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Review article

# Ripened cheese as a source of bioactive peptides

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***Abstract:*** *Cheese has a long history in the human diet. In ancient times, cheese was primarily a concentrated form of milk with the benefit of a prolonged shelf life. Recent advances in nutrition science have highlighted the contribution of cheese to nutrition and health. It is a rich source of essential nutrients, in particular, proteins, vitamins, minerals, and also short chain fatty acids. Bioactive peptides from proteins, which are the main subject of this paper, can be generated either by fermentation processes during cheese-manufacturing or by gastrointestinal digestion after consumption. As cheese is a complex food matrix containing a large number of different peptides which change with the ripening time, they are produced during secondary proteolysis through the action of proteinases and peptidases. As long as they are bound in the proteins they are biologically inactive and can only be active after they have been released from their parent protein. Then, they can exert a wide range of biological activities such as antimicrobial, opioid, blood pressure-lowering, cholesterol-lowering, mineral binding, immunomodulating, and anti-carcinogenic activities.*
*The article discusses the characteristics of cheese, distinguishing it as an important source of bioactive peptides with various activities. Bioavailability of bioactive peptides from cheese was described. The blood-pressure lowering activity was highlighted as milk proteins are the main source of this kind of bioactive peptides, and it is the best-studied and in vivo-confirmed effect. Attention has also been paid to three other activities expressed by bioactive peptides from cheese: anti-carcinogenic, antioxidative, and opioid.*

***Keywords:*** *bovine milk; milk protein; cheese; bioactive peptides.*

## Introduction

Cheese is a nutritious, versatile dairy food. A wide variety of cheese types are available to meet specific consumer requirements and allow convenience use. It is said that cheese-making could go as far back as 10 000 BC when sheep and goats were first domesticated in the Middle East, and early herdsmen would have consumed milk. These days, the development of the dairy industry plays an important role in the economy of a given country. For example, the dairy industry in Poland is one of the most developed in Europe. Over the years, most dairy companies have been modernised and their products are often exported [49]. The production of high quality cheese in Poland has been significantly growing as well. According to Seremak-Bulge [56], in 2015 the production of ripened cheeses was 309 500 tonnes and in 2016, the production rose up to 319 000 tonnes. About 40% of the cheeses were exported to various countries in the EU [57].

Thanks to such high cheese production, the Polish people are consuming more cheese. It is so not only because of the prevention of hunger but also because of the supply of important nutrients. This fact makes cheese an interesting field to investigate as a natural source of bioactive components. From a nutritional point of view, cheese is a rich source of many essential nutrients such as proteins, vitamins, minerals, and also short chain fatty acids that are important as a part of a healthy diet. In this paper, we will focus on bioactive peptides from proteins and their chosen activities. Peptides can be generated either by fermentation processes during cheese-manufacturing [45] or by gastrointestinal digestion after consumption [9, 10]. Some of the cheese-origin peptides are structurally similar to endogenous peptides that play a crucial role in the organism as hormones, neurotransmitters or antibiotics. Some of them can also survive gastrointestinal digestion or serve as precursors of the final peptide form [38]. The increasing number of publications that appeared during the last years, show that the interest in bioactive peptides in food is growing. Due to the wide range of functions, bovine milk derived peptides are regarded as potent ingredients in health-promoting functional foods. It is known that milk and its products are a source of nutrients that support the physiological functioning of the human body as well as intense mental activity [14]. Those biocomponents may offer effective protection against various diseases and can be potentially used in their prophylaxis or even treatment [8]. Since more and more individuals would like to control their health via custom-made food, the worldwide market for functional foods is one of the fastest-growing markets in the world.

