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Whey proteins – as nutraceuticals of health benefits

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Abstract: Whey, which remains a by-product after coagulation of casein in milk during cheese production, contains a wide variety of proteins characterised by high nutritional value as well as attractive functional and biological properties. For this reason, their use has been steadily increasing both in prevention and therapy of many diseases, especially those diet-related. In this aspect they meet all the criteria set for nutraceuticals, i.e. substances of both nutritional and pharmaceutical value used for the production of functional foods. In this article, the properties of major whey proteins and products of their modifications, including β-lactoglobulin, α-lactalbumin, glycomacropeptide, immunoglobulins, serum albumin, lactoferrin, lactoperoxidase, lysozyme and osteopontin are presented.

Keywords: whey proteins, bioactive proteins, health benefits

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1. Introduction

In recent years we have witnessed widespread development of acquired metabolic diseases in societies resulting from improper diets. This phenomenon urgently needs some preventative steps based in promotion of a healthy lifestyle, which along with physical activity also requires changes in human diets. Functional foods enriched with natural bioactive substances have a beneficial impact on functioning of the body and can play an important role in prevention of diet-related diseases, e.g. obesity, type-2 diabetes, osteoporosis, hypertension and dyslipidaemia. Sources of these ingredients, also referred to as nutraceuticals, are found in both plant and animal raw materials.

Among the latter, whey is especially significant, since a troublesome by-product obtained during cheese production has become a valuable co-product of that process [Papademas & Kotsaki, 2020]. Whey constitutes approx. 85–90% of milk volume used for cheese production with the use of rennet or acid coagulants (quarks). Depending on the method used for cheese production, two types of whey can be obtained: rennet whey (sweet, pH 5.5–6.5) and acidic whey (pH between 4.6 and 4.7). Approximately half of the total solids remains in whey (approx. 6.5%), among which a major portion consists of lactose, while the remaining compounds are mostly mineral salts and proteins containing approx. 20% of all milk proteins [Smithers, 2008; Yalcin, 2006]. Despite their low concentration in whey (merely 0.6–0.7%), their nutritional and biological value is the highest not only among all milk proteins, but also among other nutritional proteins [Madureira et al., 2007; Tsusumi & Tsusumi, 2014; Borad et al., 2017; Khan & Selamoglu, 2019]. The index of their nutritional value, expressed as the biological value (BV) is about 15% higher than that of egg protein used as a standard reference [Smithers, 2008]. It is due to the fact that their amino-acid composition is much richer in essential amino-acids as compared to other nutritional proteins, such as casein or egg white (Table 1). Worth noting in their composition is a very high content of branched-chain amino-acids (BCAA), i.e. leucine, isoleucine and valine and sulphur amino-acids, especially cysteine. Branched-chain amino-acids play an important role in nutrient metabolism and glucose homeostasis in blood as well as metabolism of lipids. Leucine itself plays a key role in the regulation of synthesis of skeletal muscle proteins [Chen et al., 2014; Naclerio & Seijo, 2019; Minj & Anand, 2020]. Cysteine is a precursor of glutathione, an important compound reducing oxidative stress in cells [Bell, 2000] and also enhancing the immune system of the body and protecting it from cancer [Tseng et al., 2006; Madureira et al., 2007; Trachootham et al., 2008].

Today, whey proteins are available on a commercial scale. The use of membrane filtration techniques and chromatographic methods enables their production on a larger scale in form of preparations of varied concentrations, such as whey protein concentrates (WPC) containing whey proteins within the ranges from 34% to 80%, whey protein isolates (WPI) containing more than 90% of those proteins, and besides, their individual fractions are also isolated [Baldasco et al., 2011; Kassem, 2015; Smithers, 2015]. Hydrolysates of those proteins are of great importance on the markets, since they turned out to be extremely attractive functional food additives [Meisel, 2005; Khan, 2013; Brandelli et al., 2015; Corrachano et al., 2018; Khan & Selamoglu, 2019]. Controlled hydrolysis of the proteins reduces their allergenicity and increases their biological activity as a result of releasing the peptides with a wide spectrum of activity from their sequence. The hydrolysates are frequently used in food products for children and athletes as well as medical foods or “slim food” [Ha & Zemel, 2003; Zimecki & Kruzel, 2007; Korhonen, 2009; Madureira et al., 2010; Pihlanto, 2011; Artym & Zimecki, 2013; Brandelli et al., 2015; Li-Chan, 2015; Kassem, 2015; Gupta et al., 2016; Madadlou & Abbaspourrad, 2018].

The preparations containing whey proteins also show good functional properties, related to their gelling and emulsifying properties as well as strong ability to bind water and create foams [Baldasco et al., 2011; Patel 2015a; Soltani et al., 2017; Batista et al., 2018; Minj & Anand, 2020]. They are also able to bind fragrant compounds to a greater extent than casein preparations [Livney, 2010]. Whey proteins, due to these properties, have been widely used in different industries, especially in food industry [Costa et al., 2021]. Some of them are also used as substitutes for synthetic surfactants in composition of many cosmetics [Król et al., 2014]. Various enzyme or chemical modifications of these proteins improve their functional properties [Li et al., 2005; Smithers, 2015; Patel, 2018b; Wefers et al., 2018].

Whey proteins are becoming increasingly important in medicine, either directly as therapeutic substances [Marshall, 2004; Artym & Zimecki, 2005, 2013; Sousa et al., 2012; Pal & Radavelli-Bagatini, 2013; Ng et al.,

Table 1. Essential amino acids in proteins [g/100g protein]

Amino-acids	**Types of preparation**			**Standards**	
	[1]WPC 80 aw	**[1]WPC 80 sw**	**[3]Calcium Caseinate**	**[2]Egg protein**	**[3]WHO/FAO UNU 2007 Standard**
Isoleucine	5.9	6.6	4.5	5.9	2.8
Leucine	10.7	11.4	9.4	8.5	6.6
Lysine	12.5	9.8	7.1	6.3	5.8
Methionine	1.9	1.9	3.3	5.9	2.5
Phenylalanine	3.7	3.7	10.5	9.6	6.3
Threonine	5.0	7.6	3.8	4.7	3.4
Tryptophan	2.7	2.8	1.3	0.7	1.1
Valine	5.3	6.4	6.0	6.9	3.5
Histidine	2.6	2.5	3.2	5.4	

aw-acid whey, sw- sweet whey

[1] De Boer (2014)

[2] Wereńska, M., & Okruszek, A. (2011). Wartość odżywcza różnego rodzaju jaj. Engineering Sciences & Technologies/Nauki Inżynierskie i Technologie.

