden infant death, postnatal psychosis in women and food allergic reaction. They can also stimulate some physiological processes as: stimulation of endocrine activity of pancreas, prolongation of the gastrointestinal transit time. For this reason, it is important to determine the content of the opioid peptides in human and cow milk and milk products.

The main idea of this project was to determine the content of the opioid peptides in milk and its products, and on this ground to propose a new indicator of dairy products quality, called – "opioid indicator " or "opioid activity".

The investigations were carried out using the following products: women colostrum, women milk, raw, pasteurized and UHT cow's milk; fermented products: acidophilic cow milk, kefir, yoghurt and acidophilic buttermilk; moulded cheeses: "Rokpol", "Valbon", "Brie"; hard cheeses: "Edam", "Gouda", "Mazdamer", "Kasztelan".

Peptides were extracted from milk and milk products with a chloroform/methanol mixture (2:1 v/v), according to the Halwarkar method. The peptide extracts were purified by SPE method. The purified peptide extracts were separated by SDS-PAGE electrophoresis and HPLC chromatography (gradient of acetonitrile, 0–30%). The opioid activities of the peptide extracts were determined by the Magnus method as described by Jaske with hybrid rabbits. Contractions of the isolated intestine were registered with the apparatus for registration of the isotonic contractions, Hugo Sach's company. The polyclonal and monoclonal antibodies against β-casomorphin-7 for the immunometric analysis by ELISA method were obtained.

The results obtained by SDS-PAGE electrophoresis and HPLC chromatography proved the significant qualitative and quantitative differences in the peptide extracts derived from women and cow milks, fermented milk products and cheeses. All investigated peptide extracts displayed the opioid activity, except for the peptide extract derived from yoghurt. The presence of β-casomorphin-4, 5 and 7 was proved in the investigated peptide extracts. But, not all mentioned β-casomorphins were present in all peptide extracts. β-Casomorphin-4 was absent in the peptide extracts derived from cow raw milk, acidophilic milk, yoghurt and Gouda cheese. β-Casomorphin-5 was absent only in yoghurt. While, β-casomorphin-7 was absent in six peptide extracts derived from UHT milk, acidophilic milk, buttermilk, kefir, yoghurt and Mazdamer cheese. The following amounts of β-casomorphin-4, 5 and 7 in peptide extracts were determined (expressed in ng/g of peptide extract): women colostrum (1.16, 5.97, 2.36), women milk (0.17, 6.39, 0.93), raw cow's milk (0.00, 5.70, 0.56), pasteurized milk (1.65, 7.40, 0.33), UHT milk (2.50 , 0.78, 0.00), acidophilic milk (4.26, 1.75, 4.60), acidophilic buttermilk (0.03, 0.26, 0.50), "Mazur" kefir (0.36, 0.16, 0.00), "Bakoma" yoghurt (0.00, 0.00, 0.00), "Edam" cheese (4.69, 1.17, 5.38), "Gouda" cheese (0.00, 6.30, 1.72), "Mazdamer" cheese (0.82, 2.86, 0.00), "Kasztelan" cheese (2.64, 1.82, 0.76), "Brie" cheese (1.12, 0.86, 2.45), "Valbon" cheese (2.44, 9.50, 0.06), and "Rokpol" cheese (0.32, 2.27, 0.12).

In the context of the above results, it was very interesting to determine the opioid activity of the investigated peptide extracts. The opioid activity was measured by examining the effects of morphine, naloxon and peptide extract on the motor activity of isolated rabbit intestine. Morphine (agonist) increases the amplitude of intestinal contractions, proving their interaction with opioid receptors. Naloxon, which is morphine antagonist, softened the intestinal con-

