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Physiological significance, structure and isolation of α-lactalbumin

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Abstract

Along with the constant increase in the cheese milk production, the world whey production is increasing constantly too (>2 % per year). The excellent nutritional properties attributed to whey are mainly conditioned by the presence of highly valuable proteins with wide range of biological and functional properties. The main whey proteins are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) which are extensively used in functional foods and beverages, infant formulas, sport diets, but are a very good source of bioactive peptides too. Along with casein, β-Lg is most commonly made responsible for causing food allergies, especially in infants whose digestion system isn't completely developed. Hence, there is a great interest for removing β-Lg prior to whey utilization in certain products. At the same time α-La was recognized as the nutritionally most valuable protein and might be regarded as an ideal ingredient for infant formulas. Thus, the aim of the present paper was to give an overview of the currently available methods for α-La isolation, and to highlight their advantages and disadvantages as well. Also, this paper reviews the most recent insights related to the structure and physiological significance of α-La.

Key words: α-lactalbumin, molecular structure, isolation, physiological significance

Introduction

α-lactalbumin (α-La) is a relatively small globular protein with a molecular weight of 14.2 kDa in cow's milk, and 14.07 kDa in human milk. α-La present in the mammalian milk usually contains 123 amino acids (Permyakov and Berliner, 2000) with theamino acid sequence of α-La being very similar to that of lysozyme (Lönnerdal and Lien, 2003) which is often referred to as a "natural antibiotic". Structure of α-La is variable and greatly depends on environmental conditions, but the so called *holo form* is most often found in milk. *Holo* α-La is characterized by the presence of calcium and plays an important role in the lactose synthesis occurring in milk gland cells. Under certain environmental conditions α-La takes the so called "molten globule state", which is of great importance in proteolysis (Edwards et al., 2009).

Considering the average contents, α-La makes 20-25 % of the total whey proteins, while the amounts found in human milk are much higher and usually come to 75 % (Kamau et al., 2010). However, the amino acid sequences of human and bovine α-La are very similar and overlap in 72 %. Hence, the bovine α-La might be an excellent substitute for human α-La in infant nutrition. Differences in amino acid composition in bovine and human α-La are presented in Table 1. Bovine α-La contains higher amounts of histidine, tryptophan and valine, while

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human α-La contains higher amounts of isoleucine, leucine and methionine. The amounts of the remaining essential amino acids are equal in both α-Las. Considering the non-essential amino acids, bovine α-La contains higher amounts of asparagine and aspartic acid while human α-La contains higher amounts of alanine, glutamine, serine and glutamic acid. The rest of the non-essential amino acids are present in equal amounts.

Generally, α-La is a rich source of essential amino acids, especially regarding contents of tryptophan, lysine and cysteine. Consequently, food industry has shown an increasing great interest for optimising isolation methods of α-La and developing novel intended for use by target consumers groups such as population suffering from different health disorders or infants.

The physiological significance of α-lactalbumin

Lactose synthesis

Both, cow's and human milk, contain milk sugar - lactose, which is a disaccharide synthesized within the mammalian milk glands where α-La serves as a regulatory unit of the enzyme galactosyltransferase which is responsible for the lactose synthesis from galactose and glucose. More precisely, after α-La has attached to galactosyltransferase, the conversion of galactose to N-acetylglucosamine is enabled and followed by the synthesis of lactose from the uridyldiphosphate-galactose and glucose (Kamau et al., 2010). This reaction occurs in the Golgi apparatus and requires the presence of Mn^{2+} (Lönnerdal and Lien, 2003). Galactosyltransferase can also facilitate synthesis of other disaccharides, but with α-La as a regulatory unit, only lactose is being produced.

Table 1. Differences in the amino acid composition between the bovine and the human α-La (Lönnerdal and Lien, 2003).

