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Biologically active peptides in milk proteins

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Summary: Bioactive peptides have been identified as digestion products of several food proteins. All the bioactive sequences are hidden in an inactive state inside the polypeptide chain of the larger protein. Milk proteins are a rich source of biologically active peptides such as exorphins (casomorphins), phosphopeptides and immunopeptides. Such peptides are released during intestinal digestion of caseins and whey proteins. They may be involved in regulation of nutrient entry and influence the postprandial metabolism via stimulation of the secretion of hormones. Furthermore, they may exert a stimulating effect on the immune system. These findings offer new aspects for evaluating the nutritive value of food proteins. Moreover, bioactive peptides have already found interesting applications as dietary supplements and as pharmaceutical preparations.

Zusammenfassung: Bioaktive Peptide treten als Verdauungsprodukte verschiedener Nahrungsproteine auf. Alle bioaktiven Sequenzen liegen in inaktiver Form in der Primärstruktur des höhermolekularen Proteins vor. In Milchproteinen sind zahlreiche biologisch aktive Peptide enthalten, beispielsweise Exorphine (Casomorphine), Phosphopeptide und Immunopeptide. Solche Peptide können während der intestinalen Verdauung der Caseine und Molkenproteine freigesetzt werden. Die möglichen physiologischen Wirkungen bestehen in der Beteiligung an der Regulation der Nährstoffaufnahme sowie der Beeinflussung des postprandialen Stoffwechsels durch Stimulierung der Sekretion verschiedener Hormone. Außerdem können sie einen stimulierenden Effekt auf das Immunsystem ausüben. Diese Erkenntnisse bieten neue Aspekte zur Beurteilung der nutritiven Qualität von Nahrungsproteinen. Darüber hinaus ergeben sich für bioaktive Peptide sowohl diätetische als auch pharmazeutische Anwendungsmöglichkeiten.

Key words: milk proteins; bioactive peptides; exorphins (casomorphins); phosphopeptides; immunopeptides, nutritive value of food proteins

Schlüsselwörter: Milchproteine, bioaktive Peptide, Exorphine (Casomorphine), Phosphopeptide, Immunopeptide, nutritive Qualität von Nahrungsproteinen

Nutritive value of proteins

The use of milk and dairy products in the human diet takes advantage of their content of essential nutrients. Moreover, milk from breast or baby formula is the exclusive source of nutrition for newborn infants.

Cow's milk proteins constitute up to 30 % of the total dietary protein intake. On the basis of nitrogen balance techniques and amino acid scoring procedures, it is clear that milk proteins serve as a high quality

source of both nitrogen and indispensable amino acids in the nutrition of young children and adults. Compared to the amino acid scoring pattern proposed recently cow's milk protein as a "complete" protein source exeeds the individual amino acid requirements (1).

Milk proteins are not usually consumed alone but rather as part of a food and a component of a complex diet. Consequently, when discussing the nutritive value the supplementary effect of a protein to that of other proteins has to be taken into consideration. Furthermore, it should be noted that both digestibility and absorbability are influenced by antagonistic and synergistic interactions with other dietary components.

It is now accepted that peptides and not amino acids are the main degradation products of protein digestion (2, 3, 4). So it becomes increasingly evident that a consideration of protein value must take into account the relationship between protein structure and the composition of peptides released during digestion in the gastrointestinal tract. In particular, the identification of several bioactive sequences in food proteins introduced a new criterion in evaluating the nutritive value of a protein or a mixture of proteins: Peptides which are hidden in an inactive state within the protein sequence may be released by proteolytic processes in vivo and may act, for example, as physiological modulators of gastrointestinal functions during digestion of the "precursor protein".

Although animal as well as vegetable proteins contain potentially bioactive sequences (Table 1) the following overview refers only to milk proteins because these are currently the main source of biologically active peptides.

Table 1. Bioactive peptides as intestinal digestion products of food proteins.

