Bioactive Peptides in Milk: From Encrypted Sequences to Nutraceutical Aspects

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Abstract: Milk provides a wide range of biologically active compounds that protect humans against diseases and pathogens. The purpose of this work is to describe the main aspects and research lines concerning bioactive peptides: from their chemistry, bioavailability, and biochemical properties to their applications in the healthcare sector. In this context, the uses of bioactive peptides in nutraceutical and functional foods have been highlighted, also taking into account the perspective of innovative applications in the field of circular bioeconomy.

Keywords: bioactive peptides; milk; nutraceuticals; bioavailability; biorefinery

1. Bioactive Components of Milk: Focus on BPs

Milk provides a wide range of biologically active compounds that protect humans against diseases and pathogens such as immunoglobulins, antimicrobial proteins and peptides, oligosaccharides, lipids, as well as many other components at low concentrations with significant potential health benefits [1] (Figure 1). Among these compounds, proteins play a key role as nutrients and as promoters of the physiological function and health role as source of Bioactive Peptides (BPs) [2].

In cow’s milk, around 80% of the protein present is casein (CN) and 20% is whey protein. CN consists of $\alpha_s$-1-, $\alpha_s$-2-, $\beta$- and k-CN families in the approximate ratio 38:11:38:13 [3]. Whey proteins are typically a mixture of beta-lactoglobulin (~65%), alpha-lactalbumin (~25%), bovine serum albumin (~8%), lactoferrin, and immunoglobulins. The value of proteins as an essential source of amino acids...
has been well documented, but in the 1980s, it was recognized that dietary proteins can exert many other in vivo functions through BPs. The isolation of endogenous opioid peptides called enkephalins, which occurred for the first time in 1975, led to the discovery of the opioid peptide activity derived from partial enzymatic digestion of milk proteins [4]. Since then, the bioactive components derived from food proteins, in particular from milk proteins, have been subjected to numerous studies focused on their structural and biochemical properties.

2. Biochemical Properties

BPs peptides are encrypted and inactive in the parent protein sequence but can be released and activated through the enzymatic proteolysis (gastrointestinal digestion, in vitro hydrolysis using proteolytic enzymes) of proteins and during food processing (cooking, fermentation, ripening). Once released, the BPs can act as regulatory compounds such as β-casomorphins and casein-derived phosphopeptides and are released in vivo during the gastrointestinal transit of milk and dairy products [5]. It is important to underline the strong connection between the structure and the biological activities: the amino acid sequence, the hydrophobicity, the charge of BPs determine a specific activity.

For instance, in the case of β-casomorphins (BCMs), the presence of a tyrosine residue at the amino terminal end (except δ-casein opioids) and the presence of aromatic amino acids in the third and fourth positions of the peptide represents a structural feature of the opioid activity; in particular, the phenolic hydroxyl group of tyrosine gives rise a negative potential, essential for the opioid activity. On the other hand, the Pro residue maintains the proper orientation of the Tyrosine and Phenylalanine side chains [6,7]. In regard to the importance of the amino acid sequence on the activity of BPs, human BCMs (Tyr-Pro-Phe-Val-Glu-Pro-Ile) were found to be 3 to 30 times less potent than bovine BCMs (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) [7].

The production and properties of BPs have been reviewed in many articles [8–10]. After ingestion, BPs can affect the cardiovascular, nervous, gastrointestinal, and immunological systems. BPs have been defined as specific protein fragments having a positive influence on the physiological and metabolic functions or conditions of the body. Therefore, they may have crucial beneficial effects on human health [11].

Furthermore, it is worth mentioning the recent review of Nielsen et al. [12], where a comprehensive database of milk protein–derived BPs that can be used to search for specific functions—those of peptides or proteins—was developed. A new visual arrangement was proposed: BPs were visually mapped on the basis of parent protein sequences, providing information on sites with the highest abundance of these compounds (http://mbpdb.nws.oregonstate.edu/).

