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REVIEW PAPER

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THE INFLUENCE OF HIGH TEMPERATURES ON MILK PROTEINS

High temperatures induce certain changes in milk constituents, but the degree of these changes depends on both the temperature and time of heat treatment. The most pronounced changes take place in milk proteins.
The forewarming of milk causes an increase in acidity, the precipitation of soluble Ca–phosphate, whey protein denaturation and coagulation, as well as the interaction with casein micelles, the Maillard browning reaction, the dephosphorylation of casein, the hydrolysis of casein micelles, changes in whey proteins, an extension of the rennet coagulation time and an exchange of the rheological properties of the acid and rennet casein gels, changes in the zeta–potential and casein micelle hydration, the interaction between the milk proteins and proteins of milk fat globule membrane.

Heat treatments of milk are commonly used for thermisation, pasteurisation, sterilisation, in the production of fermented milk products, condensed sweetened and condensed milk, concentrated and dried milk products.

High temperatures induce certain changes in milk constituents, but the degree of these changes depends on both the temperature and time of heat treatment. The most pronounced changes occur in proteins, especially whey proteins. Consequently, the technological properties of milk are altered, as well as the milk nutritive value when severe heat treatments are used.

The forewarming of milk causes an increase in acidity and a decrease of the pH [1–8]; the precipitation of soluble Ca–phosphate [1, 2, 9, 10]; whey protein denaturation and interaction with casein micelles [5–8, 11–17]; the Maillard browning reaction [3, 18, 19, 20]; the dephosphorylation of casein [2, 21, 22]; the hydrolysis of κ–casein [23–28]; the aggregation and dissociation of casein micelles [29–32]; changes in whey proteins [33–41]; extension of the rennet coagulation time and an exchange of the rheological properties of the acid and rennet casein gels [6, 42–59]; changes in the zeta–potential and the hydration of casein micelles [60–65]; the interaction between milk proteins and the proteins of the milk fat globule membrane [7, 66–69].

The functional properties of milk proteins are very important, because they determine the structural and textural characteristics of milk products. Also, milk proteins represent 20–30% of the proteins in the human diet [70].

According to Rowland, casein represents 74.68% of the total milk proteins whey proteins 17.73%, proteose–peptone 3.31%, while non–protein represents 7.53% [39].

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THE INFLUENCE OF HIGH TEMPERATURES ON CASEIN

Casein is the major protein component of milk, which represents 75–80% of the total milk proteins [31, 71–75]. Due to its complex composition, casein belongs to the group of phospho–glycoproteins [31, 76]. Casein is composed of the following elements: C (52.96%), H (7.13%), O (22.47%), N (15.60%), P (0.86%) and S (0.78%) [31, 77]. Casein is a polyvalent ampholyte because it behaves as both a base and acid under different environmental conditions, so casein may form salts with both bases and acids. It is known that about 20 amino–acids come into the composition of casein. About 25.5% of the amino–acids present in casein are monoamino–dicarboxylic, while diamino–monocarboxylic amino–acids represent 11.7%. The ratio of free carboxylic and amino groups is 144:83, so casein has pronounced acidic properties and a negative net charge [31, 71, 74, 77]. The primary structures of the casein micelle are represented by the primary structures of four electrophoretic fractions, α_{s1}–, α_{s2}–, β– and κ–casein, as well as by several minor proteins (γ–, R–, S– and TS–casein) [73, 75, 78–82]. According to Walstra [83] the molar ratio of the electrophoretic fractions present in the casein micelle is about α_{s1} : α_{s2}: (β+γ): κ = 4:1:4:1.3.

The structure of the casein micelle arises as a result of the interactions between the casein electrophoretic fractions, which differ from one another by their amino–acid composition and degree of reactivity. All the casein fractions are phosphorylated to varying degrees, which influences micelle formation and stability. The fractions α_s and β–casein are sensitive to the presence of Ca^{2+} due to the higher content of phosphoseryl residues, while κ–casein does not coagulate in the presence of Ca^{2+} because, beside other stabilizing factors, it has only one phosphoseryl residue [80, 82].

The stability of the casein micelle is described by the presence of κ–casein on the surface of the micelle,

where it forms a boundary layer between the hydrophobic micelle core and the sera phase surrounding the micelle [31, 71, 78, 79, 84–86].