## What is cheese?

Cheese is a food consisting of the coagulated, compressed, and usually ripened curd of milk separated from the whey. The various cheese types can be classified according to the milk used (cow, sheep, goat, buffalo), their manufacture (rennet, sour milk cheese, ultrafiltration), consistency (extra-hard, hard, semi-hard, semi-soft, soft, fresh cheese), fat content (double cream, cream, full fat, three-quarters fat, half fat, quarter fat cheese), fermentation type (lactic acid, lactic and propionic acid, butyric acid), surface (hard, soft, with smear, moulds), and interior (eyes, moulds). Additionally, they differ both in flavour and also in some bioactive components, which are mainly created during the different stages of ripening when the main ingredients, which are lactose, protein, and fat, are broken down by fermentation, proteolysis, and lipolysis [66]. At the beginning of the cheese ripening process, lactose is partly washed out with the whey and the rest is fermented into lactic acid and further into diacetyl, acetylaldehyde, acetic acid, ethanol, and carbon dioxide. These volatile compounds make cheese more attractive to most consumers. Therefore, the varieties of cheese on the market are enormous, which is also reflected in the variability in the composition of the different types of cheese [66]. As far as lactose is concerned, all types of cheese except fresh and, in a few cases, soft cheese, are free of this ingredient. Thus, lactose-intolerant people can consume these cheeses, which contribute to a healthy diet with their indispensable ingredients such as calcium. One portion (50 g) of semi-hard or hard cheese supplies one-third to a half of the recommended daily intake of 1200 mg calcium [59].

Another main component of cheese is fat. It varies between 20 and 35% of the dry mass. One portion (50 g) of full-fat cheese provides about two-thirds of the recommended daily intake of fat. The fact is during ripening, fat content does not change [66]. Given the popularity of cheese and the seeming ubiquitous goal towards eating less fat, it is no surprise that reduced- and low-fat cheeses have great market potential. However, recent studies have proved that consuming full-fat cheese does not impact negatively, among others, on traditional cardiovascular disease risk markers [11]. What is more, many flavour compounds found in cheeses happen to be fat-soluble. It is worth mentioning fat is a great flavour carrier. This is what causes some reduced- and low-fat cheeses to taste “off” compared to full-fat cheeses [32].

Apart from the sensory properties of ripened cheese, this product is an important source of proteins and amino acids – it is well documented that cheese provides all essential amino acids. Our body makes its own nonessential amino acids, although we must obtain essential amino acids from food sources. The amino acids that our body cannot make include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Cheese protein is almost 100% digestible, as the ripening phase of cheese manufacture involves a progressive breakdown of casein to water-soluble peptides and free amino acids. Among the amino acids content in cheese, it is important to highlight the high lysine content as this amino acid is of high bioavailability in cheese due to the absence of Maillard reactions [19].

## Bioactive peptides of cheese

Until recently, milk proteins were regarded mainly as a source of amino acids essential for the proper functioning of the human body. However, not only the proteins and amino acids play an important role in human nutrition. An intermediate product of proteolysis, peptides, has moved to the centre of interest. The increasing number of publications that appeared during the last years, show that bioactive peptides in food is undoubtedly the subject of intensive research. These are special amino acid sequences within the proteins, produced via certain modifications or cleavage from parent proteins [58]. Bioactive peptides represent an interesting alternative to pharmaceutical drugs without known side effects, as they are present in a variety of protein-rich foods that are consumed on a daily basis, such as ripened cheese. The interesting thing is that in 1950 Mellander was the first to report the bioactive peptides when he suggested that caseinophosphopeptides (bovine milk casein-derived phosphorylated peptides) enhanced vitamin D-independent bone calcification in rachitic infants [39]. Generally, the size of bioactive peptides ranges from 2-20 amino acid residues depending upon their type, nature and composition [35]. As long as they are bound in the proteins they are biologically inactive [33]. They can only be active after they have been released from their parent protein by proteolysis and can exert a wide range of biological activities such as opioid, blood pressure-lowering, mineral binding, antimicrobial, immunomodulating, cell-modulating, anti-carcinogenic, anti-cariogenic, anti-thrombotic, anti-inflammatory, and cholesterol-lowering activities [16]. These activities are governed by several factors, the major being structural properties, amino acid composition, and sequences within the peptide [54]. In addition, such peptides show a much lower allergenicity than the protein from which they were formed. It is related to their lower molecular weight [29].

Presently, milk proteins are considered as the most vital resource of a range of bioactive peptides [50]. Because of their physiological and physicochemical versatility, milk-derived peptides are regarded as very important constituents for incorporation in functional and novel foods, dietary supplements, and even pharmaceuticals with the purpose of targeting specific disease [43]. An adequate intake of milk and milk products, such as ripened cheese, as a part of a healthy, balanced diet is needed throughout life to promote health. Scientific studies signify that milk is undoubtedly a source of bioactive peptides that can positively have an impact on human health. The truth is milk proteins are available in great amounts with a high degree of purity at low price, which, under a technological aspect, makes them attractive in the search of biologically active peptides.