Their main regulatory effects concern the following:

- the transport of minerals (caseinophosphopeptides), such as calcium, and intestinal transport of amino acids, such as leucine, through the beta-casomorphin receptors;
- the transport of intestinal fluid (beta-casomorphine);
- the motility of the gastrointestinal tract (beta-casomorphine);
- the stimulation of the postprandial hormone secretion (insulin, somatostatin) (beta-casomorphine);
- the regulation of insulin secretion based on glucose concentration;
- immunostimulant peptides (alpha and beta casein fragments);
- anti-hypertensive peptides enzyme inhibitors converting angiotensin I (ACE) (casoknine);
- antithrombotic peptides such as ADP-activated platelet aggregation inhibitors, as well as fibrinogen binding (γ-chain) to ADP-treated platelets (casoplateline);
- opioid activities;
- antioxidative functions;
- hypcholesterolemic activities;
- antitumor activities.
<table>
<thead>
<tr>
<th>Precursor Protein</th>
<th>Fragment</th>
<th>Peptide Sequence</th>
<th>Name</th>
<th>Biological Activity</th>
<th>Preparation</th>
<th>References</th>
</tr>
</thead>
<tbody>
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<td>Casein Protein</td>
<td>60–70</td>
<td>YPFPGPIPNSL</td>
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<td>60–66</td>
<td>YPFPGPI</td>
<td>β-casomorphin-7</td>
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<td>Mixture of gastro-intestinal enzymes Trypsin</td>
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<td>β-casokinin-7</td>
<td>ACE Inhibitory Immunomodulatory</td>
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<td>ACE inhibition</td>
<td>Hydrolysis with proteinase</td>
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<td>63–68</td>
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<td>Immunomodulatory</td>
<td>Trypsin or chymosin</td>
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<td>(1–25)4P</td>
<td>RELEELNVPEIEVESLSSSEISITR</td>
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<td>Ca++ binding</td>
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<td>αs1-casein</td>
<td>90–96</td>
<td>RLYGYLE</td>
<td>α-casein exorphin</td>
<td>Opioid</td>
<td>Hydrolysis with pepsin</td>
<td>[22]</td>
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<td>RLYGYL</td>
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<td>ACE-inhibition</td>
<td>Trypsin or chymosin</td>
<td>[24]</td>
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Table 1. Cont.

<table>
<thead>
<tr>
<th>Precursor Protein</th>
<th>Fragment</th>
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<th>Name</th>
<th>Biological Activity</th>
<th>Preparation</th>
<th>References</th>
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<td>αs₂-casein</td>
<td>94–103</td>
<td>QKALNEINQF</td>
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<td>ACE inhibition</td>
<td>Hydrolysis with chymotrypsin</td>
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<td></td>
<td>163–176</td>
<td>TKKTTLTEEEKNRL</td>
<td>ACE inhibition</td>
<td>Hydrolysis with chymotrypsin</td>
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<tr>
<td>k-Casein</td>
<td>33–38</td>
<td>SRYPSY</td>
<td>Casoxin 6</td>
<td>Anti-Opioid</td>
<td>Hydrolysis with pepsin</td>
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<tr>
<td></td>
<td>25–34</td>
<td>YIPIQYVL5R</td>
<td>Casoxin C</td>
<td>Anti-Opioid</td>
<td>Hydrolysis with trypsin</td>
<td>[27]</td>
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<td></td>
<td>106–116</td>
<td>MAIPPKKNQDK</td>
<td>Casoplagnetin</td>
<td>Antithrombotic inhibition of platelet aggregation</td>
<td>Hydrolysis with trypsin</td>
<td>[28]</td>
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<td></td>
<td>YPSY</td>
<td>Casoxin 4</td>
<td>Opioid agonist</td>
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<td>50–53</td>
<td>YGLF</td>
<td>α-lactorphin</td>
<td>Opioid agonist ACE inhibition</td>
<td>Hydrolysis with gastric and</td>
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<td>TLLF</td>
<td>β-lactophin</td>
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<td>142–148</td>
<td>ALPMMHGR</td>
<td>ACE-inhibition</td>
<td>Proteolytic digestion</td>
<td></td>
<td>[32,33]</td>
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<td>146–149</td>
<td>HIRL</td>
<td>β-lactotensin</td>
<td>Ileum contraction, hypocholesterolemic activity</td>
<td>Synthetic</td>
<td>[31,34]</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>208–216</td>
<td>ALKAWSVAR</td>
<td>Albutensin A</td>
<td>Ileum contraction, ACE inhibition</td>
<td>Hydrolysis with proteinase</td>
<td>[32]</td>
</tr>
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<td>399–404</td>
<td>YGFQDA</td>
<td>Serorphin</td>
<td>Opioid</td>
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<td>[35]</td>
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<tr>
<td>Lactoferrin</td>
<td>17–41/42</td>
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<td>Lactoferricin</td>
<td>Antimicrobial</td>
<td>Hydrolysis with pepsin</td>
<td>[36]</td>
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</table>
Recent studies have suggested that milk BPs may also contribute to reducing the risk of obesity and development of metabolic disorders. The size of these peptides may vary from two to more than 20 amino acids residues, and each defined peptide bioactivity is strictly linked to its structural features. In Table 1, some examples of the main BPs derived from casein and whey milk proteins are reported.