The structure of the casein micelle is not yet fully described. According to the *coat–core model*, the core of the micelle is composed of proteins that differ from those on the surface. According to Heertje et al. [87], the bearing framework of the casein micelle is composed of α_s–casein, while β–casein and κ–casein are reversibly bound to the framework. Rollema and Brinkhuis [88] discussed both the framework and submicelle model. According to the *submicelle model*, the casein micelle is defined as an aggregate of smaller particles, sub–micelles, linked to one another by calcium–phosphate. It is assumed that the submicelle has a hydrophobic core and a hydrophilic surface [71, 74, 78, 83, 89, 90]. The *Internal structure model* is described by specific interactions between caseins and considers micelles as a porous protein network [91–93].

In the submicelle model, the bonds between casein and colloidal calcium phosphate (CCP) are electrostatic, because of the positive charge of CCP and the negative net charge of casein [31, 79, 90, 94]. A casein micelle, with an average particle weight of 10^{-8} g consists of 93.3% casein and 0.83% ester–bound phosphorus. Investigations showed the presence of 25000 ester phosphate groups. It was detected that the casein micelle possesses about 70600 Ca atoms and 30100 inorganic phosphate residues, which could form 5000 CCP clusters, existing as $Ca_9(PO_4)_6$. That means that about 25500 Ca atoms remain free and could be linked to ester phosphate groups, while $Ca_9(PO_4)_6$ clusters are linked to 40% of the ester phosphate groups [79].

It could be concluded that CCP forms a complex ion with calcium that is linked with organic phosphate or simply forms complex ion with calcium from calcium–caseinate.

There are different values for the casein micelle diameter available in the literature: 20–600 nm [79, 82], 30–300 nm [31] and 40–300 nm [83]. According to Morr [95], casein subunits (fractions) polymerize during the synthesis of milk due to the influence of Ca^{2+} and form submicelles with an average diameter of 10–20 nm. Thus formed submicelles could further associate by CCP to form porous and hydrated micelles with a diameter of 100–300 nm. The molecular weights of subunits ranged from 19000 to 25000 [95], while Dalgleish [85] assumed an interval of 19000–23000. Numerous electron–microscopy studies have reviewed the heterogeneity of both natural and artificial casein micelles, because they are composed from several hundred and even several thousand subunits of the average diameters 10–15 nm [90]. Ono et al. [96, 97] proposed submicelles as spheres with a diameter of 10–15 nm, containing about 15–25 casein molecules, while submicelles contain 22 casein molecules according to Walstra [98].

Casein belongs to the group of heat–stable proteins, because it does not coagulate when subjected to high heat treatment. The large number of prolyl residues situated along the polypeptide chains of caseins influence the so–called "random" conformation or unordered structure. The lack of secondary and tertiary structure makes casein heat–stable, but in the literature casein is designated as a naturally denatured protein [80]. This has practical importance, because raw milk with normal physico–chemical and technological properties has remarkable heat–stability with regard to the heat–treatment regularly applied in the dairy industry. This is confirmed by investigations which have shown that casein in milk coagulates after 12 hours at 100°C, after 60 min at 130°C and after 3 min at 150°C [1, 31, 92]. Fox and Morrissey [2] found that Na caseinate in water at pH 6.7 withstands heat treatment at 140°C over 60 min before coagulation, while milk coagulates in 20 min at the same temperature.

Particular hydrolytic transformations occur when casein is subjected to the influence of high temperature for a longer time [1, 2, 49]. About 10–20% of the total nitrogen is exchanged into non–protein nitrogen (NPN) [1] after 5 hours at 120°C or after 60 min at 135°C.

When a solution of κ–casein or whole casein was heated at 120°C for 20 min, peptides of similar amino–acid composition to those released by the action of chymosine were released [1, 49, 99].

Hindle and Wheelock [49] found that the release of peptides and glycopeptides soluble in 12% trichloracetic acid (TCA) occurs when milk is subjected to high heat treatment. The amounts of these peptides increases with temperature and duration of heat treatment. They also noticed the existence of N–acetyl neuraminic acid (NANA), D–galactose, 2–acetamido–2–deoxy–D––galactose in the peptides released by heat, but in smaller amounts tham those released by the action of rennin. The presence of D–mannose was also noticed. According to the aforementioned they suggested that peptides and glycopeptides released by heat differ from those released by the action of rennin.

This is a way of explaining the loss of the stabilizing ability of κ–casein during heat–treatment. Loss of the stabilizing ability of κ–casein could be simply explained by the decomposition of N–acetyl neuraminic acid [100–103].

Fox and Morrissey [2] concluded that almost 30% of sialic acid is evolved from casein at 140°C. The amount of evolved acid and the 12% TCA–soluble sialic acid linearly increase up to 90% during of heat treatment. At the point of heat–induced coagulation 20–30% of κ–casein is degraded, which indicates that as a critical point [1].