[3] Jerzy Szpendowski, Krzysztof Siemianowski Engineering Sciences And Technologies 3(10) 2013 Właściwości Odżywcze I Funkcjonalne Oraz Zastosowanie Kazeinianów W Przetwórstwie Spożywczym

2015; Gupta & Parakash, 2017; Amirani et al., 2020] or indirectly as substances used for encapsulation of drugs and other bioactive substances because of their ability to form nanocomposites [Livney, 2010, Janser et al., 2017; Varlamova & Zaripow, 2020].

As has been proven, those proteins have anti-inflammatory properties and beneficial influence on functioning of the immune system, regulation of arterial blood pressure, blood glucose and cholesterol levels and also exhibit anti-cancer properties [Marshall, 2004; Krissansen et al., 2007; Sousa et al., 2012; Zhang et al., 2016; Cicero et al., 2017; Corrochano et al. 2018; Teixeira et al., 2019; Yang et al. 2019; Vasilyev et al. 2021]. Their consumption reduces a risk of metabolic syndrom and related diseases [Shertzer et al., 2011; Amirani et al., 2020]. Positive metabolic effects caused by the consumption of those proteins are also manifested in body weight losses, which is likely due to increased secretion of some hormones, such as glucagon like peptide (GLP1), leptins, cholecystokinin and reduction of ghrelin [Coker et al., 2012; Gillespie et al., 2015; Adams & Broughton, 2016; Sanchez-Moya et al., 2020; Kondrashina et al., 2020].

Whey proteins are globular proteins. They consist of a mixture of many proteins of unique nutritional and biological characteristics as well as multifaceted applications [Krissansen et al., 2007; Smithers, 2008; Batista et al., 2018]. Quantity-wise, the greatest fractions consist of the following proteins, listed in descending order: β-lactoglobulin (β-Lg), α-lactoalbumin (α-La), immunoglobulin and serum albumin (BSA). Other proteins which occur at lower concentrations (<1%), but are also significant commercially are as follows: lactoferrin (LF), lactoperoxidase (LPO) and lysozyme [Kassem, 2015]. Moreover, other bioactive proteins are present in whey, including osteopontin and proteins capable of binding vitamins, growth factors and around 60 enzymes [Steijns, 2001]. Additionally, 10-15 % of all the proteins found in the rennet whey are made up by glycomacropeptide, which is also isolated on an industrial scale [Neelima et al., 2013].

2. Bioactive whey proteins

2.1. β-Lactoglobulin

The main protein present in whey is β-Lg, which in ruminants makes up 50–60% of its total proteins. β-Lg is also present in some monogastric animals, such as pigs, horses, dogs and cats, but not in the milk of humans [Kontopidis et al., 2004]. However, it exhibits homology with human plasma retinol-binding protein (RBP) [Papiz et al., 1986]. β-Lg is synthesized in epithelial cells of mammary gland as one of approx. 10 genetic variants, of which the most common are genotypes A and B, characterised by different amino-acid substitutions at positions 64 and 118, where Asp and Val are present in β-Lg -A, while Gly and Ala are present in variant B [Le Maux et al., 2014; Broersen, 2020]. The structure of β-Lg contains 162 amino-acid residues, including 5 cysteine residues, of which four are engaged in the formation of disulphide bonds between Cys66 and Cys160 as well as Cys106 and Cys119. Cys121 is a free residue, buried within the structure of the protein in its native form, which when exposed as a consequence of denaturation can interact through such interactions with other β-Lg molecules or different proteins [Sava et al., 2005; Broersen, 2020]. Cysteine 121 also plays a significant antioxidant role of this protein and determines 50% of antioxidative properties of milk [Liu et al., 2007].

β-Lg is characterised by a high content (approx. 25.1%) of branched-chain amino acids, [Sousa et al., 2012]. In physiological conditions, this protein is present in bovine milk as a dimer (of MW 36.7 kDa) as a consequence of electrostatic interactions between Asp130 and Glu134 in one of the monomers and corresponding lysine residues in the other, but at pH<3 and pH>8 it dissociates to monomers [Xu, 1996]. Within the pH ranges between 3.5 and 5.2 it forms octamers of MW approx. 140 kDa [Madureira et al., 2007]. During heating to 65°C, reversible structural changes occur, it denaturates at 70–75°C, while at 78°C to 82°C it forms aggregates [Sava et al., 2005]. Its thermostability depends on the genetic variant and pH of the environment, but it is the highest at pH 3 [Boye et al 1996, 2004]. β-Lg belongs to a family of lipocalins, which contain a central cavity capable of binding various ligands, including small hydrophobic compounds, such as retinol, vitamin D2, cholesterol, aromatic compounds as well as saturated and unsaturated fatty acids and phospholipids, which increase bioavailability of those compounds [Perez et al., 1995; Flower et al., 2000; Kontopidis et al., 2004; Le Maux et al., 2014; Teng et al., 2016; Broersen, 2020; Samoes et al., 2020]. Binding of retinol and β-carotene to β-Lg protects them from thermal degradation and oxidation [Futterman & Heller, 1972; Hattori et al., 1995]. Curcumin, which is poorly absorbed and quickly metabolised in the body, becomes more bioavailable and its antioxidative activity increases when it is bound to β-Lg [Li et al., 2013]. Moreover, it has been proven that docosahexaenoic acid (DHA) when conjugated with this protein is less susceptible to oxidative processes at pH 7 and 40°C than free acid [Puyol et al., 1991; Zimet & Livney, 2009].