Some milk BPs show multifunctional properties [37]; for example, some regions in the primary structure of caseins contain overlapping peptide sequences that exert different biological effects (Table 1). These regions have been considered as ‘strategic zones’ which are partially protected from the proteolytic breakdown [38,39]; e.g., peptides from the sequence 60–70 (β-casomorphin-11) and 60–66 (β-casomorphin-7) of β-casein show immunostimulatory, opioid, and ACE-inhibitory activities. These sequences are protected from proteolysis because of their high hydrophobicity, which is due to the presence of proline and other hydrophilic amino acids. Neutral and basic amino acids are, instead, rapidly hydrolyzed. Moreover, it is interesting to highlight that some bioactive sequences include proline in their domain.

3. Bioavailability

The amount of peptides released upon digestion, as well as the beneficial effects of human health, are hardly predictable. It was estimated that the theoretical yield of opioid peptides encrypted in milk proteins ranged between 2% (β-Casomorphin-5 from β-Casein, f60–64) and 6% (α-Lactorphin from α-Lactalbumin, f50–53) starting from the precursor peptide [40]. BPs bioavailability is the ability of peptides to exert physiological effects in vivo after oral ingestion. Thus, it is of crucial importance that milk-derived BPs remain active during the gastrointestinal digestion and absorption and reach the target site intact. This means that milk-derived BPs have to be resistant to hydrolysis in the gastrointestinal tract in order to reach the peripheral organs [41]. The bioavailability of peptides depends on a variety of structural and chemical properties, i.e., resistance to proteases, charge, molecular weight, hydrogen bonding potential, hydrophobicity, and the presence of specific residues [42–44]. Indeed, proline- and hydroxyproline-containing peptides are relatively resistant to degradation by digestive enzymes [45–47]. The composition of the intestinal content, including food, significantly varies. The time that a peptide is present in the GI tract, as well as its absorption, are significantly affected by gastric emptying and intestinal transit. In addition, the peptide transport could be inhibited or favored (related to pKa of the peptide) by the physiological pH.

For example, some milk-derived peptides have an in vitro inhibitory ACE activity compared to the relative synthetic ACE inhibitor, but exhibit high in vivo activity. This behavior was attributed to a greater affinity of BPs for tissues and their slower elimination [48], but other modes of action were hypothesized [16]. In contrast, some ACE-inhibitory peptides exhibit high activity in vitro but have no effects in vivo. For example, the peptide FFWAP derived from αs1-CN [49–51] is a potent ACE inhibitor in vitro but has no hypotensive effect in vivo [15]. Generally, the difficulty of establishing a direct relationship between in vitro and in vivo activity may depend on different reasons but it is clear that bioavailability after oral administration plays a key role. Despite numerous “in vitro” studies, further research is needed to clarify the relationship between the activity of BPs and their bioavailability. In this regard, the recent review by Nongonierma and FitzGerald, [52] summarizes the scientific evidence for the role of milk protein-derived BPs in humans and pointed out how double-blind randomized clinical trials based on the use of universal guidelines for the evaluation of BPs in humans are needed.

4. Nutraceutical Aspects

Numerous bioactive peptide fragments can be obtained through the hydrolysis of whole milk or via the precursor protein by digestive enzymes. This strongly leads us to hypothesize the production of such peptides in the GIT after the consumption of milk.