The dephosphorylation of casein and the formation of smaller polypeptide chains occur when milk

is subjected to heat treatment at 120°C for 5–6 hours [1, 31, 49, 101, 103]. The degree of dephosphorylation of Ca–caseinate at 139°C in 5 hours is about 80% [1]. The extent of Na caseinate dephosphorylation is greater at 120°C after 5 hours Na caseinate undergoes 100% dephosporylation and 50% after 1 hour [2]. According to Fox [1], dephosporylation of casein is slower in milk (12% in 90 min) than in Na caseinate (18% in 30 min) at 120°C. Casein is completely dephosporylated in 1 hour at 135°C [99]. Dephosporylated casein is less heat–stable than natural casein and could bind less Ca^{2+}. The acidity of milk increases during heat–treatment and about 30% of the developed acidity originates from the dephosphorylation of casein [1, 2]. Fast heat–induced coagulation occurs when 70% of the casein is dephosphorylated [2].

Colloidal calcium phosphate (CCP) greatly influences the heat stability of casein micelles. The solubility of CCP decreases with increasing temperature and pH. The sharp decrease in the solubility of CCP at 139°C and pH 6.8 coincides with the minimum in the HCT/pH curve (HCT/pH – heat coagulation time at a certain temperature as influenced by the pH). It has been suggested that CCP precipitates during heating probably as a hydroxy–apatite $Ca_{10}(PO_4)_6(OH)_2$, which differs from natural CCP which is probably a Ca–phosphate/Ca–citrate complex of the apatite type. CCP precipitated by heat is protected against sedimentation in milk most likely by association with casein micelles through links with carboxylic groups. Precipitated CCP forms a surface layer on the casein micelles, which influences the reduction of the zeta–potential and the hydration of casein micelles [1].

The investigations of Andrews and Cheeseman–a [104] showed that the ability of κ–casein to stabilize a casein micelle was damaged when a casein solution was heated at 100°C for 6 hours. Under these conditions a mixture of α_{s1}– and κ–casein coagulates in the presence of calcium ions.

The negative net charge on the surface of the micelle influences (in part) the casein micelle stability. The net charge of the casein micelle surface (indicated as the zeta–potential) increases with increasing temperature up to 50°C, so any destabilization of the casein micelle in this temperature range could be attributed to other factors. When the temperature increases, the electroforetic mobility, as well as the zeta–potential, increase [60].

Factors that influence the value of the zeta–potential and degree of micelle hydration include κ–casein glycoprotein domains and charged areas of α_s– and β–casein. Different factors, such as the hydrolysis of κ–casein, dephosphorylation and a pH drop influenced by the changes in lactose and the precipitation of CCP, influence the reduction of the zeta–potential and hydration, as well as destabilization of the system and the rate of coagulation of casein [2].

However, the most pronounced changes during heat treatment occur in whey proteins, which are thermo–labile and could be easily denatured and precipitated during heat treatment, due to their chemical character.

THE INFLUENCE OF HIGH TEMPERATURE ON WHEY PROTEINS

Serum proteins or whey proteins represent about 18–20% of the total milk proteins. Whey proteins are compact globular proteins, which differ from one another as a result of different amino–acid composition [31, 95, 105–107]. The major whey proteins are β–lactoglobulin (β–lg), α–lactalbumin (α–la), bovine serum albumin (BSA) and immunoglobulins (Ig). Beside these proteins, other proteins such as lactoferrin, lactollin, glycoprotein, blood transferrin and the proteoso–peptone fraction are present in milk sera [31, 74, 75, 80].

β–Lactoglobulin and α–lactalbumin are the most important whey proteins due to their high content in the total whey proteins and significance for the food industry.

Whey proteins do not coagulate by chymosine or other proteolitic enzymes, so they remain in whey during cheese production.

Whey proteins are markedly more hydrated than casein. About 30% of the total solvent water is bound to whey proteins, while casein binds about 50% [31]. These facts, as well as the fact that milk has 5–5.2–fold less whey proteins than casein, indicate how much more are whey proteins hydrated. Contrary to casein, whey proteins do not precipitate at their isoelectric point. Even at this condition there is a large amount of present solvent water, which keeps a sufficient intensity of repulsive forces preventing the aggregation and precipitation of whey proteins.

