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A Review on Proteolysis Rate in UHT Milk: Its Mechanism, Pattern, Assessment and Enzymatic Changes during Storage

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Abstract

With the increasing awareness of people about food consumption, the demand of supplying aseptically packed food such as ultra-high temperature (UHT) milk is increasing. Some native and microbial proteases are relatively heat stable and cannot be inactivated during UHT treatment. The traces of these enzymes are responsible for various proteolytic changes (e.g. age-gelation and bitterness) in the stored UHT milk. There are various methods reported in the literature to measure proteolysis in milk and milk products like UHT milk. All the methods have their own merits and demerits and depending upon requirement, one has to select one or more methods for the measurement of proteolysis. The comparison of these methods are carried out in this paper.

Keywords: *UHT milk, proteolysis, age-gelation, proteolysis assessment*

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INTRODUCTION

Milk is a complex product and complete food for all. With the increasing consciousness of people about food consumption, the demand of supplying aseptically packed food like ultra-high temperature (UHT) milk gets increases. The treatment of milk at UHT for a short time renders it effectively sterile, so that when packaged aseptically, is bacteriologically stable at ambient condition for several months [1]. Longer shelf life of UHT milk is the main advantage and this make able supply to longer distance. UHT milk is also favoured for other situations involving lack of refrigeration or requiring added convenience, for example, camping, traveling, emergency preparedness, disaster response, and space travel.

More than sixty different indigenous enzyme activities have been reported in clean, freshly drawn milk (Andrews, 1991) [2] and the levels of enzymes are not steady and depend on breed and age, stage of lactation, diet and health status of the cow (Andrews, 1991) [3]. Only a few of the indigenous milk enzymes (i.e. enzymes in fresh unprocessed milk) have a substantial impact on the quality and shelf life of milk and milk products [2], proteases is among them. UHT milk stored for longer period at normal room temperature, by this time, it can be reactivation of high heat stable native and bacterial proteases occurs. Certain high heat stable bacteria, which are survive during UHT process can also grow and produce proteases enzymes. These are responsible for age-gelation and bitterness in UHT milk. The proteases enzymes are mainly responsible for protein degradation.

It is necessary to check the extent of proteolysis in UHT milk during the storage. Proteolysis is inversely related to quality of milk, higher the proteolysis poor quality UHT milk. There are wide ranges of methods reported in literature like Folin-Ciocalteu phenol reagent method, trinitrobenzenesulfonic (TNBS) acid method, fluorescamine method, O-phthaldialdehyde method, ninhydrin method, gel electrophoresis, RP-HPLC etc., all methods measure the protease activities based on the protein degradation products like free amino acids and peptides.

ULTRA HIGH TEMPERATURE (UHT) MILK

Milk is highly perishable and very susceptible to microbial and chemical degradation even at

refrigeration temperatures. UHT processing of milk makes sterile milk that will retain good sensory characteristics and physical stability during storage at room temperature for several months. Ultra-high-temperature (UHT) processing involves heating milk in a continuous-flow system to a high temperature (~135–145°C) and holding it at that temperature for a short time (1–10 seconds) followed by rapid cooling. Aseptic processing involves UHT processing followed by filling the product into sterile containers in a sterile environment and sealing the containers in a sterile manner in a continuous process.

According to IDF (1981) [4], "UHT milk is a type of sterilized milk produced by a single uninterrupted continuous-flow heating process involving a high-temperature short-time combination of at least 130°C for a few seconds—or any other combination which will give the same results—associated with aseptic packaging, and should pass the following tests:

- keeping quality test as described in IDF standard 48 (1969) [5], and
- give turbidity when subjected to the turbidity test by a modified Aschaffenberg Test.

In this definition, the keeping quality test is designed to establish the sterility of the product and the turbidity test is used to ensure the product has not been subjected to excessive heat treatment [6].

UHT MILK PROCESSING

UHT processing systems can be divided into direct and indirect systems. In indirect systems, the product is heated by counter flow of cold milk and hot water or steam in a tubular or plate heat exchanger. In direct systems, the product is heated by directly mixing it with steam under pressure, which is later removed using vacuum cooling [7]. A direct systems in UHT process has the very fast heating rate, only a fraction of a second and that results in minimal chemical changes to the product compared to indirect systems. In this system, product gets direct contact with steam so pure quality steam required and energy recovery is very poor. While in indirect systems, the energy recovery is approximately 90%, it is only 45% when using direct systems, making direct UHT systems costlier to operate [7, 8]. UHT processes with ultra-short holding times have been developed, UHT treatment >150°C with holding times of 0.2 s result in a sufficient spore reduction to obtain a commercially sterile product [9, 10]. The homogenisation step can be placed before or after the UHT treatment in indirect systems, while it is always placed after the UHT treatment in direct systems to avoid the formation of aggregates [11].