By binding free fatty acids, which inhibit pre-gastric lipase, β-Lg can influence the process of digesting milk fats during infancy by increasing the activity of that enzyme [Perez et al., 1992]. In turn, β-Lg can inhibit oxidative processes by binding metal ions, such as Cu and Fe in which a key role is played by a free sulfhydryl group [Liu et al., 2007]. β-Lg can also bind polyphenol compounds, such as resveratrol and epigallocatechin gallate (EGCG), forming complexes which modify biological and functional properties of the protein [Livney, 2010, Shpigelman, et al. 2010; Kanakis et al., 2011].

In addition to its main function as a transport protein, it also plays a role in passive immunity in infants and in regulation of phosphorus metabolism in the mammary gland [Broersen, 2020]. By inhibiting adhesion of the microorganisms to the surface of the epithelium in the intestine, it prevents its colonisation by pathogens at an early phase of an infection, either preventing or reducing it [Ouwehand et al., 1997]. β-Lg also has an inhibitory effect on replication of rotaviruses, the degree of which is dose-dependent [Chatterton et al., 2006]. Antiviral properties, including the activity against HIV, are increased by chemical modifications of the protein, such as acetylation and succinylation [Pan et al., 2005; Chatterton et al., 2006; Ng et al., 2015]. As has been shown, β-Lg exhibits protective anti-cancer properties when administered orally, which is related to high content of sulphur residues in its structure [Pepe et al., 2013]. Sun et al. (2018) observed increases in anti-cancer and immunoregulatory activities of this protein after introduction of selenium ions to its structure. Using a mice model, they confirmed in vivo a stronger action of Se- β-Lg against sarcoma cells from the line S180 and higher immunoregulatory activity. The ability of this protein to bind mutagenic heterocyclic amines blocks their cancerogenic effects [Yoshida et al., 1991].

A disadvantageous feature of of β-Lg is the fact that it is the strongest allergen among all proteins present in milk, being responsible for 70–80 % of allergic reactions to milk in children [Rahaman et al., 2015]. They affect around 2-3 % of children who usually lose the sensitivity to this protein after 3 years of age [Sousa et al., 2012; Petrotos et al., 2014].

β-Lg contains many epitopes of varying degree of allergenicity, which are present along the whole length of its polypeptide chain. Some of them are linear sequences, while other, larger ones are conformational epitopes [Golkar et al., 2018; Villa et al., 2018]. Jarvinen et al. (2001) identified 7 epitopes (fragments: f1-16, f31-48, f47-60, f67-78, f75-86 and f127-144) which bind to IgE and four (fragments: f49-60, f119-128, f129-138 and f143-152) binding to IgG. The research studies by Cong and Li (2012) confirmed that four epitopes in β-Lg binding to IgE, sequences f17-36, f72-86, f92-106 and f152-166 as well as two epitopes identified to be sequences f22-36 and f127-141 bound by IgG. They also demonstrated that threonine, methionine and aspartic acid residues at positions 20, 23 and 27, respectively were critical for β-Lg epitopes binding with IgE while leucine residue at position 26 and valine residue at position 31 were critical for the epitopes binding with IgG. Cerecedo et al. (2008) found that among different epitopes of β-Lg, two of them (corresponding to sequences f58-77 and f121-140) were recognised in 75% of patients allergic to milk.

β-Lg, due to its high nutritional value, is an important component of baby food, in which it is used in a form of a hydrolysate [Quintieri et al., 2017]. In addition, binding of this protein with other compounds, such as reducing sugars in the Maillard reaction and various technological processes, including long-term heating at high temperatures (85–100°C), reduce its allergenic potential [Bu et al., 2010; Rahaman et al. , 2015; Golkar et al., 2018; Villa et al., 2018].

Wu et al. (2018) showed that covalent binding of β-Lg with polyphenolic compounds, such as EGCG and chlorogenic acid also decreases its allergenicity.

Native β-Lg is generally resistant to enzymatic hydrolysis. Besides, it is not susceptible to degradation by pepsin, but it is digested by pancreatic enzymes in the small intestine [Pelligrini et al., 2001; Creamer et al., 2004; Bossios et al., 2011]. Proteolytic enzymes of other origin also degrade this protein, although usually in a denatured form [Babij et al., 2014]. The peptides released from its structure exhibit a number of biological activities. They are antioxidant biopeptides (containing Leu, Pro, His residues in their structure), antimicrobial and hypotensive peptide able to decrease blood pressure by inhibiting enzyme converting angiotensin I to angiotensin II (Angiotensin Converting Enzyme- ACE) , i.e. lactokinins, such as β-lactosin peptide of the sequence Ala-Leu-Pro-Met f (142–145). β-Lg is also a source of opioid peptide β-lactorphin: Tyr-Leu-Leu-Phe f (102–105) and the memory-improving peptide β-lactotensin: His-Ile-Arg-Leu f (146–149) [Meisel, 2005; Hernández-Ledesma et al., 2008; Madureira et al., 2010; Brandelli et al., 2015; Świątek et al., 2019]. Nagaoka et al. (2001) identified a hypocholesterolemic peptide β – lactostatin (with a sequence: Ile-Ile-Ala-Glu-Lys f (71–75) in the trypsin hydrolysate of β-Lg, which suppressed cholesterol absorption by Caco-2 cells in vitro and also exhibited hypocholesterolemic activity in vivo in rats.

β-Lg exhibits attractive functional properties due to its amphiphilic structure and high stability in an acidic environment. It is able to form gels when heated. It has been used for encapsulating bioactive substances which can be safely transported through the acidic environment of the stomach directly to the small intestine by using β-Lg as the carrier [Madureira et al., 2007; Świątek et al., 2019; Shpigelman et al., 2010;

Teng et al., 2016; Wilde et al., 2016; Simoes et al., 2020]. Using various chemical modifications to β-Lg, either through phosphorylation or glycation, which affect its conformational changes, it is also possible to change the functional properties of β-Lg [Van Teeffelen et al., 2005; Enomoto et al., 2007; Medrano et al., 2009].

2.2. α-lactalbumin

α-lactalbumin (α-La) is the second whey protein, which, like β-Lg, is synthesized in the secretory cells of the mammary gland. It is found in the milk of almost all mammals. In human milk it is the dominant protein (75%), but in cow's milk it constitutes 20–25% of all whey proteins [Kamau et al., 2010].