Amino acid	Cow's α-La (%)	Human α-La (%)
Essential:		
Arginine	1.1	1.1
Cysteine	5.8	5.8
Histidine	2.9	2.0
Isoleucine	6.4	9.7
Leucine	10.4	11.3
Lysine	10.9	10.9
Methionine	0.9	1.9
Phenylalanine	4.2	4.2
Threonine	5.0	5.0
Tryptophan	5.3	4.0
Tyrosine	4.6	4.6
Valine	4.2	1.4
Non-essential:		
Alanine	1.5	2.5
Asparagine	6.4	3.2
Glutamine	5.4	6.4
Glycine	2.4	2.4
Proline	1.4	1.4
Serine	4.3	5.0
Aspartic acid	10.6	9.8
Glutamic acid	6.4	7.4
Total:	100	100

Anticancer activity

Recently, a new genetic variant of the human/cow's α-La was discovered. The so called HAMLET/BAMLET refers to a protein-lipid complex and contains *apo* forms of the α-La "molten globule state" stabilized by a fatty acid. According to the current knowledge, the interaction between α-La and fatty acid is stereospecific and only cis-unsaturated fatty acids (like oleic acid) bind with α-La (Chatterton et al., 2006). Several in vitro studies suggested HAMLET/BAMLET to act lethal on tumour cells (Fast et al., 2005). More precisely, when α-La is attached to a particular fatty acid, it becomes capable of entering tumour cells and causing apoptosis (cell self-destruction). Thereat it most probably binds the histones and interferes the organization of chromatin in the cell nucleus (Chatterton et al., 2006). Thus, the future application of HAMLET/BAMLET could be related to treatments of malignant cancer cells. However, it hasn't been established yet whether these complexes (HAMLET/BAMLET) were naturally present in milk or arise during digestion of dairy products in humans.

Antimicrobial activity

It was also suggested that HAMLET complex could exert antimicrobial activity, particularly against strains such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. Clinical studies in α-La enriched infant formulas with showed activity against *E. coli* strain O127 and reduced the incidence of diarrhoea when compared to human milk (Bruck et al., 2003). Such findings were most probably related to the formation of α-La derived bioactive peptides with antimicrobial effects. As Chatterton et al. (2006) stated, hydrolysis of α-La by chymotrypsin generated one (f61-68 bound by a disulfide bond with f75-80), while trypsin hydrolysis generated two (f1-5 and F17-31 bound by a disulfide bond with f109-114) bioactive peptides with antibacterial activity against Gram-positive bacteria.

Influence of α-lactalbumin to stress

Tryptophan derived from α-La hydrolysis very often served as a precursor in the synthesis of serotonin and might therefore be related to stress reduction. Markus et al. (2000) investigated the influence of α-La at stress symptoms in adults. Respondents who were suffering from various forms of stress, were divided into two groups. The control group received casein, and the test group received α-La. According to the obtained results, the level of stress decreased for approximately 48 % in the test group. Furthermore, some studies focused on individuals susceptible to stress also indicated that supplementation with α-La might favourably affected the sleeping behaviour. Results of several studies performed on animals suggested that α-La might act protective in gastric disturbances caused by alcohol and/or stress, such as stomach ulcers (Markus et al., 2000).

Immunoactive properties

Immunoactive α-La peptides released during the digestion process should theoretically influence the immune response and cellular function. However, the liberated amounts are too low to affect the immune system, especially when therapeutic effects are expected. Numerous studies focused on immunoactive properties of proteins and α-La derived peptides or enzyme hydrolysates indicated specific immune functions such as lymphocyte activation and proliferation, cytokine secretion and antibody production (Gauthier et al., 2006). "In vivo" studies conducted on rats proposed both forms of α-La, native or hydrolysed, to enhance the antibody response after systematic stimulation (Bounous and Kongshavn, 1982). More precisely, the hydrolysed α-La was suggested to directly affect the function of B-lymphocytes, and also suppressed responses of T-dependent or independent cells. Jaziri et al. (1992) identified the peptide with immunoactive properties as tripeptide Gly-Leu-Phe originating from trypsin or chymotrypsin hydrolysis of α-La. That specific peptide was found to also stimulate macrophages and consequently to promote phagocytosis.