Heat-induced Changes in Milk Protein

Whey Proteins: The effect of heat treatment on the whey proteins distinction between reversible and irreversible denaturation. It was found that β-lg and α-la unfold and lose their tertiary globular structure at 75–80°C and 65°C, respectively [12]. The kinetics of irreversible heat induced denaturation of α-la and β-lg consist of two reactions with different activation energies. Below 80°C and 90°C, the activation energies are high, indicating that a large number of bonds are broken and the rate limiting factor for irreversible denaturation is the unfolding of the whey proteins. Above 80°C and 90°C, the activation energy is much lower and the whey proteins are completely unfolded and the speed limiting factor is the aggregation of the whey proteins [13, 12].

Besides the formation of aggregates between whey proteins, β-lg can react with κ-casein on the surface of the casein micelle via thiol-disulphide interchange reactions [14]. Association of whey proteins with the casein micelles is lower in direct systems compared to indirect systems; β-lg denaturation is lower in UHT milk processed with direct systems (35–80%) than indirect systems (79–100%) due to the faster heating rate in direct systems [11]. Basically, all whey proteins are instantly unfolded during direct UHT processing, giving the reactive monomers a greater opportunity to react with themselves than associating with the casein micelles [14]. In the indirect UHT milk and the direct UHT milk 95°C/180 s + > 150°C/< 0.2 s, β-lg is almost completely denatured.

Caseins: Caseins and casein micelles are very heat stable and the micellar conformation is preserved even during severe heat treatments

[15]. The most important heat induced change of caseins during heat treatment is an increase in the casein micelle size due to the association of denatured whey proteins [14]. The increase in casein micelle size depends largely on the degree of whey protein denaturation.

PROTEASES IN MILK

Native Proteases

Native milk protease have been studied widely, the milk alkaline proteases (''plasmin'', a serine proteases) and milk acidic proteases ("cathepsin D", an aspartic proteases). These indigenous proteases arise from mammary tissue cells, blood plasma or leucocytes [16, 17] (Andrews, 1991). Milk alkaline proteases, which is plasmin most thoroughly studied of all indigenous milk proteases, and has been shown to be the same enzyme as plasmin (isolated from bovine blood) on the basis of many characteristics, e.g. pH optimum and pH stability, heat stability, sensitivity to various inhibitors, molecular mass, specificity on caseins, and, conclusively, identical amino acid sequences [18, 19].

Plasmin is a significant enzyme and it is part of complex system; which is plasmin, plasmin inhibitors (PI), the inactive zymogen plasminogen (PG), plasminogen activators (PA) and inhibitors of plasminogen activators (PAI) [18]. The optimal pH and temperature for this protease are 7.5 and 37℃, respectively [20]. In milk, plasmin is derived from blood serum and it is associated with casein micelles (Andrews, 1991) [16]. It is also found in the milk fat globule membrane (MFGM) due to the presence of casein [19, 21]. Along with plasmin other elements of system also found in milk, plasminogen associated with casein, plasminogen activators are found in casein micelles and somatic cells, plasmin inhibitors and plasminogen activator inhibitors only occur in the serum phase of milk [18].

Plasmin occurs mainly as its active form as well as inactive precursor plasminogen. The bulk raw milk contains 0.07-0.15 μg mL^{-1} plasmin and 0.7–2.4 μg mL^{-1} plasminogen [22]. The levels of plasmin and plasminogen depend on the stage of lactation, age of animal, type of breed, age and mastitis [23]. Plasminogen is converted into plasmin by proteolysis by plasminogen activators [24]. Plasminogen activators are present in somatic cells this is the reason why mastitic milk and late lactation milk shows high proteolytic activity. In the plasmin system, PA cleave the Arg^{557}-Ile^{558} bond in bovine plasminogen which is chain of 786 amino acid residues with molecular weight 88,092 Da, and it convert into active plasmin. Plasminogen has an aspartic acid at N-terminal and removal of the first 77 residues by plasmin gives an arginine at N-terminal [25]. The N-terminal 77 residue fragment of plasminogen has been called the preactivation peptide [18], release of this peptide does not activate plasminogen, and it causes a conformational change in plasminogen that exposes Arg^{557}-Ile^{558} bond for PA [26]. PAs are likely native to milk or produced by microorganisms [27]. Two major types of PA are present in fresh milk: a tissue type associated with casein and a urokinase type associated with the somatic cells. Along with these plasmin activators inhibitors and plasmin inhibitors cause structural change and retard the plasmin activators and plasmin activity [28].