α-La plays a very important physiological role by participating in the process of lactose biosynthesis. By attaching to β-galactosyltransferase, it increases its affinity to glucose in the final stage of milk sugar production from UDP-galactose and glucose in the mammary gland [Grobler et al., 1994; Qasba & Kumor, 1997; McSweeney & Fox, 2013].

α-La constitutes a 40% identity in the amino acid sequence and shows high similarity in three-dimentional structure to lysozyme, which indicates that those proteins diverge from a common ancestor, but their functions vary, since α-La takes part in the synthesis while the lysozyme in the hydrolysis of the β-1,4-glycosidic bond [Permyakov & Berliner, 2000].

α-La is a small protein with 123 amino acid residues (MW 14.175 kDa) in its structure, including 8 cysteine residues which form four disulfide bridges (Cys6-Cys120; Cys28-Cys111; Cys61-Cys77; Cys73-Cys91) stabilizing its spatial structure. It is also a metalloprotein that binds calcium which improves its molecular stability and protects it against thermal denaturation [Hiraoka et al., 1980]. Removal of calcium decreases thermal stability of α-La, although it shows the greatest heat resistance among all whey proteins. It denaturates at 62–65°C, but 90% of it renaturates after cooling. It becomes completely denatured at 70–80°C [McGuffey et al., 2005]. At higher temperatures, α-La free of calcium ions forms a classic molten globule, similarly to native protein in an acidic environment [Hochwallner et al., 2014]. Native α-La is able to bind and transport also other ions, e.g. Mg, Zn, Co, although calcium substitution for zinc decreases its thermal stability [Permyakov & Berliner, 2000]. α-La is a monomer at the pH of milk and a reversible aggregation occurs at acidic pH.

The structure of α-La is 72% homologous with human α-lactalbumin. For comparison, bovine α-La contains more His, Trp and Val than human α-La. On the other hand, human α-La is richer in Ile, Leu and Met residues (Table 2). The concentrations of other essential amino acids in these proteins are at similar levels [Lönnerdal & Lien, 2003; Kamau et al., 2010]. α-La is the most valuable milk protein in nutrition. Among all food proteins, α-La exhibits the highest content of tryptophan (5.9%), a serotonin precursor [Ruddick et al., 2006].

It can, therefore be concluded that consumption of this protein increases the level of this neurotransmitter in the brain, reduces the concentration of cortisol, has an anti-stress effect and improves cognitive abilities and mood [Markus, Olivier, Panhuysen et. al., 2000; Markus, Olivier, De Haan et. al., 2002; Orosco et al., 2004].

α-La, next to β-Lg, is the second important allergen among whey proteins, which often causes allergic reactions to milk in children. As shown previously, its structure contains many epitopes capable of binding to specific IgE and IgG antibodies (Jarvinen et al., 2001; Hochwallner et al., 2014; Golkar et al., 2018). Jarvinen et al., (2001) identified four fragments of this protein that react with IgE: f (1-16), f (13-26), f (47-58), and f (93-102) as well as three fragments binding to IgG, corresponding to the sequences: f (7-18, f (51-61) and f (89-108). On the other hand, Cong et al., (2016) showed five fragments that react with IgE: f (1-15), f (6-20), f (46-60), f (71-85), f (101-115) and four IgG binding epitopes, corresponding to the sequences: f (6-20), f (21-35), f (36-50) and f (86-100).

As has been found α-La has beneficial effects on the organism's physiological processes. Matsumoto et al. (2001 found that it had protective effects on ethanol-induced stress and gastric diseases (gastric ulcer) by increasing prostaglandin levels. Administration of this protein in an optimal dose of 200 mg/kg body weight per day proved to be effective in the treatment of these diseases. Krissansen (2007) found α-La to be effective in preventing the binding of enteropathogenic bacteria, i.e. *E. coli*, *Salmonella typhimurium* and *Shigella flexneri* to intestinal epithelial cells. Its antibacterial activity against both Gram-positive and Gram-negative bacteria is enhanced by interaction with lysozyme, with which it associates under physiological pH conditions [Expósito & Recio, 2006].

α-La also demonstrates anti-cancer properties. Bruck et al. 2014 found its ability to inhibit proliferation of certain tumor cell lines. Many other authors have confirmed that complexes of human and bovine α-La, free of Ca ions (apo-form), with oleic acid (C18:1:9cis), abbreviated as HAMLET and BAMLET, respectively, exhibit strong anti-cancer activities [Fast et al., 2005; Barbana et al., 2011; Delgao et al., 2015]. These complexes can enter neoplastic cells, bind to histones and disrupt chromatin organization in the cell nucleus. As a result, they induce apoptosis of many cancer

Table 2. Amino acid composition of bovine and human α-lactalbumin (%) (Lönnerdal and Lien, 2003).

Amino-acids Essential and conditionally essential	Bovine	Human	Amino-acids Nonessential	Bovine	Human
Isoleucine	6.4	9.7	Alanine	1.5	2.5
Leucine	10.4	11.3	Asparagine	6.4	3.2
Lysine	10.9	10.9	Glutamine	5.4	6.4
Methionine	0.9	1.9	Glycine	2.4	2.4
Cysteine	5.8	5.8	Proline	1.4	1.4
Phenylalanine	4.2	4.2	Serine	4.3	5.0
Threonine	5.0	5.0	Aspartic acid	10.6	9.8
Tryptophan	5.3	4.0	Glutamic acid	6.4	7.4
Tyrosine	4.6	4.6	-	-	-
Valine	4.2	1.4	-	-	-
Histidine	2.9	2.0	-	-	-
Arginine	1.1	1.1	-	-	-

lines, such as human skin papilloma and glioblastoma [Fischer et al., 2004; Lišková et al., 2010; Fontana et al., 2013; Rath et al., 2015; Delgado et al., 2015; Hsieh et al., 2015]. α-La hydrolysates free of allergic effects also exhibit a number of biological activities. Pellegrini et al. 1999 reported that its hydrolysates obtained by trypsin and chymotrypsin digestion exhibited bactericidal activity, mainly against pathogenic Gram-positive bacteria. Besides, they also had a stimulating effect on the growth of bifidobacteria.