Antiviral activity

It has been shown that α-La derived peptides formed during hydrolysis by trypsin, chymotrypsin or pepsin, might possess specific activity against herpes disease. Trypsin or chymotrypsin hydrolysis was performed at 37 °C, pH 7.0, with the ratio of enzyme/substrate 10 %, while the pepsin hydrolysis was performed at pH value 2.0. The obtained peptides were fractionated using high performance liquid

chromatography with reversed phase (RP-HPLC), and were further modified with 3-hydroxyphthalic anhydride (3-HP). In vitro studies have demonstrated that α-La derived peptides modified with 3-HP acted antiviral against herpes simplex virus type 1 and human immunodeficiency virus type 1 (HIV-1) as well (Berkhout et al., 1997; Oevermann et al., 2003). Hence, α-La derived peptides could be a promising alternative for commonly applied antiviral drugs, since most of those are accompanied by certain side effects.

The molecular structure of α-lactalbumin

Native form of α-La has a globular structure stabilized by four disulfide bonds (^{6}Cys-Cys^{120}, ^{60}Cys-Cys^{77}, ^{90}Cys-Cys^{73}, ^{28}Cys-Cys^{111}) (Figure 1). Tertiary structure of the protein is composed of two domains: α-domain (large domain) and β-domain (small domain) (Figure 1). α-domain (amino acids (AA) 1-34 and from 86 to 123) contains four α-helixes, of which three are pH stable: helix H1 (5-11 AA) helix H2 (23-34 AA) and helix H3 (86-98 AA), and one is pH dependent: helix H4 (105-110 AA). α-domain also contains two shorter helixes h1 (18-20 AA) and h3 (115-118 AA). The flexible spot of the protein is placed between amino acids 105 and 110 where it takes helical conformation at pH values between 6.5 and 8.0 (Pike et al., 1996). β-domain is composed of three antiparallel β-pleated sheets: plate S1 (41-44 AA), plate S2 (47-50 AA) and plate S3 (55-56 AA) and one short α-helix h2 (77-80 AA).

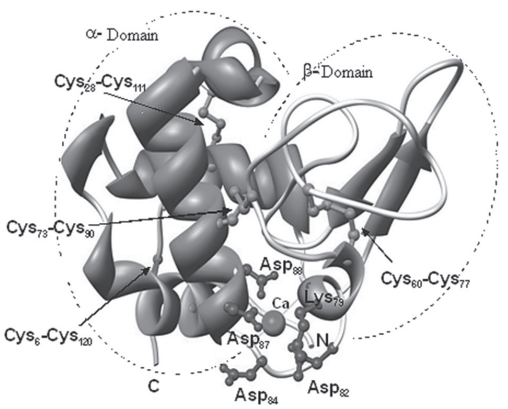

Figure 1. The model of the α-lactalbumin structure (Kulozik, 2011)

Physical characteristic of α-lactalbumin

Solubility

The isoelectric point of α-La is between pH 4.2 and 4.6 (Kamau et al., 2010). α-La's solubility depends on temperature, pH and protein concentration. α-La is soluble in water and saline chloride solutions, while the maximum solubility is reached at temperatures up to 50 °C and pH values between 3.0 and 9.0. A certain turbidity of α-La solutions were observed already at 55 °C, whilst the greatest tendency for aggregates formation was recorded at temperatures higher than 50 °C and pH values close to the isoelectric point i.e. between 4.2 and 4.6. The tendency to form aggregates increases in proportion to the protein concentration of the solution (Bramaud et al., 1997).