Plasmin hydrolyses all caseins in milk except k-casein. It has a preference for β- and α_{s2}-caseins [29]. It has a preferential specificity for bonds on the C-terminal side of lysine (Lys) and arginine (Arg) residues, cleaving Lys-X bond much faster than Arg-X bonds [30]. Plasmin does not hydrolyse α-lactalbumin and β-lactoglobulin [31]. Three elements of the plasmin system (plasmin, PG and PA) have been reported to have very similar, and relatively high, heat stabilities. For plasmin and plasminogen, high heat stability is attributed to protection by casein [32]. PI and PAI have low heat stability and thermally unstable. Plasmin activity will survive pasteurisation, level of plasmin and plasminogen survive after pasteurisation remain 0.14–0.73 μg mL-1 and 0.55–2.73 μg mL-1, respectively [33]. Milk processing like UHT of milk imbalance the ratio of the activators and inhibitors, increase the activators and this can lead to an enhanced proteolysis in heated milk [27]. Plasmin undergoes reversible denaturation at high

temperature: firstly unfolding at high temperatures (denaturation), then refolding to an active form after cooling [34]. Severe heat treatment of milk (115°C/20 min or 120°C/15 min) is required to ensure the absence of plasmin induced proteolysis in milk products [35]. The storage of UHT milks at ambient temperature leads favourable condition for protease activity. The optimum pH for one protease ranges from pH 7 to 8 with 85% to 90% of maximum activity at pH 6.5, the pH of milk [36].

Cathepsin D (EC 3.4.23.5) is a glycoprotein with mannose-containing oligosaccharides attached at positions Asn^{67} and Asn^{183} [37] and located in the lysosomes of all mammalian cells. It is an aspartic proteases which is a bi-lobed molecules and each lobe contain Asp active site to substrate binding cleft formed between two. Cathepsin D mostly synthesised as proenzymes with an N-terminal propeptide which is removed to form the mature enzyme it is mainly found in the whey fraction of raw bovine skim milk at a level of 0.4 μg mL-1 [17]. It has optimum pH and temperature for this 39 kDa-enzyme is 4 and 37°C, respectively [38] and can degrade all milk proteins except β-lactoglobulin. Cathepsin D produces the glycomacropeptide (k-CN (f106–169)) that is also produced by chymosin by the enzymatic cleavage of the Phe_{105}-Met_{106} bond [39]. The milk clotting potential of cathepsin D, however, has been reported to be very poor so it cannot to be regarded as clotting enzyme [17]. It is completely inactivated by heat treatment of 70° for 10 min at pH 4 in acetate buffer [37] and by pasteurization at 65°C for 30 min in skim milk, due to this relatively low heat stability, not acquired much attention in UHT milk and other dairy products [40].

Bacterial Protease

Along with indigenous enzymes, milk also contains enzymes come from contaminating bacteria. Microbial proteolytic enzymes produce by various genus, e.g. *Pseudomonas, Bacillus, Clostridium, Proteus, Escherichia, Micrococcus, Microbacterium, Flavobacterium,* etc., while the first two are the most important because production of enzymes like proteases and lipases, some species of these genera are capable to grow at pasteurization temperature and many enzymes produce by them are high heat stable. They all classify on the basis of their optimum growth temperature like mesophiles (20–45°C) or thermophiles (>45°C). Three main sources of bacterial contamination have been found in raw milk *viz.* the interior of the udder, cows' teats as well as milking and storage equipment [41].

Freshly drawn milk from the udder may not contain detectable levels of contaminant bacteria. The contaminants increase during storage is mainly psychrotrophs (especially species of *Pseudomonas*). *Pseudomonas* acquired 70–90% and 87% of psychrotrophs isolated from raw and pasteurised milk, respectively, stored for 1 week at 4°C (Adams *et al.,* 1975) [42]. The majority of *Pseudomonas* species produce only one type of proteinase, typically a neutral zinc metallo-proteinase with pH optimum of 6.5–8, when their density reaches 10^7–10^8 CFU/ml [39, 43]. Proteinase production by psychrotrophs is normally at a maximum in the late exponential or stationary phase of growth [44, 45]. Their proteinases usually have optimal conditions at temperature 17.5–30°C and pH 6.5–7.0 [46]. Adams *et al.* (1975) [47] reported that the proteinases produced by *Pseudomonas* isolates were survived under heat treatment of 149°C for 10 s in buffers at pH 7.5. Griffiths *et al.* (1981) [48] showed that the proteinases from *pseudomonas* retained 20–40% of their activity after the exposure to 140°C for 5 s. Most proteinases from psychrotrophs preferentially attack casein over whey proteins. The β-casein and k-casein are more susceptible than α_{s1}-casein (Gebre-Egziabher *et al.,* 1980) [49].

Bacillus species are widely distributed in the environment and can be introduced into milk and milk products during production, handling and processing [50–52]. An additional problem with *Bacillus* species is that they sporulate during milk processing, and the spores are extremely resistant to heat and chemical cleaning reagents [53]. Phillips and Griffiths (1990) [53] reported that 86% of thermoduric psychrotrophic bacteria which can survive pasteurisation, isolated from raw milk were *Bacillus* species and can produce a

large variety of extracellular enzymes (e.g. proteinases and lipases), maximum production normally occurring in the late exponential and early stationary phases of growth, before sporulation [54]. Many mesophilic and thermophilic *Bacillus* species are also isolated from processed milk and milk products. Bacillary proteases usually have a pH optimum in alkaline zone and a temperature optimum at 30–37°C [55]. *Bacillus* species have higher extracellular and intracellular proteolytic activities and are producing more than one type of proteases known to be capable of producing more diverse proteolytic activities than *Pseudomonas* ones.