Whey proteins belong to the group of thermo–labile proteins, which irreversibly denature and coagulate when are exposed to high temperatures. Numerous authors have investigated the influence of high temperature on whey proteins in milk [39, 40, 108–110], in whey [34, 41, 105, 111–116], in whey–based products [117, 118] and in model systems [119–122, 142]. Contrary to casein, whey proteins are fully denatured in 5 min at 90°C. The solubility of whey proteins is reduced at the isoelectric point [1, 108].

According to de Wit and Klarenbeek [105] middle temperatures, up to 60°C, induce reversible changes in the structure of whey proteins, which are result of hydrophobic interactions. The intensity of hydrophobic interactions increases with increasing temperature to 60°C and decreases by decreasing the temperature. These reactions could be intra– and/or intermolecular. Reversible changes in the whey protein structure influence the association or dissociation of some whey proteins. These changes are often explained by some

kind of "pre–denaturation" influenced by the partial loss of three–dimensional structure and the exchange of hydration.

Irreversible changes in whey protein structure occur at the denaturation temperature and above, but are additionally influenced by environmental conditions, such as pH, ionic strength and protein concentration. Reduced protein solubility is the most important consequence of irreversible changes [105]. The heat denaturation of whey proteins is a two–step process. The first phase includes denaturation as a result of breaking hydrogen, hydrophobic and other non–covalent links in polypeptide chains. In the second phase, subsequent to the first, proteins aggregate and form precipitate or gel, as influenced by pH, ionic strength and protein concentration [105, 106, 123, 124].

The extent of whey protein denaturation increases in the temperature range between 60 and 90°C. Only 3% of the total whey proteins denature in 10 min at 60°C, 15% at 70°C, 66% at 80°C and 85% at 90°C [125]. Larson and Rolleri [39] investigated (by electrophoresis) the heat sensitivity of whey proteins and concluded that almost 29% of the whey proteins denature in 30 min at 70°C. Under these conditions 89% of the immuno-globulins, 52% of BSA, 32% of β–lactoglobulin and only 6% of α–lactalbumin are denatured. Ghosh et al. [46] assumed that in 2, 3, 5 and 9 min at 80°C, the degree of denaturation of whey proteins in milk was 20%, 30%, 50% and 80%, respectively. The heat–treatment of milk at 90°C/6 min induces a greater extent of denaturation, which for β–lg accounts for more than 99%.

According to Donovan and Mullvihill [125] the heat–stability of whey proteins decreases in the following order: PP > α–la > β–lg > BSA > Ig. Lyster [40] investigated the denaturation kinetics of whey proteins and assumed that the denaturation of α–la is a first order reaction. On the other hand, the denaturation of β–lactoglobulin in milk is a second order reaction. However, some authors have suggested that the kinetics of α–la denaturation is more complex and exhibits pseudo first order kinetics [33, 109].

The degree of whey protein denaturation is often determined by the degree of β–lg denaturation, since it represents more than 50% of the total whey proteins [31, 95, 106, 126].

THE INFLUENCE OF HIGH TEMPERATURE ON B–LACTOGLOBULIN

β–Lactoglobulin is the most important whey protein. Native β–lactoglobulin is a globular protein with a monomer molecular weight of 18300 [127]. It contains two disulphide bridges and one free sulfohydryl (thiol or –SH) group at the position 121 [128, 129], the activity of which occurs above pH 7.0 [75, 105, 106, 127, 130].

β–Lactoglobulin may associate and form aggregates of different size depending on the pH and temperature of the solution [71, 80, 127, 130, 131].

β–Lactoglobulin occurs as a dimer between pH 5.2 (isoelectric point) and 7.5 [127] and at room temperature and a pH between 5.0–7.0 [105]. According to Farell and Thompson [71], β–lactoglobulin occurs as a dimer with a molecular weight of 36000 (36700 according to Swaisgood [80]) between pH 3.5 and 7.5. β–lg occurs in milk as six genetic variants [A, B, A_{DR}, B_{DR}, C and D) [72, 106], of which variants A and B are the most represented. On the other hand Fox [132] reported five genetic variants of β–lg (A, B, C, D and D_r).

β–lactoglobulin dissociates into monomers from the dimeric form between 30 and 55°C [127]. In highly acidic conditions, at pH below 3.5, β–lactoglobulin dissociates from dimer to monomer due to strong electrostatic repulsive forces [74, 80, 127, 133]. According to Farell and Thompson [71], β–lactoglobulin exists as a monomer only at pH below 3.5 and above 7.5. β–Lactoglobulin, between pH 3.5 and 5.2, reversibly forms tetramer/octamer associates in which hydrophobic interactions dominate [80, 127]. According to Boye et al. [133] in the pH range 3.7–6.5, β–lg reversibly associates and forms octamers. β–lg does not denature in acid conditions [127]. It is resistant to denaturation even at pH 2.0 [134]. The polypeptide chains of β–lg unfold during denaturation, the globular structure is loosened, which results in the exposure of buried thiol groups and their increased activity [95, 127, 135].