Ion binding capacity

One of the most interesting properties of α-La is the capability of binding metal ions. Thus, the molecular stability of α-La (tertiary structure) is directly affected by the binding site for calcium ions. Calcium binding site is formed by oxygen ligands of carboxyl group of three aspartic acid residues (Asp^{82}, Asp^{87}, Asp^{88}), two carbonyl bonds between lysine and aspartic acid (Asp^{84} and Lys^{79}) and one or two water molecules (N`Negue et al., 2006, Permyakov and Berliner, 2000). X-ray analysis of the human α-La confirmed the presence of a second binding site for calcium ions which is coordinated by four amino acid residues Thr^{38}, Gln^{39}, Asp^{83} and Leu^{81}. However, the second binding site showed to be less stable, and to be located at the α-La surface, 7.9 Å away from the first binding site of Ca^{2+} (Wu et al., 1996; Lönnerdal and Lien, 2003). α-La has a zinc binding site too, which is in human α-La located between Glu^{49} and Glu^{116} (Permyakov and Berliner, 2000). Binding of Ca^{2+} to the primary binding site can enhance the protein stability, which might be further supported by other metal ions such as Mg^{2+}, Na^{+} and K^{+}. The stronger the interaction between ions and proteins, the higher stability of the protein towards denaturing agents (temperature, pH and pressure) is achieved (Kamau et al., 2010).

The influence of pH and temperature on α-lactalbumin

Under certain conditions like the calcium removal, the disposal to high temperatures, high acidity (pH≤3.0) and/or other denaturing agents, α-La is losing functional properties, it retains the secondary structure while the tertiary becomes unstable (N`Negue et al., 2006). This condition is referred to as the "molten globule" (MG) state. Radius of the tertiary structure of α-La with bounded calcium is 15.7 Å, while the radius of the MG molecule is 17.2 Å. In the MG state α-La retains a globular structure. However, the MG state is often described as "swollen" because approximately 270 water molecules are usually attached to α-La causing the protein size to increase for 5-7 % in comparison to the native molecule (Permyakov and Berliner, 2000). Consequently, the MG state of α-La is more susceptible to proteolysis which can be of great importance for optimizing selective isolation and fractionation by hydrolysis of whey proteins. The differential scan colorimetry (DSC) analysis confirmed α-La to be thermostable in the presence of saturated amounts of calcium ions is whereas the thermal denaturation started at temperatures above 68 °C. In contrast to that, the *apo* form of α-La (without Ca^{2+}) was rather unstable and began to denature already at 43 °C (Chatterton et al., 2006; Edwards et al., 2009). Thus, the capacity to bind calcium is the most important property concerning the structure and the stability of α-La.

Isolation of α-lactalbumin

Numerous methods have been established for purposes of isolating pure α-La from substrates such as whey protein concentrate (WPC), whey protein isolate (WPI), whey hydrolyzates (HS), liquid whey or milk. The choice of a specific method depends on factors like the amount of present α-La in the substrate, the expected purity and yield, and the intended use as well. Due to complex properties of α-La, a combination of several isolation methods is most commonly applied. However, owing to very high costs many difficulties occur when transfer from the laboratory to the industrial scale is considered. Isolation methods can be divided into several groups: isolation by 1) chromatography and gel filtration, 2) membrane filtration, 3) precipitation and aggregation, and 4) enzymatic hydrolysis. Summary of available methods of isolation of α-La is given Figure 2.