MECHANISM OF PROTEOLYSIS

Proteins are polymers of amino acids which are held by the peptide bonds between them. During proteolysis these peptide bonds are broken by protease enzymes and generate the various peptides. Furthermore these peptide split into small peptide and releases free amino acids. According to Svendsen (1976) [56] the catalysis by the major class of proteases occurs as three consecutive reactions:

(i) Formation of the Michaelis complex between the original peptide chain (the substrate) and the enzyme.
(ii) Cleavage of the peptide bond to liberate one of the two resulting peptides and
(iii) A nucleophilic attack on the remaining of the complex to split off the other peptide and to reconstitute the free enzyme.

As stated by Svendsen (1976), it is generally accepted that for serine protease acting on peptide bonds and the second step is the rate-determining step. Plasmin is serine proteases and it also includes the digestive enzymes like trypsin, chymotrypsin, elastase etc. Different serine proteases differ in substrate specificity, like plasmin has specificity for the lysine and arginine residues. An acyl-enzyme intermediate is transiently formed and hydrolysis of the ester linkage yields the second peptide product. The active site in each serine protease includes a serine residue, a histidine residue, and an aspartate residue and during hydrolysis, a proton is transferred from the serine hydroxyl to the imidazole ring of the histidine, as the adjacent aspartate carboxyl is H-bonded to the histidine.

Cathepsin D is an aspartic proteases, it tends to cleave dipeptide bonds that have hydrophobic residues as well as a beta-methylene group. Unlike serine protease these proteases do not form a covalent intermediate during cleavage, proteolysis therefore occurs in a single step. The acid-base mechanism for aspartic proteases is widely accepted. In this mechanism, the involvement of coordination of a water molecule between the two highly conserved aspartate residues takes place. One aspartate activates the water by abstracting a proton, enabling the water to perform a nucleophilic attack on the carbonyl carbon of the substrate scissile bond, generating a tetrahedral oxyanion intermediate. The rearrangement of this intermediate leads splitting of the substrate peptide into two product peptides.

PATTERN OF PROTEOLYSIS IN MILK

Milk contains average 3.3% milk protein and it also contains all 9 essential amino acids required by humans. Milk protein divided into casein and whey proteins. Casein contains phosphorus while whey proteins do not contain of it. Casein get precipitate at pH 4.6 while whey proteins remain soluble at this pH. The 80% of the milk protein is casein whereas remaining 20% is the whey (serum) protein. The casein family of protein consists of several types of caseins (α_{s1}, α_{s2}, β, and γ) and each has its own amino acid composition, genetic variations, and functional properties. The whey protein contains β-lactoglobulin, α-lactalbumin, immunoglobulin, blood serum albumin, transferrin, lactoferrin, and enzymes.

Earlier time, the most research on hydrolysis of casein by plasmin focused on β-casein and it has trypsin like activity [57,58]. These workers found that there are three plasmin-sensitive bonds present in β-casein; Lys^{28}–Lys^{29}, Lys^{105}–His^{106} and Lys^{107}–Glu^{108}. When plasmin act on these bonds, the following peptides are released; γ_1-casein (f 29-209), γ_2-casein (f 106-209), γ_3-casein (f 108-209), proteose-peptone component 8-fast (f 1-28), proteose-peptone component 8-slow (f 29-105 and f 29-107) and proteose-peptone component 5 (f 1-105 and f 1-107). Rauh *et al*. (2014) [59] found that, plasmin hydrolysed β-

casein in UHT milk at Lys^{29}-Ile^{30}, Lys^{32}-Phe^{33}, Lys^{48}-Ile^{49}, Lys^{97}-Ala^{98} and Lys^{99}-Glu^{100} bonds; initially plasmin hydrolysed at Lys^{105}-His^{106} and Lys^{107}-Glu^{108} and sequentially continued to hydrolyse β-casein at Lys^{97}-Ala^{98}, Lys^{99}-Glu^{100} and Lys^{113}-Tyr^{114} and resulting various small peptides.

Plasmin also shows the activity on α_{s1}-casein, when it is incubated with bovine plasmin at 37°C, several band having high electrophoretic mobilties were found with disappears of α_{s1}-casein band in gel electrophoresis [60, 61]. The α_{s1}-casein has lower susceptibility than that of β-casein [61]; this may be due to relatively low concentration in milk. Aimutis and Eigel (1982) [62] indicated that peptides derived from α_{s1}-casein incubated with plasmin have identical electrophoretic patterns of crude λ-caseins. McSweeney *et al*, (1993) found that hydrolysis of α_{s1}-casein by plasmin results in 26 different peptides. Le Bars and Gripon (1993) [63] also found that the seven Lys-X and four Arg-X bonds, and resulting in 15 peptides. The major cleavage sites found in α_{s1}-casein are Arg^{22}-Phe^{23}, Arg^{90} Tyr^{91}, Lys^{102}-Lys^{103}, Lys^{103}-Tyr^{104}, Lys^{105}-Val^{106}, Lys^{124}-Glu^{125} and Arg^{151}-Gln^{152}. Both these research groups did not determine which of these peptides corresponded to the λ-casein fraction.