The denaturation of β–lg during heat treatment undergoes through two distinct groups of reactions, marked as type I and type II, which induce the formation of small and large aggregates with the sedimentation coefficients 3.75S and 29S, respectively [106]. Sawyer [121] defined these phases as the primary and secondary phase of denaturation.

In the first phase, small aggregates are formed from four monomers by intermolecular disulphide bridges, the oxidation of thiol groups or by thiol–disulphide interchange [121]. The primary phase (type I reaction) begins at 65°C and achieves maximum in the temperature region between 75°C and 85°C [106, 136, 137].

Small aggregates may react with one another to form larger aggregates (type II reactions). According to Sawyer [121] this type of aggregation is designated as non–specific due to the absence of thiol group reactions and becouse it occurs at a lower temperature than primary aggregation. On the other hand Elfagm and Wheloock [136] suggested that the formation of larger aggregates occurs at higher temperatures. A third type of reaction occurs when the thiol groups are blocked by thiol–blocking agents such as N–ethylmaleimide (NEM) prior to heating, which prevents the formation of primary aggregates (type I reaction) and therefore secondary "non–specific" aggregates [106]. According to Mulvihill and Donovan [106] the dynamic equilibrium between the dimer and monomer forms of β–lg at lower

temperatures is directed toward the formation of monomers. Consequently, reversible conformational changes occur, which include the ionization of certain groups, the greater exposure of tyrosine and tryptophane residues to the solvent, thus increasing the activity of thiol groups. Hydrogen and hydrophobic bonds are broken and thiol groups became exposed by increasing of the temperature, which leads to loosening of the secondary and tertiary structure of β–lacto-globulin. The first phase of aggregation and the formation of primary aggregates of β–lactoglobulin starts at temperatures above 70°C [95, 135].

THE INFLUENCE OF HIGH TEMPERATURES ON α–LACTALBUMIN

a–Lactalbumin is the second major protein present in whey, which represents 20% of the total whey proteins or 2–5% of the total nitrogen matter of milk [31, 75, 80, 106, 138]. Like β–lg, α–la has a compact globular structure and relatively low molecular weight, 14000. α–La has the affinity to bind different ions, to associate and polymerize. Also, it possesses good solubility. α–La occurs in two genetic variants A and B, with molecular weights of 14146 and 14174, respectively [72, 75, 80, 105, 139, 140]. However, Fox [132] has reported that three genetic variants of α–la, A, B and C, have been identified in milk.

The primary structure of α–la consists 123 amino–acid residues. It also has a high content of tryptophane and aspartic acid, only one residue of arginine and methionine, four disulphide bridges inside the polypeptide chain, but free thiol and phosphate groups are absent [105, 106, 132, 141, 142]. It has been suggested that the secondary structure of α–la contains 26% of the α–helix, 14% of the β–structure and 60% of unordered structure at the natural pH value of milk [80].

Hiraoka et al. [139] confirmed that α–la belongs to the group of metalloproteins by calcium chelation with EDTA (ethylendiamine–tetraacetate).

α–Lactalbumin, excluding the proteoso–peptone fraction, shows the most pronounced heat stability among whey proteins [39, 106, 126, 143, 144].

In earlier works of several authors, who investigated the heat–stability of whey proteins, α–la was ranked as one of the least heat–stable whey proteins due to its low temperature of denaturation [105, 139, 145, 146]. These authors concluded that the denaturation of α–la started at a considerably lower temperature (at 62°C) compared to other whey proteins and that the removal of calcium additionally decreased the temperature of denaturation. The same authors also investigated the heat–stability of α–lactalbumin in model systems and assumed that α–la had the ability to recover its original structure up to a level of 90% after heating at 62°C.

Mulvihill and Donovan had a different point of view [106], and assumed that α–la was the most heat–stable whey protein, based on α–la solubility at pH 4.5. The phenomenon of renaturation or reversible denaturation of α–lactalbumin could explain the small degree of denaturation measured on the base of its solubility at pH 4.5. However, the renaturation of α–la has not been estimated in whey protein concentrate [105, 145].