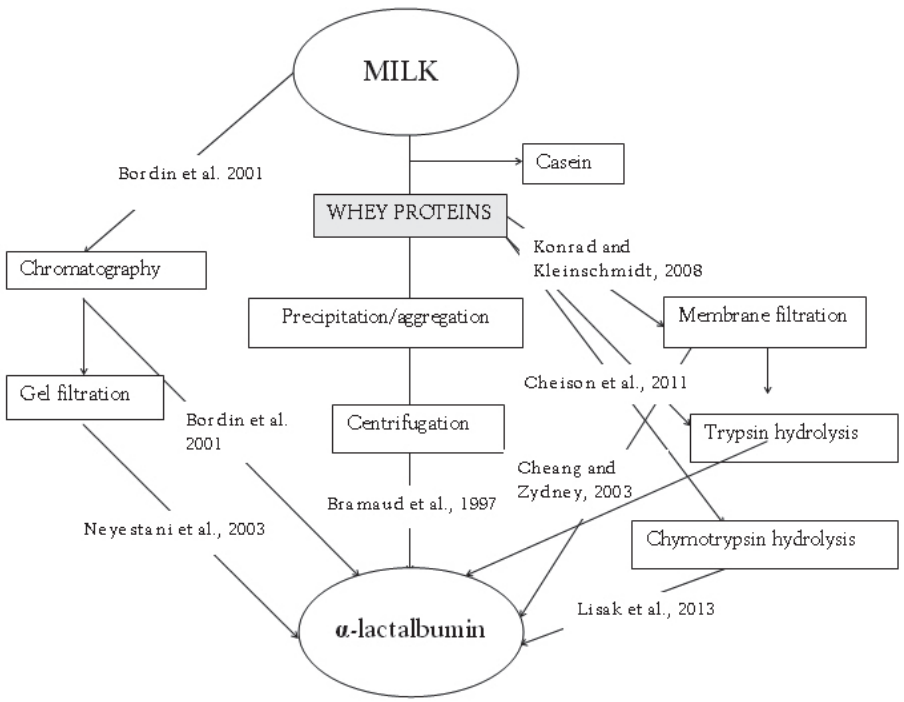

Figure 2. Scheme of the available isolation methods of α-lactalbumin (Lisak, 2013)

Chromatography and gel filtration

Chromatographic isolation method relies on the separation of proteins due to differences in their affinity to the mobile or stationary phase. Gel filtration (type of chromatography) separates proteins due to difference in their sizes by passing through a gel. The proteins do not interact with the used buffer and their biological activity is maintained. Also, prior to isolation, the substrate needs to be prepared depending on its nature i.e. applying operations such as the pH, temperature and/or ionic strength adjustment are required. Neyestani et al. (2003) separated α-La from blood serum albumin by Sephadex G-50 gel filtration based on various physico-chemical properties in a laboratory scale. In the first step of isolation, β-Lg was separated from whey by ion chromatography. The method appeared to be very efficient since the final product was of high purity and with completely preserved bioactivity. Bordin et al. (2001) successfully isolated α-La by chromatographic method from milk without removing caseins and the rest of the whey proteins. The applied method relied on ion chromatography with reversed phase (C_4 column) and photodiode detection. The identification was based on the composition of aromatic amino acid of α-La (phenylalanine, tyrosine and tryptophan) and calculating the area under the peaks at 214 and 280 nm. Many researchers focused on the isolation of α-La by variation of chromatography and gel filtration (Minor et al., 1985; Outinen et al., 1996; Ye et al., 2000; Conrado et al., 2005). Chromatographic methods are usually quite expensive and limited to laboratory scale. There is not chromatographic method that allows 100 % utilization of the substrate, and the final yield depends on the number of steps during the isolation process. By optimizing each step and combining different methods, yield and purity of α-La could be improved without affecting the biological activity.

Membrane filtration

The second group of isolation methods refers to membrane filtration (MF) process which are the most commonly used due to the possibility of industrial scale implementation and lower costs when compared with chromatographic methods. Membrane filtration isolation methods are based on the difference in the molecular weight of the proteins. The final product should be a pure α-La with no or extremely small amount of other whey proteins. Membrane separation process mostly implies removal of β-Lg by microfiltration (MF) or ultrafiltration (UF) with 50 or 100 kDa "cut-off" membranes, while α-La goes into the permeate (Muller et al., 2003; Konrad and Kleinschmidt, 2008; Chatterton et al., 2006). Muller et al. (1999) used a 30 kDa "cut-off" membrane ultrafiltration membranes to isolate α-La from a whey protein concentrate (WPC). The obtained yield of α-La was about 90 %, but the purity was not satisfactory. Despite the pH adjustment of the WPC solution to 7.0 at which β-Lg was assumed to be a dimer (36.6 kDa), the membrane apparently allowed an undisturbed permeation of the α-La (about 14.2 kDa) and β-Lg as well. Also, the authors came to the conclusion that ceramic membranes were more suitable for protein filtration because due to higher resistance to cleaning agents and the reduced fouling as well. To increase the purity of the end product, preliminary steps in sample preparation are necessary. For this purpose, two-stage or multi-stage cascade of membranes with different pore diameters might be used. For instance, Cheang and Zydney (2004) combined a process of ultrafiltration by applying a 100 kDa and 30 kDa "cut-off" membranes. The most significant drawback of membrane processes is related to the decrease of protein transmission (transmission α-La/transmission β-Lg), and consequently a clogging of the membranes, concentration polarization, adsorption of proteins to the membrane and protein-protein interactions.