## Bioavailability of bioactive peptides from cheese

Bioactive peptides are already present in high abundance in many different types of cheeses after technological transformation or fermentation processes, and even more often are generated during the process of digestion. Transport mechanisms and the bioavailability of bioactive peptides are often studied with identified and isolated peptides or in cell culture models [20]. In order to investigate the properties of a certain pharmaceutical drug, it is administered as a single compound in a dose-dependent manner, which allows the analysis of its physiological effects. This procedure is much more complex for bioactive peptides that are inactive and hidden in the intact protein within the food matrix. What is more, in the past, the analytical tools for such complex questions were not sensitive or selective enough, or were even completely lacking. These days, however, if and how many of the ingested peptides are absorbed and reach the blood *in vivo* is a subject of current research. It is so because the biological activity of *in vitro* milk-derived peptides does not always imply physiological effect *in vivo*. It is very difficult to establish direct relationship between *in vitro* and *in vivo* activity mainly due to the bioavailability of the bioactive peptides after oral administration. Apart from bioavailability, oral tolerance to proteins or peptides is a critical point toward generation of new nutraceuticals as alternatives for pharmaceutical drugs. So far, the different literature resources reported that oral administration of isolated proteins or peptides was well tolerated and no side effects were observed. However, this needs to be investigated for each case in a similar way as for Lactoferrin (b-LF) from bovine milk which was approved by the US and European Food Safety authorities as dietary supplement [25].

To exert physiological effects *in vivo* after oral ingestion, it is of crucial importance that bioactive peptides remain active during digestion by human proteases and absorption as well as reach the bloodstream in an active form [15]. Physicochemical properties are one of the most important barriers in limiting the absorption of food peptides. The bioavailability of peptides depends on a variety of structural and chemical properties; that is, molecular weight, hydrophobicity, charge, hydrogen-bonding potential, and solubility [44]. It is worth mentioning that biological potency of peptides at the tissue level decreases as the chain length increases [52]. Another barrier limiting the absorption of food-originated peptides is a metabolic one. It is, depending on food bioactive peptides structure and properties, susceptibility to brush-border peptidases [24]. Resistance to proteases throughout the gastrointestinal tract is a prerequisite for food-derived peptides to exert biological activities. Currently, with the development of the different formulation approaches, the obstacles for effective delivery of peptide and protein therapeutics have been slowly overcome [4].

## Blood pressure-lowering effects of bioactive peptides

Many different *in vitro* and *in vivo* functions of bioactive peptides have been already described in the literature. However, the best-studied and *in vivo*-confirmed effect of bioactive peptides is their blood pressure-lowering function [6]. One of the major public cardiovascular diseases worldwide is hypertension, which usually occurs with other metabolic disorders such as obesity, prediabetes, and atherosclerosis [63]. Hypertension affects up to an average of 30% of the adult population in the developed countries, and in most western countries, it is a major risk factor for both coronary heart disease and stroke. The fact is that the relationship between hypertension and coronary heart disease is well established [46]. The main factors related to the growth of this problem are excessive consumption of high-energy foods as well as reduced physical activity of consumers. There is scientific evidence that diet has a direct relationship to cardiovascular diseases and the treatment of hypertension is no longer limited to the simple prescription of pharmaceuticals [31]. Although antihypertensive drugs are available on the market, nutritionists claim that peptides which lower blood pressure found in food are safer than “traditional” drugs and can be used as preventive agents. Numerous food components have been used for many years without any negative side effects [65].