Plasmin hydrolyses α_{s2}-casein at approximately the same rate as β-casein (Kelly and McSweeney, 2003). Rapid proteolysis of α_{s2}-casein by plasmin cause 80% of α_{s2}-casein got hydrolysed after 4 h incubation (Snoeren and van Riel, 1979). The two independent groups found eight identical plasmin cleavage sites in bovine α_{s2}-casein, seven Lys-X and one Arg-X residues [64, 65]; it includes Lys^{21}-Gln^{22}, Lys^{24} Asn^{25}, Arg^{114}-Asn^{115}, Lys^{149}-Lys^{150}, Lys^{150}-Thr^{151}, Lys^{181}-Thr^{182}, Lys^{188}-Ala^{189} and Lys^{197}-Thr^{198} were susceptible to plasmin. There were very low reports available on cleavage of α_{s2}-casein by plasmin this due to its relatively low concentration in milk and the dimeric nature, which complicates peptide analysis of α_{s2}-casein hydrolysates.

The κ-casein was resistant to hydrolysis by plasmin which is because of carbohydrate moieties attached to it [66] and polymeric nature. When carbohydrate was absent; κ-casein became slightly susceptible to hydrolysis by plasmin. Whey proteins do not hydrolyse by plasmin because of globular structure and show some inhibitory effects on plasmin activity [67]. However, Rauh *et al.* (2014) identified 6 peptides originating from β-lg in UHT milk. The cleavage sites were Lys_8-Gly_9, Lys_{71}-Thr_{72}, Lys_{75}-Ile_{76}, Lys_{71}-Thr_{72}, Lys_{83}-Ile_{84} and Lys_{91}-Val_{92}. This might be due to partial denaturation of β-lg on high heat treatment.

Kaminogawa *et al.* (1980) [68] investigated the action of partially purified cathepsin D on milk casein. The proteolysis product of casein was similar to those is produced by chymosin [17, 39]. Cathepsin D acts on α_{s1}-casein at Phe_{23}-Phe_{24} and produces a peptide with the same molecular mass as α_{s1}-CN f24-199 (α_{s1}-I-casein), this peptide also further hydrolyses at Phe_{24}-Val_{25}, Leu_{98}-Leu_{99} and Leu_{149}-Phe_{150} and releases various peptides. Cathepsin D first hydrolyses Leu_{99}-Tyr_{100} in αs_2-casein and coverts into αs_2-casein monomer. It also cleaves Leu_{123}-Asn_{124}, Leu_{180}-Lys_{181} and Thr_{182}-Val_{183} bon in αs_2-casein [17]. Proteolysis of β-casein by cathepsin D is similar to that by chymosin, with β-CN (f 1–192) being the primary product and β-CN (f 1–163/165/167) being secondary products. Cathepsin D hydrolyses κ-casein and produce *para*-κ-casein like peptide [40]; it also acts on κ-casein at Leu_{32}-Ser_{33} and Leu_{79}-Ser_{80}. Whey protein generally is resistant to cleavage but α-lactalbumin slightly hydrolysed [17].

In contrast to plasmin, extracellular bacterial proteinases predominantly attack κ-casein with the formation of material similar to para-κ-casein [69, 70] followed by extensive non-specific hydrolysis. β-casein is also readily hydrolysed while α_{s1}-casein is slowly hydrolyzed by bacterial proteinases [69, 71]. Bacterial proteinases preferentially hydrolyze the κ-casein which is located on the micelle surface. Driessen (1983) [72] suggested that proteolysis by bacterial enzymes is accompanied by an increase of non-protein *N* and the formation of *para*-κ-casein, while the plasmin produces an increase of non-casein *N* and the formation of γ-caseins. The susceptibility of the caseins to hydrolysis by bacterial proteinases and milk plasmin are $\kappa > \beta > \alpha_{s1}$ and $\beta = \alpha_{s2} > \alpha_{s1} > \kappa$, respectively.

ENZYMATIC CHANGES OF PROTEOLYSIS IN UHT MILK DURING STORAGE

In processed milk, proteolysis has been measured by monitoring changes in nitrogen levels or release of free amino acids like tyrosine. Release of tyrosine leads to bitterness, unclean, sour and tenuous defects [73]. The actual effects of plasmin in UHT milk are not fully understood because the mechanisms of age gelation remain unresolved [20]. Plasmin activity is the major factor affecting the storage stability of UHT milk [74]. Proteolysis also causes lower the heat stability of proteins leading to greater fouling in heat exchangers [75, 76]. Age gelation and bitterness are the important factors to assess quality of UHT milk.