Bioactive peptide	Protein source	
Exorphins	Milk:	α-, β-caseins *(casomorphins)* α-lactalbumin β-lactoglobulin *(lactorphins)*
	Wheat:	gluten
	Maize:	zein
	Barley:	hordein
Exorphin-antagonists	Milk:	κ-casein *(casoxins)* lactoferrin *(lactoferroxins)*
	Wheat:	gliadin
	Soy:	α-protein
Antigastric peptide	Milk:	κ-casein *(glycomacropeptide)*
Neuroactive peptides	Wheat:	gliadin
Immunostimulating peptides	Milk:	α-, β-casein
Antibacterial peptides	Milk:	α-, β-casein
Binding peptides for minerals	Milk:	α-, β-casein *(phosphopeptides)*

Strategies for seeking bioactive sequences

Chiba and Yoshikawa (5) have described two strategies for identifying new bioactive peptide sequences in milk proteins:

1) searching proteins for amino acid sequences similar to those known to be bioactive, e.g., opioid peptides, followed by synthesis of these peptides and evaluation of their bioactive properties;
2) isolation and characterization of bioactive peptides from in vitro digests of proteins.

Additionally, a third strategy of investigation was employed in the Federal Dairy Research Centre in Kiel (3, 6–11):

3) isolation and characterization of bioactive peptides from the gastrointestinal chyme of Göttingen minipigs fed with animal or vegetable proteins.

Bioactive peptides in casein

Most of the known bioactive peptides are derived from caseins (12). Casein fragments have been shown to behave like opioid agonists, carriers for minerals and immunomodulating agents.

β-casein fragments

The heptapeptide Tyr-Pro-Phe-Pro-Gly-Pro-Ile isolated from a peptone digest of bovine β-casein was the first opioid peptide described (13–15). This fragment and other C-terminally shortened peptide sequences derived from it were named β-casomorphins. Since they originate from exogenous food sources and exert morphine-like activities they are also termed exorphins or formons (from "food hormones"). The common features among opioid peptides (exorphines and endorphines) are the presence of tyrosine residues at their amino termini and the presence of another aromatic residue, Phe or Tyr, in the third or fourth position. Table 2 lists a series of peptides which correspond to the 60th–70th residues of bovine β-casein. These compounds were isolated from in vitro digests (13, 16, 17) or from in vivo duodenal chyme samples (8), or they were synthesized (18). With the exception of the tripeptide Tyr-Pro-Phe, all of these β-casomorphins (including β-casomorphin-11, the only one which could be isolated in vivo from duodenal chyme of minipigs (8)), exert naloxone inhibitable opioid activities. Morphiceptin, the amide of β-casomorphin-4, is the most active opioid peptide so far known (19, 20). Lack of the N-terminal Tyr as in des-Tyr-β-casomorphin-7 results in an total absence of bioactivity (18).

Similar peptides were identified in human milk proteins; the appropriate sequences correspond to residues 41–44, 51–58 and 59–63 of human β-casein (23, 24). Among other bioactive fragments, the tetrapeptide amides valmuceptin residues 51–54 and β-casorphin residues 41–44 show opioid activities similar to morphiceptin.

It should be noted that a number of caseolytic bacterial species used in the production of some types of cheese and other milk products are able to liberate β-casomorphin-like substances from β-casein (21). Several cheese peptides with N-terminal tyrosyl groups have, indeed, been found in *Harz cheese* (22); preliminary characterization showed that two of these β-

casomorphin-like peptides have identical chromatographic properties as β-casomorphin-5 and -4 amide.

Among several active fractions, an immunostimulating peptide has been characterized from enzymic digests of bovine (residues 191–193) as well as human β-casein (residues 54–59) (25). Furthermore, it has been found that β-casomorphin-11 isolated from in vivo digests includes the immunostimulating bovine hexapeptide Pro-Gly-Pro-Ile-Pro-Asn (residues 63–68).

Three caseinophosphopeptides from the N-terminal region of β-casein (residues 1–28) could be produced by tryptic hydrolysis of sodium caseinate (26, 27).