To date, milk proteins are the main source of this kind of bioactive peptides [27]. At this respect, antihypertensive peptides have been found in processed dairy products, including several types of cheese. These peptides are usually built with 2 to 12 amino acid residues, although there are longer sequences [2]. The identification of antihypertensive peptides was initially focused on the search for angiotensin-converting enzyme (ACE, EC 3.4.15.1) inhibitors. ACE is a key enzyme in the regulation of blood pressure in the renin-angiotensin system by converting angiotensin I to a potent vasoconstrictor, angiotensin II, which also induces the release of aldosterone and therefore increases the sodium concentration and blood pressure. ACE also takes part of the kinin-kalicrein system as it hydrolyses bradykinin, which has a vasodilator action [38]. By inhibiting the effect of ACE, these peptides have a positive influence on hypertension [8]. In cheese, which is a complex food matrix containing a large number of different peptides which change with the ripening time, they are produced during secondary proteolysis through the action of proteinases and peptidases. Several studies have shown ACE-inhibitory activity in various cheese varieties with different bioactive peptides being responsible for that effect. The examples of ACE inhibitors from cheese are casokinins identified in the water-soluble extracts from Gouda, Cheddar, Gamalost, Gorgonzola, Grana Padano, Gruyere de Comté, Maasdam or Manchego cheese [1, 40, 48, 61]. Among these different ACE-inhibiting peptides, the tripeptides valyl-prolyl-proline (VPP) and isoleucyl-prolyl-proline (IPP) belong to the most potent ACE-inhibiting peptide group and are encrypted in the β-casein of milk. They are easily absorbed by the intestine and it has been shown that peptides containing a C-terminal Pro-Pro sequence are rather resistant to further degradation by digestive proteases and peptidases [23].

Recent work in spontaneous hypertensive rats demonstrated that after oral administration, the pentapeptide HLPLP, from β-casein, was, after rapid absorption, further degraded by plasma enzymes to LPLP, HLPL and HLP. The biological function was conserved in all fragments, although with a different kinetic behaviour, showing peaks from 2 hours for the parent peptide to 8 hours for the smallest fragment. All peptides decreased blood pressure by 15-21 mmHg [55]. Another study was a human double-blind clinical trial involving subjects with metabolic syndrome. It showed a mild but significant mean decrease in systolic and diastolic BP after treatment with lactotripeptides [12].

## Anti-carcinogenic activity of bioactive peptides from cheese

Proteins, peptides, and amino acids have been implicated in preventing the development of different types of cancer. Dairy milk proteins and their peptide derivatives play a role in cancer prevention. Cancer is characterised by a group of diseases that involve uncontrolled or abnormal cell growth, potential to invade or spread to other parts of the body [28]. There are various factors involved in the occurrence of cancer, but diet imparts a central role in the ethology of cancer. It has been observed that breast, prostate, colon, and rectum cancer are common in developed countries because of poor dietary habits. However, about one-third of all cancers can be reduced by modifying the diet plans. Dairy proteins from cheese and their fractions have potential to cure various cancers [34].

Antitumour peptides were defined as those having the ability to decrease or halt cell proliferation *in vitro* or *in vivo* [7]. The advantage of these peptides is their low intrinsic toxicity and high tissue-penetration, cell diffusion, and permeability. Proliferation of tumour cells can occur through the inhibition of cell migration, inhibition of tumour angiogenesis, inhibition of transcription, induction of apoptosis or disorganisation of tubulin structure [13]. The fact is that cytomodulatory peptides inhibit the growth of cancer cell and stimulate the activity of immune competent cells. The peptidic fractions from milk fermented with *Lactobacillus helveticus* showed cytomodulatory effects in mice [37]. Moreover, it has been proved that β-casomorphins released from casein by induction of apoptosis can inhibit the proliferation of colorectal cancer cells or diseased white blood cells [53]. Additionally, cytomodulatory effects in a human breast cancer cell line were reported from the peptides obtained from the milk fermented with probiotic microorganisms [43].

## Antioxidative activity of bioactive peptides from cheese

Several milk peptides play a regulatory role in oxidative metabolism which is essential for the survival of cells and causes oxidative changes by producing free radicals. Reactive oxygen species and other free radicals can be produced in the body and can oxidatively damage DNA, proteins, and other important cellular components [3]. They have been implicated in the occurrence of diabetes, neurodegenerative diseases, and cancer. Moreover, they are generally associated with aging [17]. Thus, a control of oxidative stress seems to be one of the crucial steps in slowing down the progress of these diseases or preventing their complications.