A broad spectrum of activity is exhibited by the biopeptide α-lactorphine with the sequence Tyr-Gly-Leu-Phe, a fragment of 50-53 α-La, released as a consequence of digestion by pepsin, which shows a structural similarity to the human opioid peptide Leu-enkephalin (with the sequence Tyr-Gly-Gly- Phe). By stimulating the opioid receptor, it relieves pain, alleviates blood pressure and shows immunomodulatory and antibacterial activity against E. coli Q127. It also exhibits anti-cancer activity. Peptides released from the C-terminus of α-La, which are fragments of its sequence: f 99-108 and f 104-108, exhibit high antihypertensive activity. Another biopeptide Gly-Leu-Phe f 51-53 shows an immunomodulatory effect by stimulating phagocytosis in macrophages [Chatterton et al., 2006; Madureira et al., 2010]. In general, α-La hydrolysates exhibit immunostimulatory properties through the activation and proliferation of lymphocytes, stimulation of cytokine secretion and antibody production [Gauthier et al., 2006].

α-La, due to its high nutritional value and its positive effects on gastrointestinal microbiota, immunomodulating action and stimulation of brain development and growth in children, is widely used in their diets [Layman et al., 2018; Nielsen et al., 2020]. It is also a component of many foods for adults. α-La also exhibits attractive functional properties. When subjected to limited hydrolysis with the use of serine protease from Bacillus licheniformis under appropriate pH conditions and in the presence of a divalent cations, it shows the ability to form self organized nanotubular structures which can be widely used as carriers of various substances, including those with a therapeutic effect [Graveland-Bikker et al., 2005; Ipsen & Otte, 2007].

2.3. GMP

GMP, a glycomacropeptide is another major protein present in rennet whey, processed on a larger scale than acid whey. It is a product released from κ-caseins via the action of a coagulating enzyme, that is chymosin or its substitutes. It is also released under physiological conditions after milk consumption as a result of protein hydrolysis by pepsin. In recent years this peptide has become especially interesting due to its unique physicochemical, nutritional and health-promoting properties. GMP is a fragment of the C-terminal sequence (f 106-169) of κ-casein with a MW of about 7.50 kDa and a pI at pH 3.15 [Córdova-Dávalos et al., 2019] for glycosylated GMP and 4.15 for non-glycosylated form [Kreuß et al., 2009].

It exhibits strongly hydrophilic properties, mainly due to the presence of sugar components in its structure which also contribute to its heterogeneity. They are most often (approx. 75%) in the form of tri - and tetra-oligosaccharides, composed of galactose, N-acetyl-galactosamine and sialic acid, connected with GMP by O-glycosidic bonds with threonine residues and one serine [Brody, 2000; Sawin et al., 2015]. The presence of sugars, especially sialic acid, has a significant influence on biological activity of this oligopeptide [Fernando & Woonton, 2010]. Amino acids: Pro, Glu, Ser and Thr are abundant residues in GMP composition, while aromatic residues (Phe, Tyr and Trp) as well as His, Cys and Arg [Brody, 2000] have not been found. Due to the lack of phenylalanine in its composition, it is a valuable dietary product, produced on an industrial scale for people suffering from phenylketonuria [Laclair et al., 2009]. Its special nutritional value is also due to the high content of branched-chain amino acids (mainly Ile and Val), which together with a low content of methionine, makes GMP a valuable component of the diets for individuals with diagnosed liver diseases [Korhonen, 2009]. Many authors reported its significant impact on functioning of the digestive tract [Brody, 2000; Manso and Lopez-Fandino, 2004; Tulipano, 2020]. GMP has been shown to inhibit gastric acid secretion. It is also believed to stimulate the secretion of cholecystokinin, the satiety hormone regulating the appetite, although this was not clearly confirmed in other studies [Poppit et al., 2013]. This oligopeptide exerts positive impact on the microflora of the gastrointestinal tract, especially the growth of bifidobacteria, which is related to the presence of sugar moiety in its structure [Manso and Lopez-Fandino, 2004; Li and Mine, 2004; Sawin et al., 2015; O 'Riodan et al., 2018]. The beneficial effects of GMP on brain development and improvement of learning ability, as demonstrated by the authors who conducted studies on animal models, are attributed to its high content of sialic acid [Wang et al., 2009]. On the other hand, the high content of threonine in the sequence of GMP present in dietary supplements for infants (especially premature infants) are likely to cause hypertreoninemia [Rigo et al., 2001].

GMP also exhibits antimicrobial properties. In vitro studies showed that it inhibited the adhesion and growth of *Streptococcus mutans* and *Streptococcus sobrinus* bacteria involved in plague formation, therefore it is added to commercially available anti-caries dental preparations [Brody, 2000; Setarehnejad et.al., 2010]. Rhoades et al. (2005) found that GMP inhibited adhesion of pathogenic *E.coli* strains (VTEC and EPEC) to HT29 human colon cancer cells. Zimecki et al. (2006) reported a protective effect of glycomacropeptide against endotoxemia and bacteraemia in mice. Moreover, GMP is able to inactivate *Vibrio cholerae* toxins as well as *E. coli* LT I and LT II enterotoxins, and also inhibit hemagglutination caused by four strains of human influenza virus [Kawasaki et al., 1992; Zimecki et al., 2005, 2007].

GMP also affects functioning of the immune system [Brody, 2000; Zimecki et al., 2006; Cordova-Davalos et al., 2019]. It has been shown that it stimulates phagocytosis and proliferation of human macrophage cells of the U937 lineage [Li and Mine, 2004] and accelerates proliferation of human B cells, but not T cells [Brody, 2000]. It shows anti-inflammatory effects and also increases production of the cytokines in human monocytes [Sawin et al., 2015; Requena et al. 2009, 2010]. Numerous authors reported therapeutic properties of GMP, including its preventive and therapeutic effects on metabolic processes and associated diseases [Thomä-Worringer et al., 2006; Sauve et al., 2021].