Age Gelation

Age gelation in UHT milk has been proposed to be a two-stage process [77]. The gel forms in UHT milk is a 3-dimensional protein matrix formed by the interaction of whey proteins, particularly *β*-lactoglobulin, with casein, chiefly κ-casein, of the casein micelle. According to enzymatic mechanism, proteinases are responsible for the release of the *β*-lactoglobulin-κ-casein complexes (*β*κ-complex). The proteinases do not act directly on the *β*κ-complex but cleave the peptide bonds which anchor the κ-casein to the casein micelle, facilitating release of the *β*κ-complex [78, 79]. This dissociation of *β*κ-complexes considered to be the first stage of age gelation [80]. The second stage involves the subsequent aggregation of the *β*κ-complexes and formation of a three-dimensional network of cross-linked proteins. When a critical concentration of cross-linked *β*-κ-complexes and entrained proteins is attained, a semi-rigid gel is produced.

Heat-stable proteinases produced by psychrotrophic bacterial contaminants of raw milk are also capable of causing gelation in UHT milk (Cogan, 1977) [81]. A major difference is the lower specificity of bacterial proteinase for the phe_{105}-met_{106} bond of κ-casein; this causes a relatively slower gelation/clotting process during age gelation in UHT milk. At the point of gelation, more than 95% of the α_S- and β-CN were hydrolysed [59]. Datta and Deeth (2003) [82] found that the initial gel was usually formed at the bottom of the container and has been described as fragile and custardy. Many factors like proteolysis, mode and severity of heat treatment, milk production factors, microbiological quality of raw milk, storage temperature, additives and fat content are also affect the age gelation and these are discussed at below:

Proteolysis: A widely held view is that gelation is caused by proteolysis of casein caused by the natural milk proteinase, plasmin, and/or heat-stable proteinases produced by psychrotrophic bacterial contaminants in the raw milk before processing. Milks containing these bacterial proteases are known to be particularly susceptible to gelation; however, milks without these enzymes but containing plasmin can also gel. Plasmin is one of the few natural milk enzymes that are resistant to heat and may withstand UHT conditions. Furthermore, it can increase during storage as its inactive precursor, plasminogen, which is also present and heat-stable, is converted to the active plasmin by the plasminogen activator. Mastitic milk and milk from cows in late lactation have elevated levels of plasmin.

Severity of heat treatment: The more severe the heat treatment, the longer age gelation is delayed. Thus milks sterilized by indirect heating methods are less susceptible to gelation than milks treated by the direct steam injection and infusion methods. Furthermore, retort sterilized milk is more stable than UHT milk.

Storage temperature: The rate of gelation of UHT milk is markedly influenced by the temperature of storage. Gelation occurs at a maximum rate at ~25–28°C. The gelation at 35–40°C is lower; it may be due to a high degree of proteolysis by proteases, which prevents the degraded proteins from forming a strong gel matrix.

Bacteriological quality of milk: Milk of poor bacteriological quality at the time of processing is much more susceptible to gelling than good quality milk. The enhanced gelation milk is largely attributable to the production of heat-stable bacterial proteases in the milk.

Milk composition: Milks with high solids contents such as concentrated milks are much more susceptible to gelation than normal milks.

Addition of sodium hexametaphosphate (SHMP) (0.05% and 0.1%) to milk before heat treatment is effective in retarding gelation. SHMP does not inhibit proteolysis but prevents proteolysed milks from gelling. Its effect appears to be through interaction with calcium ions within the casein micelles, which stabilize the micelles and prevent dissociation of the *β*κ-complexes. A low temperature inactivation (LTI) method also proposed for reducing the activity of heat-resistant proteases in the milk, heating at ~55°C for 30–60 minutes [83]. The treatment is effective either before or after the high-heat treatment. Inclusion of an LTI step with UHT sterilization has been reported to significantly prolong the shelf Life of UHT skim milk. The use high-quality raw milk and indirect heating system for the high-heat treatment can minimise the age gelation. Suitable high-quality raw milk has a low somatic cell count and low bacterial count. It should be processed as soon as possible after milking and milk more than 48 hours old should be avoided whenever possible.

Bitterness

Bitterness in UHT milk is mainly attributed to formation of hydrophobic peptides. The presence of hydrophobic peptide is mainly due to hydrolysis of *β*-casein and the extent of hydrolysis of *β*-casein is positively correlated with the intensity of bitterness (Lemieux and Simard, 1991) [84]. The plasmin hydrolyzed β-CN into γ-CN fraction that can further spilt into f(203−209) which is extremely bitter [85]; but its concentration was very low in UHT milk. Bitter peptides deriving from α_{s1}-CN, that can be formed by plasmin, are α_{s1}-CN f(23−34) and α_{s1}-CN f(91−100). The smaller peptides α_{s1}-CN f(1−7) and α_{s1}-CN f(194−199) were highly hydrophobic, and could have contributed to bitterness. The peptides α_{s2}-CN f(182−207), α_{s2}-CN f(189−207), and α_{s2}-CN f(198− 207) have been correlated with bitterness and were also present early in the storage period in the UHT milk (Le Bars and Gripon, 1989). Cleavage of Arg_{205}-Tyr_{206} in α_{s2}-CN gives rise to the bitter dipeptide Tyr-Leu [86]. The concentration and sensory thresholds of the potential bitter peptides in the UHT milk are unknown, and the bitterness cannot be attributed to a specific peptide. Psychrotrophs produce heat-stable proteolytic enzymes that survive UHT processing and cause development of bitterness in the finished product [69, 87, 88]. UHT milk developed bitter flavour defects within 7 days at room temperature when it contained 2U proteases mL^{-1} [89].