α_{S1}-casein fragments

Table 3 shows the bioactive peptides derived from bovine α_{S1}-casein. The in vitro fragment which corresponds to residues 90–96 of α_{S1}-casein was named α-casein exorphin (28) because of its opioid activity, whereas the C-terminal hexapeptide acts as an immunostimulant (25).

Recently, a caseinophosphopeptide released from α-casein could be purified from jejunal chyme of minipigs fed with casein diet (10). It was

Table 2. Bioactive β-casein fragments.

Arg↓Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-SerP-Leu-SerP-SerP-SerP-Glu-Glu-Ser-Ile-Thr-Arg↓Ile-Asn-Lys↓Lys

Val↓Tyr-Pro-Phe↓Pro↓Gly↓Pro↓Ile↓Pro↓Asn↓Ser-Leu↓Pro

Phe↓Leu-Leu-Tyr↓Gln

residues	bioactivity	isolation		synthesized	references
		in vitro	in vivo		
Casomorphins					
60–62	inactive	–	–	+	13, 16, 17
60–63	opioid antagonist	–	(+)[c]	+	12, 13, 16, 17, 35
60–63[a]	opioid agonist	+[b]	–	+	13, 14, 16, 17
60–64	opioid agonist	+[b]	–	+	11–13, 15–17, 35
60–65	opioid agonist	–	(+)[c]	+	
60–66	opioid agonist	+[b]	(+)[c]	–	11, 12, 15, 17, 35
60–67	?	+[b]	–	–	14
60–70	opioid agonist	–	+[d]	–	7, 10
Immunopeptides					
63–68	immunostimulation	–	–	+	23
191–193	immunostimulation	+[b]	–	+	23
Caseinophosphopeptides					
1–28	mineral carrier	+[b]	–	+	24, 25
1–25	mineral carrier	+[b]	–	+	24
2–28	mineral carrier	+[b]	–	–	24, 25

[a]) amide of fragment 60–63. [b]) enzymatic casein digest. [c]) authentic immunoreactive materials in intestinal content after bovine milk ingestion in adult humans. [d]) duodenal chyme of minipigs after ingestion of a casein diet.

characterized as a nonapeptide corresponding to residues 66–74 of α_{S1}-casein. Three tryptic phosphopeptides were isolated from in vitro digests of whole casein and identified as α_{S1}-casein fragments (26).

α_{S2}-casein fragments

Several caseinophosphopeptides corresponding to different phosphorylated regions of α_{S2}-casein (Table 4) were obtained from tryptic digests of sodium caseinate (26). Although the phosphorylated casein sequences which had so far been analyzed contained only seryl bound phosphate groups, the isolated α_{S2}-casein fragment 126–136 includes also a phosphothreonyl residue (26).

κ-casein fragments

Table 5 lists a number of bioactive peptides derived from bovine κ-casein. The opioid peptides, named casoxins, were obtained in vitro by enzymatic digestion and by chemical synthesis, respectively (5, 29). The tetra-, penta- and hexapeptides corresponding to residues 33–39 were modified by methoxylation during the isolation procedure. These methyl esters are much more active as opioid antagonists than the non-methoxy-

Table 3. Bioactive α_{S1}-casein fragments.

Lys-↓43Asp-Ile-Gly-SerP-Glu-SerP-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-↓58Gln-59

Met-Glu-Ala-Glu-SerP-Ile-↓66SerP-SerP-Glu-Glu-Ile-Val-Pro-Asn-↓74SerP-Val-

Glu-Gln-Lys-↓79His

Glu-↓90Arg-↓91Tyr-Leu-Gly-Tyr-Leu-↓95Glu-↓96Gln

Lys-↓106Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-SerP-Ala-Glu-Glu-Arg-↓119Leu