The products of GMP hydrolysis also exhibit pro-health properties. Biopeptides with antithrombotic activity derived from its N-terminal sequence were identified among them, including undecapeptide (f106-116) called casoplatelin, which inhibited both the aggregation of ADP-activated platelets and the binding of fibrinogen chains to receptors on the platelet surface. The smaller biopeptide, constituting a 106-110 fragment of GMP called casopiastrin isolated from trypsin hydrolysates, showed anticoagulant activity by inhibiting fibrinogen binding [Clare and Swaisgood, 2000]. In addition to nutritional and biological values, GMP also exhibits desirable functional properties, such as solubility at wide pH ranges, high thermal stability, foaming, gelling and emulsifying properties, which can be improved by conjugating GMP with fatty acids or sugars [Neelima et al., 2013]. Due to all these properties, GMP is used as an attractive additive to various food products.

2.4. Immunoglobulins

Mammalian whey proteins include blood-derived immunoglobulins which are passed on as immune antibodies with mother's milk to newborns. Their composition varies depending on the species. In ruminants, immunoglobulins G: IgG1 and IgG2 are dominant, constituting approx. 75% of their total concentration [Kassem, 2015]. In addition, Ig M and Ig A are also present. Immunoglobulin content is the highest in colostrum (approx. 48 g/L), in which they constitute 70–80% of all milk proteins [Steijns, 2001]. Their concentration in whey is approx. 9% of all its proteins. It is possible to increase the level of immunoglobulins by immunization of cows against specific pathogens or antigens [Mehra et al., 2006; Pihlanto, 2011; Karhonen, 2009]. They condition specific humoral immunity of the body and play a role in fighting bacterial infections by agglutination, bacteriolysis, bacteriostasis or opsonization. They also neutralize viruses and toxins and are also capable of binding the proteins of the complement system [Mehra et al., 2006]. Numerous clinical studies confirm the effectiveness of immunoglobulin preparations against oral pathogens (*Str. mutans*, *Candida albicans*), and *Helicobacter pylori*, which causes gastric ulcers as well as *Cryptosporidium parvum*, which causes diarrhoea in immunocompromised individuals and against enteropathogenic *E. coli* infections in children [Severin et al., 2005; Steijns, 2001; Korhonen, 2009]. Oral administration of an immunoglobulin concentrate, especially in encapsulated form enclosed in gastric acid-resistant coatings, can prevent the body from bacterial infections [Steijns, 2001; Bostwick et al., 2000; El-Loly, 2007].

IgG2 is accompanied by a complex of proline-rich peptides, referred to as colostrinins. It contains a mixture of peptides of MW < 10 000 Da. Their composition is dominated by two amino-acids: proline and glutamic acid (more than 20% and 18%, respectively) [Sokołowska et al., 2008]. Their sequence shows homology to protein precursors: beta - casein and hypothetical beta - casein homologue. This complex exhibits antioxidant and anti-inflammatory effects. It was also found that it regulated the growth and differentiation of lymphocytes and inhibited pathological processes related to β amyloid aggregation in people with degenerative changes in the central nervous system [Zimecki & Kruzel, 2007; Zabłocka & Janusz, 2012; Zabłocka et al., 2020].

2.5. BSA

BSA (bovine serum albumin), which is present in blood (it accounts for 50% of all blood proteins) also constitutes a portion of albumins present in whey, in which it constitutes approx. 8% of all its proteins (0.02–0.35 mg/ml) [Maduiro et al., 2007]. It is a protein with an MW of about 66 kDa, characterized by a high content of essential amino acids and the presence of 17 disulfide bridges and one free sulfhydryl group [McSweeney and Fox, 2013]. The sequence of this protein is in 75% homologous to human serum albumin. BSA mainly functions as a transport protein for many ligands, including long-chain fatty acids, steroid hormones and metal ions [Krissansen, 2007]. BSA is also a source of bioactive peptides, such as albutensin (f208-216) and serophin (f399-404) exhibiting opioid activity [Madureira et al., 2010].

2.6. Lactoferrin

Lactoferrin (LF) is a complex, multifunctional protein present at a highest concentration in colostrum. In normal cow's milk, its content is many times lower and amounts to an average of 0.2 g/L, i.e. about 2% of whey proteins. LF is a glycoprotein from the family of transferrins involved in chelation and transport of iron. It consists of a single polypeptide chain with an MW of about 80 kDa, containing about 700 amino-acid residues, among which the most frequent are: alanine, leucine and glycine, which make up 10%, 9% and 7% of LF composition, respectively [Niaz et al., 2019]. LF contains 14 disulfide bridges in its structure. Its molecule is made up of two N- and C-terminal lobes connected by a short α-helix, which makes it very flexible. The lobes, which exhibit homology at a level of 31-41%, additionally have two domains, N1 and N2, as well as C1 and C2, respectively [Giansanti et al., 2016]. Bovine LF shows 69% structural similarity to that of human milk LF [Steijns, 2000]. A characteristic feature of Lf is highly basic character, with the pI in the range of 8.0-8.5 [van der Strate et al. 2001].

Lactoferrin contains from 6.7 to 11.2% of sugars, which also influence heterogeneity of this protein [O'Riordan et al., 2014]. They include: N-acetylglucosamine, acetylgalactosamine, galactose, fucose, mannose and neuraminic acid, N-linked with Asn233, Asn281, Asn368, Asn476 and Asn545 [Rascon-Cruz et al., 2021]. The degree of Lf's glycosylation affects its resistance to proteolysis by digestive enzymes and it;s sensitivity tolow pH of the environment. Lf can exist in three different isoforms: α, γ and β, but only the first isoform has the ability to bind iron ions. The other two isoforms exhibit ribonuclease activity, especially against mRNA [Furmanski et al., 1989; Garcia-Montoya et al., 2012; Karav et al., 2017]. The LF molecule can bind 2 ions of this metal, one in each lobe, and each ion is bound by 4 amino acid residues: Asp, two Tyr residues and His.This process is accompanied by binding of a bicarbonate anion by the arginine residue. Iron binding is a reversible process, therefore LF can also exist as apo-LF without iron or as holo-LF when bound to metal ions. Typically, LF is saturated only in 15%. Depending on the presence of iron, LF may also show different levels of biological activity [Wang et al., 2019]. LF can also bind other metal ions, such as Co, Mn, Cu, Zn, although with a lower affinity [Steijns, 2000]. Apo-LF, as compared to holo-LF, has a more open structure and thus a greater susceptibility to hydrolytic decomposition and the denaturing effect of high temperatures [Karav et al., 2017]. Form of Lf determinates not only thermal stability but also biological functions. Pasteurization of milk at 72–74°C for 15 sec has practically no effect on Lf structure, whereas ultra-high temperature (UHT) treatment at 135°C for 4 sec results in the loss of iron binding ability and antimicrobial characteristics [Sanchez et al., 1993; Karav et al., 2017].