The behavior of α–la at high temperatures is the influenced by the presence of four disulphide bridges and the absence of free thiol groups in the polypeptide chain. Heat treatment at 100°C for 10–30 min is sufficient to split 12–20% of the disulphide bridges and reactive thiol groups are formed [147].

The denaturation of α–lactalbumin is more pronounced in the presence of β–lactoglobulin. This effect is enlarged by increasing the temperature and pH of environment [136]. Reduction of the amount of α–lactalbumin has not been noticed at temperatures up to 70°C, but reduction is significant in the temperature region between 74 and 96°C. It may be assumed that α–lactalbumin reacts with the formed aggregates of denatured β–lactoglobulin in these conditions i.e. that primary aggregates of denatured β–lactoglobulin must be formed for the interaction between α–lactalbumin and β–lactoglobulin [40].

THE FORMATION OF A CHEMICAL COMPLEX BETWEEN CASEIN AND WHEY PROTEINS

The investigations of Trautman and Swanson [16], Zittle et al. [17], and Long et al. [12] showed that the chemical complex between κ–casein and β–lactoglobulin is formed during the longer heating of milk above 70°C, while other authors concluded that a–la also participates in this reaction [5, 6, 11, 37, 48, 55, 135, 136, 148–162]. Complexes formed in this fashion are known in the literature as coaggregates of milk proteins.

β–Lactoglobulin in its primary structure has two disulphide bridges and one free thiol group [72, 106, 130, 163]. The denaturation of β–lactoglobulin takes place at a higher temperature of milk treatment, and the buried thiol group become reactive and reacts with the disulphide bridges of other milk proteins to form a complex [106]. Such bridges in milk exist in β–lactoglobulin, α–lactalbumin, κ–casein, bovine serum albumin and immunoglobulins [1, 31, 40, 135].

The exposure of thiol groups begins at 72°C, but attains a maximum at 95°C. During heat–treatment the buried thiol groups from β–lactoglobulin become exposed and react faster with κ–casein [135]. Several authors consider that free and reactive groups of the previously denatured β–lactoglobulin and the disulphide bridge of κ–casein react to form a complex. At temperatures above 65°C, molecules of β–lg irreversibly associate and form aggregates of different size. It is suggested that only this form of β–lg is capable of reacting with κ–casein [16, 48, 135, 161, 164]. The presence of a thiol blocking reagent, such as

N–ethylmaleimide, 2–mercapto–ethanol and hydrogen peroxide, prevent β–lactoglobulin aggregation and the formation of a complex between κ–casein and β–lactoglobulin [16, 40, 159, 161, 165, 166]. Disulphide links between κ–casein and β–lactoglobulin split in the presence of 2–mercapto–ethanol, dissociate the previously formed complex and part of the κ–casein is released from the casein micelle [161].

At a higher temperature α–lactalbumin also forms intermolecular links with β–lactoglobulin. It has been confirmed that α–lactalbumin does not react with κ–casein in the absence of denatured and aggregated β–lactoglobulin [36, 136, 149, 167]. According to Mottar et al. [55] β–lactoglobulin denatures as a result of high temperatures and reacts through SH/SS interchange with κ–casein and radialy covers the casein micelle. The formed complex has pronounced hydrophobic properties. The newly–formed surface is ragged with numerous filaments that originate from the β–lactoglobulin located at the micelle surface. At the higher temperature (90°C/10 min) α–lactalbumin denatures and binds to the filaments of β–lactoglobulin, thus filling "gaps" on the micelle surface, which is converted from a ragged into a regular spherical form. Due to the presence of a–lactalbumin, there is an increase in the complex hydrophilic properties. The higher a–lactalbumin content influences the more pronounced hydrophilic properties of the coaggregates at pH 4.6 [168, 169].

The influence of heat treatment on the interaction between β–lactoglobulin and casein has been the objects of many investigations. On the other hand, there are few articles that investigate the influence of heat treatment on the formation of complexes between α–lactalbumin and other proteins. Elfagm and Wheelock [36, 136, 149] studied the influence of heat treatment on whey proteins and concluded that the denaturation of whey proteins was greater in milk than in whey during heating at 74°C. On the basis of this investigation, they assumed that whey proteins reacted with κ–casein and that complex formation in milk was a two–stage process. In the first phase, whey proteins denatured and formed a complex between α–lactalbumin and β–lactoglobulin without the contribution of κ–casein, while the formed complex reacted with κ–casein in the second phase [36].