Lys-↓194Thr-Thr-Met-Pro-Leu-199Trp

residues	bioactivity	isolation		synthesized	references
		in vitro	in vivo		
Casomorphins					
90–95	opioid agonist	+[a]	–	+	26
90–96	opioid agonist	+[a]	–	+	26
91–96	opioid agonist	–	–	+	26
Immunopeptides					
194–199	immunostimulation	+[b]	–	–	23
Caseinophosphopeptides					
43–58	mineral carrier	+[c]	–	–	24
59–79	mineral carrier	+[c]	–	–	24, 25
106–119	mineral carrier	+[c]	–	–	24
66–74	mineral carrier	–	+[d]	–	9

[a]) peptic digest of α-casein. [b]) tryptic digest of casein. [c]) trypic digest of sodium caseinate. [d]) jejunal chyme of minipigs.

Table 4. Bioactive α_{S2}-casein fragments.

Lys-↓Asn-Thr-Met-Glu-His-Val-SerP-SerP-SerP-Glu-Glu-Ser-Ile-Ile-
SerP-Gln-Glu-Thr-Tyr-Lys-↓Glu
Arg-↓Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-SerP-SerP-SerP-Glu-↓
Glu-SerP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-↓Ile
Arg-↓Glu-Gln-Leu-SerP-ThrP-SerP-Glu-Glu-Asn-Ser-Lys-↓Lys-↓Thr-Val-
Asp-Met-Glu-SerP-Thr-Glu-Val-Phe-Thr-Lys-↓Lys

residues	bioactivity	isolation		synthesized	references
		in vitro	in vivo		
Caseinophosphospeptides					
2–21 46–70 126–136 138–149	mineral carrier	+[a]	–	–	24

[a]) tryptic digest of sodium caseinate.

Table 5. Bioactive κ-casein fragments.

Lys-↓Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-↓Ser-↓Arg-↓Tyr-Pro-Ser-Tyr-↓Gly-Leu-Asn-↓
Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-Gln-Phe-Leu-Pro-↓Tyr-Pro-
Tyr-Tyr-↓Ala

residues	bioactivity	isolation		synthesized	references
		in vitro	in vivo		
Casoxins					
35–38[a]	opioid antagonist	–	–	+	4
34–38[a]	opioid antagonist	–	–	+	4
33–38[a]	opioid antagonist	+[b]	–	+	4
33–38	opioid antagonist	–	–	+	4
25–34	opioid antagonist	+[c]	–	–	27
35–41	opioid antagonist	–	–	+	27
58–61	opioid antagonist	–	–	+	27
Glycopeptide					
part of macropeptide fragment 106–169	antigastric activity	(+)[d]	–	–	28, 29

[a]) methoxylated. [b]) peptic diges. [c]) tryptic digest. [d]) fraction of a peptic digest; not chemically characterized.

lated natural peptides (5). Only the casoxin fragment 25–34 showed an opioid antagonistic activity comparable to the esterified casoxins (29).

An as yet unidentified glycopeptide which inhibits gastric secretion was obtained by peptic proteolysis of κ-casein (30, 31). This antigastric peptide is contained in the so-called macropeptide fragment (residues 106–169) of the κ-casein structure.

Bioactive peptides in whey proteins

Two tetrapeptides in the primary structure of whey proteins have potential opioid activities: Tyr-Gly-Leu-Phe (residues 50–53) from human and bovine α-lactalbumin and Tyr-Leu-Leu-Phe (residues 102–105) from bovine β-lactoglobulin. The corresponding amides – named α- and β-lactorphin, respectively – were chemically synthesized and their opioid activity was established (5).

Physiological effects of bioactive peptides from milk protein

The physiological role of bioactive peptides derived from food proteins is not yet understood. However, since milk protein is the only protein which is biosynthesized to be ingested, it is quite possible that bioactive sequences in milk protein are physiologically significant. Milk protein is indeed a rich source of different biologically active peptides (Table 1), which are liberated during enzymatic digestion. In particular, exorphins, phosphopeptides and immunopeptides derived from casein might have significant physiological and nutritional implications.