tractions. The investigations of the contractions of the isolated intestine caused by the peptide extracts derived from the different dairy products enabled answering a question whether the contractions of the intestine are proportional to the content of the opioid peptides in them. It is worth emphasizing that β-casomorphin-5 is more reliable in characterizing the opioid activity compared to β-casomorphin-4 and 7. The average values of the contractions of the intestine for morphine and the investigated peptide extracts were as follows (expressed in miliniuton): morphine (17.37), women colostrum (5.97), women milk (5.75), raw cow's milk (5.15), pasteurized cow's milk (6.80), UHT cow's milk (5.36), acidophilic cow's milk (5.95), buttermilk (6.75 ?), kefir (5.75), yoghurt (5.65), Edam cheese (17.30), Gouda cheese (11.89), Mazdamer cheese (18.79), Kasztelan cheese (4.17), Brie cheese (31.75), Valbon cheese (22.11), and Rokpol cheese (8.82). Analysing these results it is necessary to remember that the absolute values of the contractions of the intestine depend on its physiological condition, which can be variable. For this reason, rather the proportion of the spontaneous contraction of the intestine peptide to the contractions caused by the peptide extracts should be taken into consideration. The calculated values of these proportions were as follows: morphine (1.30, 0.83), women colostrum (1.01, 0.91), women milk (1.09, 0.78), raw cow's milk (1.06, 1.00), pasteurized cow's milk (1.08, 0.85), UHT cow's milk (1.15, 0.92), acidophilic cow milk (0.99, 1.07), buttermilk (2.22, 0.54), kefir (1.00, 0.92), yoghurt (1.09, 1.03), Edam cheese (1.18, 0.96), Gouda cheese (0.74, 1.11), Mazdamer cheese (1.29, 0.57), Kasztelan cheese (1.28, 0.44), Brie cheese (1.42, 0.70), Valbon cheese (1.28, 0.81), and Rokpol cheese (1.05, 0.96).

The above results show that the value of the contractions of the intestine is correlated only with the amount of the pure opioid, for example morphine or β-casomorphin. Such relation was not observed for the peptide extracts derived from dairy products, in which the opioid peptides present included only casomorpin-4, 5 and 7. This phenomenon can be explained in two ways. Firstly, milk proteins are not only the source of the opioid peptides but also peptides with the antagonistic properties against the opioid receptors. The presence of both the agonistic and antagonistic peptides in the peptide extracts obtained from dairy products can be the reason of their competing interactions with the opioid receptors. The effect of such interactions is the decrease of the contractions of the intestine in comparison with the pure opioid peptide. The decrease of the opioid activity of the peptide extract could also follow another mechanism. From the theoretical point of view, it is possible that the peptide extracts derived from dairy products contain peptides with the hydrophobic-hydrophilic nature similar to that of the opioid peptides (β-casomorphins). This structural similarity facilitates their concentration in the vicinity of the opioid receptors, which causes the limited access of β-casomorphins to the opioid receptors. This phenomenon could also decrease the contractions of the intestine. This mechanism suggests the important role of the hydrophobic-hydrophilic interactions in the formation of the complex between the opioid peptides and the opioid receptors. It can be assumed that the opioid receptor has at least two active centres. One is responsible for the binding of the opioid peptide (β-casomorphin) and the second one for the interaction of the opioid peptide with the hydrophobic part of the opioid receptor, which is responsible for its physiological action. The opioid peptide could bind with the opioid receptor by hydrogen bonds and electrostatic interactions. Such mechanism of the formation of the complex between opioid peptide and the opioid receptor could explain the fact that β-casomorphin-5 shows the highest opioid activity. From the stereochemical point of view, β-casomorphin-5 can form the conformation of the most similarity to morphine in comparison with other β-casomorphins. Additionally, it characterizes the most hydrophilic C-terminal amino acid. Generally, it can be said that interactions of the peptide extracts derived from dairy products with the opioid receptors located in the intestine are of diversified character. In order to characterize the total nutritional and physiological value of the dairy products, it is necessary to determine the content of the opioid peptides in them. For this reason, the poly- and monoclonal antibodies were produced against β-casomorphin-5 and 7 and began the preparation of the immunometric method for their determination in peptide extracts derived from dairy products.