Precipitation and aggregation

Isolation methods implying precipitation and aggregation are based on precipitation of either α-La or β-Lg aggregates, under certain process parameters. In order to isolate the proteins from whey, a good knowledge of their physical and chemical properties is required. Precipitation can be carried out by heating with the addition of ferrous chloride, or relying on poor solubility of β-Lg at low ionic strength at pH 4.65 or high protein concentration (Tolkach et al., 2005). Selective precipitation of α-La is a base for acquiring other whey protein fractions (Bramaud et al., 1997). Precipitation of α-La usually appears after acidification of whey or WPC by an organic acid (citric or lactic) to pH 4.0 at 50 °C, and with the simultaneous control of calcium concentration. However, there are no conditions which would enable precipitation of α-La solely (Kamau et al., 2010). Most commonly, precipitation is combined with centrifugation, which proved to be more efficient than microfiltration (Eugenia Lucena et al., 2007). Tolkach et al. (2005) optimized the pretreatment for α-La separation from WPC using a

selective denaturation of β-Lg. The best results were achieved under following conditions: 5 to 20 g/L protein, 0.5 g/L of lactose, 0.55 g/L of calcium and pH value of 7.5. The most significant advantage of this method was the high purity of the final product, while α-La retained the native structure, and thus the functional properties. Such isolation methods are promising, yet it is necessary to optimize the conditions (initial amount of protein, pH, time, the number of flushing sediment, temperature, ionic strength, etc.) under which a precipitation of α-La would be achieved. Furthermore, Toro-Sierra et al. (2013) optimized fractionation method for both α-La and β-Lg isolation in the pilot scale. The method comprises in 6 steps: (1) selective thermal precipitation of α-La, (2) aging of the formed particles, (3) separation of native β-Lg from the precipitate via microfiltration and ultrafiltration, (4) purification of β-Lg, (5) resolubilisation of the precipitate, and (6) purification of α-La. Protein fractions with a purity of 91.3 % for α-La and 97.2 % for β-Lg were produced. The method offers potential for pilot plant scale and possibly industrial application to produce pure native fractions of α-La and/or β-Lg.

Enzymatic hydrolysis

Enzymes possess a great potential to enable the production of proteins and bioactive peptides by minimal denaturation and minimal financial costs as well. Schmidt and Poll (1991) reported high selectivity of trypsin towards whey proteins. α-La was resistant to hydrolysis, while β-Lg was almost completely hydrolysed. Konrad and Kleinschmidt (2008) succeeded in isolating α-La of high purity (90-95 %) by applying the combination of membrane filtration and trypsin hydrolysis (42 °C, pH 7.7). However, only 15 % of α-La was isolated from the original substrate. Cheison et al. (2011) also used the selectivity of trypsin in order to isolate native α-La, whereas β-Lg was completely hydrolysed. More precisely, the used substrate was a whey protein isolate, while the hydrolysis was performed at pH 8.5 and 25 °C. The obtained isolate contained approximately 67 % of α-La. According to findings presented in several studies (Schmidt and Poll, 1991; Galvão et al., 2001; Custodio et al., 2005; Konrad and Kleinschmidt, 2008; Cheison et al., 2011) trypsin obviously enabled selective enzymatic hydrolysis of β-Lg while α-La remained more or less in the native form. In our recent research (Lisak et al., 2013) isolation of α-La from WPI by α-chymotrypsin hydrolysis was performed. Our work demonstrates the selective susceptibility of WPI to chymotrypsin hydrolysis. Chymotrypsin shared the same hydrolysis properties with trypsin considering the way it attacked the whey proteins. Higher α-La resistance to chymotrypsin was detected at 25 °C regardless of the pH, whereas lower resistance was detected at 50 °C. At lower substrate concentration (5 % WPI) the recovery of α-La was better in comparison to higher substrate concentration (10 % WPI). The residual β-Lg was negligible in both cases. The highest possible recovery of native α-La (81 %) was achieved at 25 °C, pH 8.5, 1 % E/S ratio, 5 % WPI (w/v) when chymotrypsin was inhibited by the Bowman-Birk inhibitor. The Bowman-Birk inhibitor offered a potential to avoid the heat-denaturation and further losses due to continued hydrolysis during heat-denaturing (Lisak et al., 2013). Enzymatic hydrolysis is usually combined with membrane filtration, which can result in high purity of isolated proteins. The advantage of this method is the low cost, especially when enzymes of microbial origin are used, and a simple conversion to the industrial scale, as well.