The range of biological functions of LF is extremely wide, which fully entitles it to be called a multifunctional protein. It is the innate immunity protein of the body which strongly influences its acquired immunity.

It is the body's first line of defence against bacterial infections and thus protects and stimulates the growth of the intestinal epithelium, especially in premature infants. Initially, its protective antimicrobial effect was attributed to the sequestration of iron, necessary for growth of many pathogenic bacteria. However, the more effective bactericidal activity of LF results from its direct interaction with the cell wall of microorganisms, both Gram-positive and Gram-negative bacteria as well as fungi and protozoa [Castri et al., 2017; El-Loly et al., 2011; Garcia-Montoya, 2012; Artym, 2012; Lodhi et al., 2019; Rascon-Cruz et al., 2021]. By binding to the structures of their wall via the N-terminal fragment of a strongly cationic nature, causes its destabilization and cell damage. LF also prevents adhesion of pathogens to host tissues by binding to bacterial adhesins, and also exhibits enzyme activity against their protein virulence factors. The direct antimicrobial action of LF is also caused by inhibition of the process of biofilm formation by bacteria, which is more resistant to the host's protective mechanisms and to antibiotic therapy. It may also result from its antioxidant properties related to the chelation of pro-oxidative metals and free radicals scavenging by its sulfur residues [Khan & Selamoglu, 2019]. LF also exhibits broad antiviral activity against animal and human RNA and DNA-viruses, including hepatitis C, HIV, herpes viruses and rotaviruses, and influenza virus [Superti et al., 2001; van der Strate et al., 2001; Berkhout et al., 2004; Pan et al., 2006; Garcia-Montoya et al., 2011; Berlutti et al., 2011; Redwan et al., 2014; Niaz et al., 2019]. The mechanism of this action is based on its interaction with viral particles or with their receptors on the surface of target cells. The ability of LF to bind to receptors, also those of the coronavirus, has recently become an incentive to conduct studies on its use for prevention and treatment of SARS CoV-2 [Wang et al., 2020; Kell et al., 2020; Hu et al., 2021; Salaris et al. 2021].

A broad spectrum of antimicrobial activity of LF also results from its interactions with other bioactive components present in whey proteins, such as lysozyme and lactoperoxidase or osteopontin [Leitch & Willcox, 1998; de Andrade et al., 2014; Nakano et al., 2019; Jiang et al., 2020]. Some chemical modifications of LF increase its biological properties as well, including antibacterial and antiviral or anti-cancer properties [Pan et al., 2007; Najmafshar et al., 2020].

LF, by inhibiting the development of pathogens, simultaneously modulates composition of the gastrointestinal microflora and stimulates growth of probiotics, mainly from the genera of *Bifidobacterium* and *Lactobacillus*. Its beneficial effects also extend to other tissues and organs of the body, including the respiratory system, urinary system and skin [Artym & Zimecki, 2020, 2021; Superti, 2020].

The bactericidal properties are found not only in the native LF protein, but also in the products of pepsin hydrolysis, which show amphipathic properties. Among them, peptides derived from N-terminal end, referred to as lactoferricins, were identified [Madureira et al., 2010; Sinha et al., 2013]. They are peptides of sequences 1-11 and 17-41. The peptide of the first sequence turned out to be effective against 5 particularly drug-resistant strains of *Acinetobacter baumanii* bacteria and *Staphylococcus aureus* resistant to methicillin, as well as against many *Candida* species [Sinha et al., 2013]. On the other hand, the peptide of the second sequence, rich in basic (Lys and Arg) and hydrophobic (Try and Phe) residues, containing a disulfide bridge (Cys 19-Cys 36) demonstrates antibacterial and antiviral activities and also inhibits tumor metastasis and induces apoptosis of blood cancer cells. It turned out to be much more active than the substrate protein. Another peptide released from LF, which is a fragment of the LF 268-284 sequence, referred to as lactoferrampin, exhibits particularly broad antimicrobial activity, including antibacterial and antifungal activities [Bruni et al., 2016].

LF may also exert indirect antimicrobial effects by stimulating the immune system. Oral administration of LF stimulates both a local immune response within the lymphatic tissue of the gastrointestinal mucosa (GALT) and a systemic response. By inducing the production of IgA and IgG antibodies, it affects the secretion of certain cytokines and the activation of B, T and NK cells [Legrand et al., 2005; Zimecki & Kruzel, 2007; Artym, 2012; Kruzel et al., 2017; Superti, 2020].

As shown by Kawashima et al. (2012), through its function in promoting secretion of tears as well as antioxidant and anti-inflammatory activity in the aging eye, LF also has a protective effect against age-related "dry eye" syndrome. This protein improves functioning of the lacrimal gland, and although the mechanism of this process is still unknown, it is believed that it is likely due to direct action of LF on the lacrimal gland and its overall effect on metabolic processes in the body. It can, therefore be recommended as an alternative dietary supplement for patients with "dry eye" syndrome.

The antitumor activity of this protein is extremely important, which, in addition to its antioxidant and immunomodulatory effects also induces apoptosis of neoplastic cells and inhibits their proliferation as well as the process of angiogenesis, ie. formation of new blood vessels necessary for tumor development and metastasis [Hsieh et al., 2015, Olszewska, et al., 2021]. LF is also one of important chemopreventive agents contributing both to neutralization of carcinogens and many repair processes, including reductions in the amount of reactive oxygen species and inhibiting inflammation [Artym, 2012].