The truth is antioxidant proteins and peptides partially originate from milk itself but most of them are formed during cheese processing. Intact caseins were shown to possess antioxidant activity [47]. Peptides derived from β-casein have free radical-scavenging activity and inhibit both enzymatic and nonenzymatic lipid peroxidation [51]. The proposed mechanism of antioxidant activity of caseins is the quenching of free radicals by the oxidation of amino acids in caseins. Cheese ripening and proteolysis are the most complex process and are identified as the major stages of antioxidant formation [5]. In a study by Igoshi et al. [30], the presence of antioxidant activity was searched in 20 types of cheese. It was revealed the ripened cheeses such as Camembert, Gouda and Parmesan, had antioxidant activities. This activity was particularly high in mouldy cheeses. Another study by Gupta et al. [26] demonstrated the antioxidant properties of Cheddar cheeses. The results indicated antioxidant activity was dependent of the ripening period. A gradual increase of this activity was observed up to the 4 months of ripening of Cheddar cheese, after which it decreased and retained similar values from the seventh to the ninth month of ripening.

The mechanism of antioxidant activity of peptides and proteins is not yet quite clear. It is discussed in the literature whether the antioxidant effect evolves from single amino acid in protein sequence or if the effect is a result of interactions between multiple amino acid side chains, although most researchers agree upon the latter [21]. Generally, antioxidant property of proteins is related first to their amino acid composition. The correct positioning of amino acids in the protein sequence plays an important role in the antioxidant activity of proteins [68]. Antioxidative properties of proteins are also related to their hydrophobicity, as well as the ability of protein to chelate, and store or transport catalytically active metals that are important prooxidants in food lipids [22]. Future research should be directed towards a more detailed characterisation of peptides responsible for this bioactivity, especially in traditional types and defining factors that affect the optimal formation during cheese production.

## Opioid activity of bioactive peptides from cheese

The physiological activity of neuroactive peptides is a very interesting consideration. These peptides are involved in the body's response to stress: they alleviate pain, have a sedative effect, dull the senses, lead to respiratory depression, lower blood pressure, change body temperature, contribute to the feeling of satiation, inhibit the secretion of gastric juices, and lead to changes in sexual behaviour [40]. Casein-derived β-casomorphins were the first peptides reported to show agonistic activity on exogenous opioid receptors and they are the most studied opioid receptor ligands. Generally, milk-derived opioid peptides are characterised by the presence of a tyrosine residue at the N-terminal and another aromatic amino acid at third or fourth position which is an important structural motif that fits into the binding site of opioid receptors [41]. Opioid peptides exert their activity by binding to specific receptors of the target cell in an agonistic or antagonistic fashion. The fact is β-casomorphins are transported across mucosal membranes of neonates that regulate physiological responses resulting in calmness and sleep in infants [62]. On other hand, β-casomorphin interacts with opiate receptors in the serosal side of the intestinal epithelium and plays a crucial role in certain activities like regulation of electrolyte transport, insulin secretion, and food absorption [64]. However, a high content of proline residues in β-casomorphins supports their low susceptibility to many proteolytic enzymes, enabling them to cross the blood-brain barrier and affect central opioid receptors nervous system. The casein-derived material was found in the brainstem of human infants who died of Sudden Infant Death Syndrome (SIDS) [67].

The formation and fate of β-casomorphins have been studied in different cheese varieties, e.g. in Gouda Cheese extract [36], in semi-hard (Edamski, Gouda, and Kasztelan) and in two ripening mould cheeses (Brie and Rokpol) varieties [60]. Another study by De Noni and Cattaneo [18] demonstrated the occurrence of β-casomorphins in some commercial cheeses (Gorgonzola, Caprino, Brie, Taleggio, Gouda, Fontina, Cheddar, and Grana Padano) and their digests. This work also provided evidence that release of β-casomorphins is mainly promoted by the action of gastrointestinal proteases during *in vitro* digestion. Thus, more research needs to be done to evaluate the absorption of these peptides in a complex food matrix as cheese.

## Conclusions

The potential health benefits of bioactive cheese peptides have been a subject of growing commercial interest in the context of health-promoting functional foods. Cheese can be considered an important component of an equilibrated diet from a nutritional point of view. Its high quality protein and the possibilities for technological transformation and fermentation present an ideal source for bioactive peptides which in general represent a promising alternative to conventional drugs due to the fact that no side effects are currently known. However, more studies are still needed that will increase the scientific knowledge of the molecular mechanisms and pharmacokinetic properties of specific bioactive peptides and will complete the picture of their impact on health.

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