ASSESSMENT OF PROTEOLYSIS IN MILK

Most of the methods used for measuring proteolysis in UHT milk during storage belong to two groups. The first group includes methods that measure aromatic amino acids in trichloroacetic acid (TCA)-soluble peptides. These aromatic amino acids are detected by reaction with the Folin-Ciocalteu phenol reagent as in the methods of Hull (1947) [90] and Lowry *et al.,* (1951) [91]. The other group of proteolysis detection methods measure free amino groups (FAG) in TCA-soluble peptides. These include the trinitrobenzenesulfonic (TNBS) acid [92], fluorescamine [93], O-phthaldialdehyde [94] and ninhydrin methods [95]. In addition to these, gel electrophoresis and RP-HPLC methods are also reported in the literatures [96, 97]. Before the performing these methods, proteolysis product get extracted with the help of TCA or by the lowering of pH to 4.6 by acetic acid.

Hull Method

Hull method is the very oldest method to determine the proteolysis in milk and milk products. In this method, TCA-soluble peptides mixed with sodium carbonate are reacted with phenol reagent. Aromatic hydroxyl groups in tyrosine and tryptophan reduce the phenol reagent and give a blue color and its intensity is measured at 650 nm in a spectrophotometer [90]. Citti *et al*. (1963) [98] reported difficulties in measuring proteolysis in autoclaved milk, which they attributed to the release of amino acids sensitive to the reagent used. In addition, there are many chemicals which can interfere with the reaction of the phenol reagent and aromatic amino acids [99]. The Hull method is not very

sensitive for measuring milk proteolysis since microbial proteases liberate only low concentrations of aromatic amino acids from casein [81]. In addition, further hydrolysis of TCA-soluble peptides, as well as peptides which do not have aromatic amino acids, are not detected by this method [93].

Trinitrobenzenesulfonic (TNBS) Acid Method

The 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was originally proposed by Okuyama and Satake (1960) [100] for determining amino acids and peptides and the reaction was more specific and the tyrosine or histidine side chains were not detected. TNBS reacts with free amino acids, yielding a yellow colour product. TNBS reacts only with amino groups in their unprotonated state. Measurements of the yellow color of the sulfite complex are made at 420 nm. The reaction is carried out in borate buffer at pH 9.5. At this pH, the reaction is stopped by lowering the pH to neutrality after the amino groups have been trinitrophenylated, or it is subtracted out continuously, using a split-beam recording spectrophotometer. This method is simple and rapid and makes able to detect lower level of proteolysis. Chove *et al.* (2013) [101] measured the proteolysis in UHT milk exposed under the various heat treatments (85°C/15s, 110, 120, 130 and 142°C for 2 s) by TNBS method and they found that the proteolysis was positively correlate with development of yellow colour; raw milk and pasteurized milk shows high activity because of survival of protease enzymes and activity decrease with increase the heat treatment; proteolysis in high heat treated milk was steady but significantly increase during storage.

Fluorescamine Method

The fluorescamine method developed by Castel *et al*. (1979) [102] is used to assess the protease activity. Fluorescamine method measures the TCA soluble peptides provided a sensitive method for measuring protease activity that is specific for primary amino groups and relatively free of interference. Fluorescence was determined with a spectrofluorometer after excitation at 390 nm and emission at 475 nm. This method was especially suitable for measuring protease activity in sterile milk. It requires small quantities of reagents; fluorescence of peptide has affected by final pH of the reaction mixture. Optimization of the final pH should give an increase in sensitivity for other proteases. This should be more reliable than the traditional Hull method for assessing proteolysis because of the higher sensitivity and the specificity of the reaction. If the maillard reaction is occurring, then some of the amino groups will not be available to react with fluorescamine and affect the final results.

O-Phthaldialdehyde Method

O-Phthaldialdehyde (OPA) method is also suitable for measuring the proteolysis in milk. The OPA reagent was prepared essentially as described by Goodno *et al.* (1981) [103]. It is a rapid, sensitive, and convenient spectrophotometric assay and characterized for measurement of proteolysis of milk proteins in buffered solutions or in milk, α-amino groups released by hydrolysis react with O-phthaldialdehyde to form an adduct that absorbs strongly at 340 nm. Specific characteristics that make the OPA assay a desirable method for monitoring proteolysis are: a) the OPA reagent is soluble and stable in aqueous solutions, b) the reaction with primary amines proceeds essentially to complete within seconds at ambient temperature and c) a single reagent solution serves both to inhibit proteolytic activities and to develop the reaction color. Milk types, treatment and storage of the milk affected the proteolysis measurement by this method so it limited suitable for comparison.