For these reasons, the potential that different BPs have to improve human health by reducing the risk of chronic diseases or by activating the immune response is gaining more and more
interest within the scientific and commercial fields. In fact, since milk proteins are a rich source of natural active compounds, they could be used as ingredients for different applications and represent nutraceutical substances with potential health benefits that make them suitable for food and pharmaceutical applications [53].

4.1. Methodology toward Innovative Aspects

Processing techniques in laboratory and on industrial scale are now being developed for the extraction, fractionation and isolation of main proteins from milk [54–57], with particular attention to modern non-thermal, clean and green methodologies [58]. It is worth stressing, as already demonstrated by several studies, that each time a nutraceutical with potential health benefits is needed for a functional food, a specific enzymatic hydrolysis of milk proteins should be planned [59,60]. It is important to mention the review by Hafeez et al. [61] on different strategies for increasing the production of BP's from milk in order to obtain functionalized fermented products; the authors reported three types of methodology approaches: (1) proteolytic system of lactic acid bacteria (LAB) or food grade enzymes or a combined dual approach, (2) supplementation of the fermented milk products with the BP's obtained outside of the product, and (3) microorganisms using recombinant DNA technology [61]. Linares et al. [62] reviewed and discussed the use of health-supporting bacteria as starters or adjunct cultures for the development of dairy foods with targeted functional properties, including BPs.

4.2. Applications

The recent review by Mohanty et al. [63] gives a preliminary classification of bioactive milk-derived peptides and their impact on human health. It describes their physiological functions, general characteristics, and potentialities for improving health, as well as their nutraceutical and/or pharmaceutical applications. Besides their biochemical and physiological efficacy and versatility, milk-derived BPs are considered as ingredients of functional foods, as pointed out in a review by Park and Nam [64]. Milk BPs in dairy and non-dairy food formulations have been exploited, as shown by several authors [65–67]. The fractionation of bioactive milk ingredients represents a new, emerging sector of the market, which offers new and innovative products [53,59,67]. FitzGerald, et al. [68] exploited the hypotensive peptides from milk proteins and reported some examples of hypotensive dairy protein-derived products on the market such as a sour milk named “Calpis” (Calpis Co., Tokyo, Japan) or a fermented milk called “Evolus®” (Valio Oy, Helsinki, Finland) containing IPP and VPP peptides (peptides with hypotensive effect) from β- and κ-casein. CalpisTM and Evolus® have been tested extensively in rats and in a clinical trial [69].

In addition, Korhonen, and Pihlanto, [70] have reported other commercial dairy products and ingredients having potential health benefits due to the presence of BPs, such as “BioZate” (Davisco Food International, Eden Prairie, MN, USA). It is a hydrolyzed whey protein that claims to reducing blood pressure. Another example is represented by “Vivinal Alpha,” an ingredient/hydrolysate (Borculo Domo Ingredients—BDI, Zwolle, The Netherlands) that favors relaxation and sleep.

Today, the recovery of BPs from milk and dairy industry byproducts has recently caught the attention of researchers in industrial biorefineries [71–73]. In order to solve the problem of the high environmental impact of byproducts deriving from cheese production industries, several new techniques are now employed to convert milk and dairy waste and byproducts into value-added products.

It is worth mentioning Patel et al. [74], who underline the emerging trends in nutraceutical applications of whey protein and its derivatives. To cite an example, Athira et al. [75] produced and characterized a whey protein hydrolysate with an antioxidant activity from cheese whey using the response surface methodology. In recent research, Abd El-Salam and El-Shibiny [76] have summarized today’s ongoing research into the preparation, properties, and uses of enzymatic milk protein hydrolysates.
5. Conclusions

The exploitation of the chemistry, bioavailability, and biochemical properties of BPs in cow’s milk represent the basis for the development of nutraceutical and functional foods, in particular in the perspective of innovative applications in the field of circular bioeconomy. Moreover, for the use of these BPs as nutraceuticals, it is important to encourage clinical trials to test their effectiveness on humans and support the research through projects funded by public and private institutions.

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Conflicts of Interest: The authors declare no conflict of interest.

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