Conclusions

Requirements of the food industry to develop new and improved products with preserved nutritional and functional properties are increasing. Specifically, the average consumer becomes more aware and requires foods with positive health effects. Whey proteins are widely used in food production for special purposes, such as the production of infant and enteral nutrition, nutrition for population suffering from allergies, etc. They are also added to various food products due to excellent functional properties such as emulsification, gelling, water binding capacity, the ability to create foam, good solubility and an increase in viscosity of the product. Addition of proteins reduces the need for additives whose quantities in human nutrition are limited. Whey proteins can be added to the products in the various forms like concentrates, isolates, hydrolysates, or as individual protein fractions for which is the food industry the most interested. The main disadvantages of separation methods and fractionation of α-La are related to a very low degree of purification and yield, significant denaturation of proteins and very high production costs when conversion into the industrial scale is considered. Choice of the isolation and fractionation method of α-La from milk or whey is essential and critical step in preserving the biologically active properties of proteins. Recent studies have shown

that the enzymes might have a great potential in the production of α-La and bioactive peptides, which caused minimal denaturation where costs are significantly reduced. The proper selection of enzyme and hydrolysis conditions can affect the selectivity and kinetics of enzyme reactions and thus isolating of each fraction of whey protein can be improved.

Fiziološki značaj, struktura i metode izolacije α-laktalbumina

Sažetak

Količina proizvedene sirutke na svjetskoj razini (>2 % godišnje) neprestano raste, s obzirom da neprekidno raste i svjetska proizvodnja sira. Visoka nutritivna vrijednost sirutke zasniva se prije svega na prisustvu visokovrijednih proteina sirutke koji se odlikuju brojnim poželjnim biološkim i funkcionalnim svojstvima. Najzastupljeniji proteini sirutke su β-laktoglobulin (β-Lg) i α-laktalbumin (α-La) koji se naširoko upotrebljavaju u proizvodnji funkcionalne hrane i napitaka, te dojenačkim formulama i sportskoj prehrani, a također su i dobar izvor bioaktivnih peptida. Uz kazein, β-laktoglobulin najčešći je uzročnik alergijskih reakcija na hranu, pogotovo u dojenčadi čiji probavni sustav nije do kraja razvijen. Stoga je od velikog značaja selektivno ukloniti frakciju β-laktoglobulina iz sirutke prije njezine daljnje prerade u postupku proizvodnje određenih prehrambenih proizvoda. Suprotno tomu, α-laktalbumin se smatra nutritivno najvrjednijim proteinom zbog čega je idealan sastojak u proizvodnji formula za dojenčad. Stoga je cilj ovog rada pružiti pregled trenutno dostupnih metoda za izolaciju α-La, uz poseban naglasak na njihove prednosti i nedostatke. Osim toga, ovaj rad pruža pregled najnovijih saznanja vezanih uz strukturu i fiziološki značaj α-La.

Ključne riječi: α-laktalbumin, molekularna struktura, izolacija, fiziološki značaj

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