Ninhydrin Method

In ninhydrin method, amino acids are reacted with ninhydrin hydrate at pH 5 and 100°C for a standard period of time, yielding a purple-blue compound. The ammonium salt of diketohydrindylidene and diketohydrindamine is the major product. The absorbance of the purple-blue product is measured at 570 nm. Proline and hydroxyproline give a yellow product. This can be measured at 440 nm. Ninhydrin is perhaps the most widely used reagent, but heating and cooling steps are required, and relatively high blank readings are obtained because of this limited use of this

method (Reimerdes and Klostermeyer, 1976) [95].

Gel Electrophoresis

Proteolysis was also monitored using gel electrophoresis to qualitatively indicate the disappearance of intact milk proteins and large polypeptides and also to observe some newly formed proteolysis products. The gel electrophoresis technique proved useful for qualitative analysis in monitoring proteolysis. However, the use of hazardous chemicals, poor resolution of lower molecular weight peptides (and probable loss of some small peptides which are not retained in the gel) and lengthy analysis, limits its application for routine laboratory analysis [97].

Reversed-Phase HPLC

Reversed-phase high pressure liquid chromatography (RP-HPLC) has proved to be a reliable method with high resolving power for separating peptides from casein hydrolysates [104]. RP-HPLC involves the separation of molecules on the basis of hydrophobicity. The RP-HPLC method is very accurate, sensitive, reproducible and sophisticated compared with the fluorescamine and TNBS methods. It has been reported that the limits of detection of proteinase activity determined by HPLC are approximately 600 times lower than those determined by the fluorescamine methods (Le *et al.*, 2006). It relies on pumps to pass a pressurized liquid solvent containing the sample through a column filled with a solid adsorbent material. The individual component in the sample interacts differently with the adsorbent materials leads to different flow rates. Peptides were separated from milks and casein digests before HPLC analyses either by precipitation of intact casein and large peptides with 10% acetic acid to pH 4.6, or by adding TCA to a final concentration of 4% (w/v). However, the requirement for expensive equipment would limit its use as a method for routine analysis in a quality assurance laboratory.

The advantages and disadvantages of various methods of proteolysis assessment are given in Table 1.

***Table 1**: Advantages and Disadvantages of various Methods of Proteolysis Assessment.*

Method	Advantages	Disadvantages
Folin-Ciocalteu phenol reagent	• A rapid and very sensitive method • Measure minute proteolysis	• Many chemicals interfere with the reaction of the phenol reagent and aromatic amino acids • Microbial proteases liberate low concentrations of aromatic amino acids from casein
Trinitrobenzenesulfonic acid method	• Possible to detect lower levels of proteases • Being simple and rapid method	• Less sensitive
Fluorescamine method	• Simple and Rapid to perform • Very sensitive to low level proteolysis	• Require expensive equipment (spectrofluorometer)
O-Phthaldialdehyde method	• A rapid, sensitive, and convenient method for measurement of proteolysis	• Milk types, treatment and storage of the milk affected the proteolysis measurement • Not suitable for comparative study
Gel-Electrophoresis method	• Less quantity of sample required • Proteolytic product can be differentiate and can be identified • Newly formed proteolysis products can be observe	• The use of hazardous chemicals, poor resolution of lower molecular weight peptides • Probable loss of some small peptides which are not retained in the gel and • Required more time for analysis
RP-HPLC method	• It is very accurate, sensitive, reproducible and sophisticated method • The limits of detection of proteolysis is very lower • Approximately 600 times lower than those determined by the fluorescamine methods	• The requirement for expensive equipment would limit its use as a method for routine analysis

CONCLUSIONS

UHT milk has great scope in India as well as in other countries because of longer shelf life. In UHT treatment, milk heated at such high temperature to ensure the complete destruction of all bacteria, but final bacteriological quality of milk is depend on the initial bacterial load. Freshly drawn milk contains indigenous enzymes, among them protease enzymes gets remarkable consideration. During handling and processing, milk acquires contaminating bacteria that also produce further heat stable proteases. UHT treatment will not be enough to inactivate all of the enzymes; certain proteases are unfolding their structure during heating and again reform their structure and get activated. These indigenous enzymes as well as enzymes originate from the bacteria are capable to fully or partially degrade all milk casein and produce variety of product like polypeptide, small peptide, free amino acids etc., during the storage period. The activities of proteinases that survive the UHT heat treatment may cause age gelation due to release of *β*-κ-complex and aggregation of them; and bitterness in milk because of release of bitter peptides.

Many methods are available for determination of proteolysis in UHT milk. Some of the methods based on measuring the levels of aromatic amino acid containing peptides, hull method is one of them which measure the tyrosine and tryptophan. Other methods are based on detection the level of α-amino groups, it include the trinitrobenzenesulfonic (TNBS) acid, fluorescamine, O-phthaldialdehyde and ninhydrin method. Along with all these method RP-HPLC and gel electrophoresis also use for proteolysis determination in UHT milk; RP-HPLC is the most advanced and very accurate method for determination but it requires expensive equipment assembly. All these methods have their own merits and demerits and depending upon requirement, one has to select one or more methods for the measurement of proteolysis.

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