The various exorphins produce distinct opioid activities in several in vitro assays (Fig. 1) where they probably represent μ-type opiate receptor agonists (14, 20). In vivo, they have been found to prolong gastrointestinal transit times and also to stimulate the release of somatostatin and insulin (32–35). It is concluded (36, 37) that somatostatin and insulin may have a common function relating to nutrient flux. Hence it is tempting to speculate that exorphins, like casomorphins, may act as exogenous regulators of the entry rate of nutrients. To exert biological effects in vivo, casomorphins could interact with opiate receptors in the intestinal tract, or they may be degraded to smaller opioid fragments in the brush border. The intestinal brush border contains high levels of peptidase activity, so that the action of several peptidases from the intestinal epithelium could contribute to the rise of several free casomorphins. The highly hydrophobic casomorphin fragments can, in principle, cross the intestinal mucosa as well as the blood-brain barrier. Therefore, opioid peptides are expected to be absorbed without degradation into the blood stream to reach opiate receptors in peripheral organs or in the brain. After milk ingestion, β-casomorphin immunoreactive materials were, indeed, detected in intestinal contents of adult humans as well as in plasma of newborn calves (38, 39). In both cases the immunoreactive material might represent a precursor of β-casomorphin-7 such as β-casomorphin-11 which

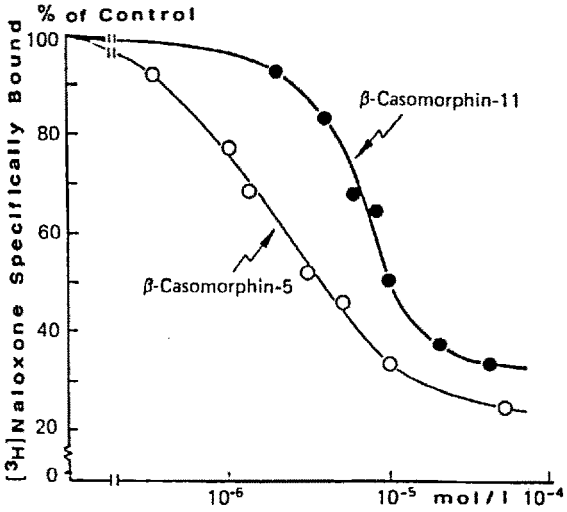

Fig. 1. Opiate receptor binding assay (8): Displacement of specifically bound [^{3}H]naloxone from opiate receptors in rat brain homogenate by β-casomorphin-11 in comparison to synthetic β-casomorphin-5. The opiate antagonist naloxone competes for the binding of opioid peptides to specific opiate binding sites. The concentration of an opioid peptide which can inhibit 50 % of the radioactivity of specifically bound naloxone is a measure for its opioid potency. Accordingly, the affinity for opiate receptors of β-casomorphin-11 is substantial but weaker than that of β-casomorphin-5 which acts as a very potent opioid peptide in several bioassays.

was identified in duodenal chyme of minipigs after ingestion of a casein diet (8).

Because of their affinity for μ-type opiate receptors of β-endorphins on human T-cells and on human phagocytic leukocytes, it seems likely that absorbed casomorphins could act not only as modulators of the postprandial hormone release but also as immunomodulating agents (25).

In addition, several casein derived peptides may be implicated in the stimulation of the immune system, especially in the newborn (25). These immunopeptides are not identical to casomorphins; however, it is remarkable that some immunopeptides are located in a part of the β-casein sequence which contains the β-casomorphins. Synthetic caseinoimmunopeptides stimulated in vitro phagocytic activity of murine and of human macrophages and exerted in vivo a protective effect against *Klebsiella pneumoniae* infection of mice (25).

Regarding the physiological function of phosphopeptides, they are likely to enhance the intestinal absorption of calcium and trace elements by increasing the concentration of soluble minerals. It is known that phosphopeptides are resistant to further proteolytic breakdown by digestive enzymes (10, 40, 41). After injection of a phosphopeptide prepared from β-casein into a ligated loop of rat small intestine, two important effects have been observed (42): firstly, the calcium absorption was enhanced, and secondly, the deposition of calcium in the femur was increased.