κ–Casein practically inhibits β–lactoglobulin coagulation. Additionally, precipitation does not take place in milk although the whey proteins present in the coaggregates are denatured, contrary to pure whey proteins, which rapidly denature, aggregate and precipitate during heat treatment in the absence of κ–casein. Coaggregates form a stabile colloidal phase in milk, which may be explained by the significantly larger mass fraction of casein in the coaggregates.

The formation of a complex between casein and whey proteins influences the heat stability of milk and the shape of the HCT/pH curve to a great extent [2, 170, 171]. When milk is heat treated at 140°C, the fastest heat induced coagulation occurs at pH~6.9, and the slowest at pH~6.5–6.7, when a stable complex between κ–casein and β–lactoglobulin is formed [2]. When milk is heated at temperatures above 90°C, at pH 6.5–6.7, a stable complex is formed between casein and the whey protein, and the surface charge and hydration of the micelles are increased. Under these conditions the dissociation of κ–casein from the micelle is suppressed, which affects complex stability and improves the heat–stability of milk [161, 172, 173]. At pH 6.9 or higher the complex is not formed or the formed complex is unstable and dissociates resulting in the reduction of the surface charge and hydration of the casein micelle. According to Singh and Fox [161, 172, 173] the presence of cross–linking agents, such as formaldehyde and dimethyl suberimidate, prevents the dissociation of κ–casein from the micelle, increases the stability of the complex and milk and eliminates the minimum in the HCT/pH curve. Reducing agents such as 2–mercapto–ethanol, dithiothreitol and Na–sulphite, change the shape of the milk heat–stability curve at pH<7.1, the HCP/pH curve loses its minimum/maximum, while milk heat stability increases with increasing pH [161, 172, 173]. That means that the maximum in the HCT/pH curve changes into a minimum under these conditions, which confirms the fact that the shape of the HCT/pH curve depends on the sulfhydryl–disulphide interactions between β–lactoglobulin and κ–casein, namely it depends on the stability of the formed coaggregates [161].

According to Long et al. [12] the largest amount of complex between κ–casein and whey protein is formed at 85°C. At higher temperatures the reaction is faster, but the amount of formed complex is smaller [12]. When a mixture of κ–casein and β–lactoglobulin is heated at pH 6.5 for 20 min, the amount of β–lactoglobulin that reacts with κ–casein is 3.4, 15.4, 48.8, 67.8, 82.9 and 76.7%, respectively, at 65, 70, 75, 80, 85 and 99°C [12, 49]. The reaction is the fastest at 99°C, but the reason for the smaller amount of formed complex must be the degradation of cysteine [49]. Maćej [6] and Maćej et al. [174] established that during heating at 87°C/10 min the same amount of complex is formed as during heating at 90°C/10 min and 95°C/10 min, while Fetahagić et al. [175] concluded that heat teatment at 95°C/10 min had the greatest influence on coaggregate formation compared with heat treatment at 85°C/10 min and 90°C/10 min.

When mixtures with different ratios of β–lactoglobulin and κ–casein are heated, it may be concluded that δ ($\delta = g_{b-lg}/g_{k-casein}$) increases when the amount of β–lactoglobulin is increased; δ is higher during heating at 85°C/20 min than at 99°C/20 min [12]. Investigations have shown that during heating at 85°C/20 min almost 2.2 g of β–lactoglobulin reacts with 1g of κ–casein, while at 99°C/20 min only 1.4 g of β–lactoglobulin reacts with

1g of κ–casein [12, 160]. Maćej et al. [176] investigated the influence of added demineralized whey powder (DWP) on the degree of complex formation and concluded that heat treatment at 85°C/20 min and 90°C/10 min did not give significantly different amounts of formed coaggregates, as well as that almost all the whey proteins from the DWP coaggregated with the casein from milk.

The amounts of whey proteins associated with casein micelles increase to a finite, maximum value during heating [177]. The degree of whey protein denaturation, as well as the degree of their reaction with casein depends on both the heat transfer conditions (processing conditions) and the temperature of heat treatment. During HTST pasteurization α–lactalbumin reacts more slowly and to a lesser extent than β–lactoglobulin [178]. Heat transfer conditions during UHT sterilization (direct or indirect) also influence the rate and degree of complex formation. The extent of whey protein denaturation, as well as the degree of interaction between whey protein and κ–casein, are lower during direct sterilization (DSI) than during indirect UHT sterilization [55, 178].

According to Mottar et al. [55] the amount of a–la associated with casein micelles increases at higher heat treatment, specifically during indirect (UHT) sterilization or by heating milk at 90°C/10 min. A larger amount of β–lg is associated during direct UHT treatment.