LF also participates in the regulation of metabolism of bone and cartilage tissues by stimulating differentiation and proliferation of osteoblasts and chondrocytes and inhibiting effects on their apoptosis [Cornish, 2004; Artym, 2012]. LF influences the metabolism of lipids, can inhibit peroxidation of fatty acids and formation of superoxide radicals [Fernandez-Real et al., 2012; Khan et al., 2019]. The result of numerous studies also confirm the antihypertensive, anti-stress and analgesic effects of LF [Artym, 2012].

All these properties of LF make it widely used in clinical practice [Artym & Zimecki 2013; Garcia-Montoya et al., 2011]. Discovery of the human and bovine LF gene sequences enabled the production of recombinant protein on an industrial scale. The granting of the GRAS status to LF by the FDA and EFSA, which allows its use as a novel food ingredient [Kelly, 2020] means that it is now widely used commercially in food for infants, as an iron supplement, in chewing gums, cosmetics and animal feed or as a nutraceutical strengthening the immune system in functional foods and therapeutic preparations but only in clinical trials [Steijns, 2001; Garcia-Montoya et al., 2011; Niaz et al., 2019].

2.7. Other whey proteins

Other proteins present in whey, which apart from lactoferrin, have an impact on non-specific immune reactions of the body, include two enzymes: lactoperoxidase (LPO) and lysozyme.

LPO is a glyco-hemoprotein containing 0.07% of Fe and approx. 10% of sugars. Its molecule with an MW of 77.5 kDa is composed of two identical subunits. In cow's milk, its content is approx. 30 mg/L. For commercial purposes, it is isolated from whey. It is one of the most heat-resistant enzymes present in milk, therefore, it is used for monitoring the effectiveness of high-temperature pasteurization processes. It is one of the most effective antimicrobial barrier component of ruminant milk. LPO catalyzes oxidation of thiocyanates by H_2O_2, resulting in the formation of hypothiocyanate, which is toxic to Gram-positive and Gram-negative bacteria. LPO was found to be particularly effective in inhibiting the growth of bacteria causing gingivitis and it also contributes to rapid gum healing [Smithers, 2015].

The bactericidal effect of the LPO system has found wide applications in the treatment of enteritis in calves and mastitis in cows and also in milk preservation, especially in the regions with warm weather conditions, as well as an additive to toothpaste. LPO is also used for preservation of a wide variety of food products [Severin, 2015; Flemmig et al., 2016; Costa et al., 2021].

The lactoperoxidase system is involved in the breakdown of many carcinogens and has been found protective against toxic effects of hydrogen peroxide. The suppressive activity of this protein against melanoma cells of the B-16 lineage has been demonstrated in a mouse model [Seifu et al., 2005]. Besides, it has a synergistic effect with other bioactive milk proteins, e.g. lactoferrin.

Lysozyme is a bactericidal hydrolase which lyses mainly Gram-positive bacteria by the hydrolysis of β 1-4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of their cell wall. Two of its amino-acid residues are involved in the process of catalysis, i.e. glutamic and aspartic acids. As has been shown, lysozyme can also affect the cell walls of Gram-negative bacteria, especially in synergy with lactoferrin [Lönnerdal, 2003; Severin & Wenshui, 2005]. *Micrococcus lysodeikticus* bacteria, are particularly sensitive to the lytic activity of the lysozyme. Antimicrobial properties of the lysozyme enable its use as a natural antibiotic for food and fodder preservation, as well as in medical treatments of various infections or in antibiotic-supporting therapy.

Lysozyme is also widely used in the treatment of viral and bacterial infections, treatment of skin and eye diseases, gingivitis, leukemia and many cancers [Benkerroum, 2008; Zimecki & Artym, 2005]. It is an element of innate immunity, e.g. in saliva, and it protects oral cavity against the development of pathogens. It also shows anti-HIV activity, reducing the frequency of replication of this virus. It is a thermostable protein, in heat treated milk at 75°C for 15 minutes or at 80°C for 15 seconds, it retains 75% of its initial activity [Zagorska & Ciprovica, 2012].

Osteopontin (OPN) is another protein present in whey, but in smaller quantities [Schack et al. 2009]. On average, its concentration in cow's milk is 18 mg/L, which is much lower than in breast milk (138 mg/L). Bovine and human OPN exhibit a 61% sequence homology, however, these proteins differ in the chain length with 262 amino acids in bovine OPN compared to 298 residues in human OPN [Demmelmair et al., 2017]. OPN is also found in numerous tissues and body fluids. It is an acidic, highly phosphorylated glycoprotein with an isoelectric point at pH 3.5 and MW ranges from 45 to 74 kDa, depending on the degree of phosphorylation and glycosylation [Denhardt & Noda, 1998]. It contains 27 phosphoserine residues and one phosphotreonine, which allows it to bind calcium, the affinity to which is extremely high (Kd = ~ 3–5 × 10−8 M). Its structure includes the Arg-Gly-Asp integrin binding sequence, which mediates binding to many different integrins. OPN is a multifunctional protein exhibiting a number of biological activities, including immunomodulatory, antiinflammatory, antitumor and antibacterial activities. Due to its ability to bind calcium it has important function in the process of biomineralization~~,~~ which is particularly important for bone growth and wound healing [Schack et al., 2009; Icer & Gezmen-Karadag, 2018; Liu et al., 2020].

Bovine OPN, similarly to human OPN, exhibits a strong affinity to positively charged LF, forming complexes of increased bioactivity [Jiang et al., 2020; Jiang et al., 2020]. OPN is obtained from GMP-free acid whey. It is used mainly for the production of humanized food, in which its concentration is increased to the level of human milk (i.e. 2.1% in relation to all proteins) and in clinical nutrition. It is also used as a dietary supplement for adults [Jang & Lorendal, 2016]. Today recombinant OPN is available commercially [Jiang et al., 2021].

3. Conclusions

Whey contains a wide array of proteins of high nutritional value, beneficial to human health, which improve metabolic processes via direct mechanisms or indirectly. For this reason, these proteins and products of their modifications, especially biologically active peptides, are very attractive nutraceuticals for food and pharmaceutical applications.

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