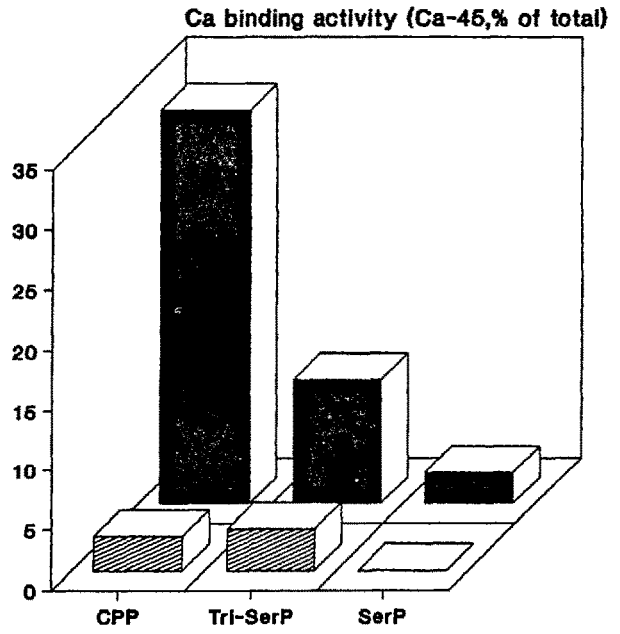

Fig. 2. Competitive Chelex resin assay (44): A solution containing a soluble calcium binder, like phosphopeptides is mixed with Chelex resin; a complex equilibrium results between calcium in the ionic form, calcium bound to the soluble binder and calcium bound to the insoluble Chelex resin. The distribution of calcium is determined with the aid of radiocalcium.
The concentration of the soluble (peptide-bound and free) calcium in relation to the calcium added (1μmol/l) is a measure for the calcium binding activity of the peptides. Filled columns: Calcium binding activity (soluble ^{45}Ca, percent of total) of a mixture of caseinophosphopeptides (CPP) obtained by enzymatic digestion of casein (43), tri-phosphoserine (Tri-SerP) prepared by chemical phosphorylation of tri-serine with phosphorus oxychloride and phosphoserine (SerP, Sigma). Hatched columns: The reduction of calcium-binding activity after treatment (4 h at RT) with alkaline phosphatase (immobilized APase, Mobitec) shows that the binding sites for $Ca^{2+}$are the phosphoseryl residues.

Casein derived phosphopeptides exhibit a potent ability to form soluble complexes with calcium (Fig. 2) and to prevent the precipitation of calcium phosphate (42). It is suggested that phosphopeptides bind to initial aggregates of calcium and phosphate ions to block their crystal growth; they therefore effectively inhibit the formation of insoluble calcium phosphate at different Ca/P ratios (27).

Caseinophosphopeptides can also form organophosphate salts with trace elements such as iron, zinc, copper and may function as carriers for the particular minerals. Accordingly, they have already found an interesting application as dietary supplements and as pharmaceutical preparations (26, 43). It is claimed that phosphopeptides obtained by tryptic digestion of sodium caseinate can be used in the treatment of dental diseases, rarefying bone diseases or to improve mineral malabsorption (43).

Conclusions

The discovery of biologically active peptides derived from food proteins has introduced a new criterion in defining the "nutritive value" of proteins. Although the real physiological role of these peptides as exogenous metabolic modulators during their gastrointestinal liberation is not yet well understood and their activities are contradictory to some extent, the following dietary and pharmaceutical applications are indicated for the future:

- supplementation of the diet with desirable synthetic bioactive peptides
- production of desirable bioactive peptides during food processing by use of genetically transformed microorganisms
- administration as pharmaceutical preparations, for example in the treatment of diarrhoea (casomorphins), dental and bone diseases as well as mineral malabsorption (caseinophosphopeptides) and immunodefiency (immunopeptides).

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