Oldfield et al. [179] used the DSI system to investigate the degree of β–lg and α–la denaturation, as well as the extent of their interaction with casein micelles in skim med. Their investigations demonstrated that the degree of β–lg and α–la denaturation and association with casein micelle increase with the time and temperature of heat treatment. On the other hand, the extent of association is smaller than the degree of whey protein denaturation. It was concluded that association between the casein micelle and β–lg occurs in the temperature range between 80 and 130°C, while the association of α–la starts after a longer time of heat treatment. However, in the case of heat treatment below 80°C, synchronized associations of β–lg and α–la with casein micelles occur. The maximum of α–la association depends on the temperature of heat treatment. It achieves ~ 40% in the temperature range 95–130°C, while at temperatures below 90°C it attains ~ 55%. The maximal level of β–lg association is ~ 55%, regardless of the used heat treatment.

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IZVOD

UTICAJ VISOKIH TEMPERATURA NA PROTEINE MLEKA

(rad)

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Visoke temperature prouzrokuju promene na sastojima mleka, a intenzitet tih promena zavisi od visine temperature i dužine njenog delovanja. Najveće promene dešavaju se na proteinima mleka.

Uticaj visokih temperatura prouzrokuje povećanje kiselosti mleka, precipitaciju kalcijum fosfata, denaturaciju i koagulaciju proteina surutke, kao i njihovu interakciju sa kazeinom, Maillard–ovu reakciju, defosforilizaciju kazeina, hidrolizu kazeinskih micela, promene na serum proteinima, produženje vremena koagulacije pod dejstvom sirišnog enzima, promene reoloških karakteristika kiselog i slatkog kazeinskog gela, promenu z– potencijala i hidratacije kazeinskih micela, interakciju između proteina mleka i proteina membrane masnih kapljica.

Kazein spada u grupu termostabilnih proteina. Veliki broj rezidua prolina u polipeptidnom lancu uslovljava tzv. neorganizovanu "prelom" konformaciju, zbog čega kazein ima slabo izraženu sekundarnu i tercijarnu strukturu, pa se u literaturi često označava kao prirodno denaturisani protein. Kazein u mleku koaguliše na temperaturi 100°C tek nakon 12 časova, na 130°C nakon 60 min, a na 150°C nakon 3 min. Na temperaturi od 120°C nakon 5 časova, a na temperaturi 135°C u toku 60 min 10–20% ukupnog azota prelazi u neproteinski azot. Zagrevanjem rastvora κ–kazeina ili celog kazeina na 120°C u toku 5–6 časova dolazi do defosforilizacije kazeina i obrazovanja manjih polipeptidnih lanaca. Ustanovljeno je da pri tim uslovima defosforilizacija kazeina iznosi 80%.

Serum proteini spadaju u grupu termolabilnih proteina. Na 60°C u toku 10 min denaturiše samo 3%, na 70°C 15%, na 80°C 66%, a na 90°C oko 85% ukupnih serum proteina. Za razliku od α–laktalbumina, denaturacija β–laktoglobulina podleže kinetici reakcije drugog reda. Stepen denaturacije serum proteina se uglavnom određuje stepenom denaturacije β–laktoglobulina, jer on čini približno 50% ukupnih serum proteina. Ponašanje α–laktalbumina prema dejstvu visokih temperatura ustanovljene su postojanjem četiri disulfidna mosta i odsustvom slobodnih –SH grupa u polipeptidnom lancu. Zagrevanjem na 100°C u toku 10–30 min, raskida se 12–20% disulfidnih veza i obrazuju reaktivne tiolne grupe. Denaturacija α–laktalbumina u prisustvu β–laktoglobulina je izraženija i povećava se sa porastom temperature i pH sredine. Zagrevanjem mleka iznad 70°C sa dužim delovanjem visokih temperatura obrazuje se hemijski kompleks između kazeina i serum proteina (koagregati proteina mleka). Zagrevanjem smeše κ–kazeina i β–laktoglobulina u odnosu 1:1 u toku 20 min pri pH 6.5, količina β–laktoglobulina koja izreaguje sa κ–kazeinom na 65°C, 70°C, 80°C, 85°C i 99°C iznosi 3.4%, 15.4%, 48.8%, 67.8%, 82.9% i 76.7%.

Ključne reči: Kazein • Koagregati • Mleko • Proteini • Serum proteini • Termički tretman •
Key words: Casein • Coaggregates • Milk • Proteins • Whey proteins • Heat treatments •