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ELISA To Detect Proteolysis of Ultrahigh-Temperature Milk upon Storage

DIDIER DUPONT,*,† DAMIEN LUGAND,†,‡ ODILE ROLET-REPECAUD,† AND JACQUES DEGELAEN§

L'Institut National de la Recherche Agronomique (INRA), Unité de Recherches en Technologie et Analyses Laitières, Place du Champ de Foire, F-39800 Poligny, France, and UCB, Chemin du Foriest, B-1420 Braine l'Alleud, Belgium

Casein proteolysis can occur in milk during storage leading to its gelation. The two main proteolytic systems suspected to be involved are the plasmin and the proteases produced by psychrotrophic bacteria. The latter have been shown to cleave κ-casein at the $Phe_{105}-Met_{106}$ bond. Although several techniques allow the determination of plasmin in milk, few rapid and easy-to-perform analytical techniques are available to check for bacterial proteolytic activity. This study presents the development of an inhibition ELISA allowing for the quantification of the κ-casein intact at the $Phe_{105}-Met_{106}$ bond. It uses a monoclonal antibody specifically directed against this peptide bond that binds to the protein as long as the molecule's cleavage site is intact but not when it is cleaved. This simple technique allows for the rapid analysis of more than 20 samples within 3 h. Applied to commercial milks, this assay allowed for the detection of unstable milk.

KEYWORDS: UHT milk; age gelation; proteolysis; κ-casein; ELISA; *Pseudomonas fluorescens*

INTRODUCTION

During the storage of ultrahigh-temperature (UHT) milk, chemical and biochemical reactions may occur and modify its constituents. In particular, casein (CN) can be hydrolyzed by residual proteolytic systems that survive to the heat treatment, leading to the age gelation of milk upon storage (*1*).

Two major proteolytic systems have been suspected to be involved in the age gelation of UHT milks. Plasmin is the major indigenous milk protease and is present in milk together with its inactive precursor, the plasminogen. Plasmin is sufficiently heat-stable to play a part in this biochemical phenomenon. Indeed, a residual plasmin activity has been detected in direct UHT milks heated to 142 °C for 4 s (*2*) and 5 s (*3*). In contrast, no plasmin activity was detectable in indirect UHT milks (142 °C, 3 s), but 19% of the plasminogen was found to remain in milk after indirect UHT treatment (*3*). Nevertheless, activation of plasminogen into plasmin during storage of UHT milk has been reported (*3*−*6*). Both plasmin and plasminogen can be quantified in raw and heat-treated milk either using enzymatic techniques (*7*) or immunological techniques (*8*), making it possible to demonstrate the role of this enzyme when age gelation occurs.

Proteinases produced by some psychrotrophic bacteria have a much higher heat stability than plasmin. For instance, *D* values at 130 °C at 8 and 11 min for the extracellular protease of *Achromobacter* spp. 1−10 and *Pseudomonas fluorescens* 22F were found, respectively (*9*). Such *D* values result in residual activity even after retort sterilization. More recently, the heat stabilities of proteases from 15 strains of *Pseudomonas* isolated from raw milk were compared (*10*). Production of proteases by psychrotrophic bacteria occurs mainly at the end of the exponential growth phase (*11*). Therefore, enzymes should normally not be present in products produced from good quality milk; however, several studies indicated the presence of bacterial proteases in commercial UHT milk (*12*, *13*). The nature of the proteases produced in milk by psychrotrophic bacteria will depend on the bacteria species involved, and within the same species will differ from one strain to another, making their detection by specific antibodies difficult (*14*, *15*).

Proteases from psychrotrophic bacteria have been shown to preferentially hydrolyze κ-CN in an action similar to that of proteinases in calf rennet (*9*, *13*, *16*). The peptide bond $Phe_{105}-Met_{106}$ is cleaved, leading to the release of the C-terminal caseinomacropeptide (CMP) (*17*). Liberation of this hydrophilic peptide from the surface of the CN micelle reinforces the hydrophobic interactions between micelles, resulting in the formation of a gel. Therefore, biochemical techniques like high-performance liquid chromatography (*17*) and capillary electrophoresis (*18*) have been developed to quantify κ-CN proteolysis in milk and to reveal milk instability.

Recently, we proposed another strategy for monitoring the proteolysis process in dairy products (*19*). This approach is based on the production of anti-peptide antibodies directed

* Corresponding author. Tel: (+33)-3-84-73-63-00; fax: (+33)-3-84-37-37-81; e-mail: dupont@poligny.inra.fr.
† INRA.
‡ Present address: ENILBIO, Place du Champ de Foire, F-39800 Poligny, France.
§ UCB.

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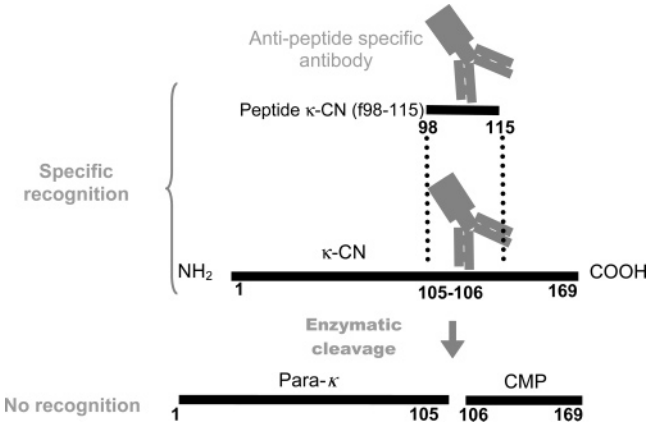

Figure 1. Strategy followed to raise antibodies specifically directed against the Phe_{105}–Met_{106} peptide bond. Peptide κ-CN (f98–115) covering the Phe_{105}–Met_{106} peptide bond was coupled to a carrier protein and used as immunogen. The antibody produced recognizes the protein as long as the 105–106 bond is intact. When enzymatic cleavage occurs, the antibody does not bind to κ-CN anymore.

against the cleavage site on the substrate of the enzyme responsible for proteolysis. As long as the molecule's cleavage site is intact, the antibody will bind to the protein. However, after cleavage of the peptide bond by the protease, the antibody will no longer be able to recognize the substrate (**Figure 1**). Thus, the development of an ELISA that uses this specific antibody allows the hydrolysis of the substrate protein to be monitored. In the present work, we developed an ELISA to detect the hydrolysis of κ-CN at the $Phe_{105}-Met_{106}$ bond, and we applied this technique to the characterization of commercially stable and unstable UHT milks.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, chemicals of analytical grade were purchased from VWR International (Fontenay-sous-Bois, France). αs_1-, αs_2-, κ-, and β-CN were purified as described previously with a minimum purity of 85% (*20*, *21*). CMP and para-κ-CN were kindly provided by Dr. Leonil (INRA, Rennes, France). Bovine serum albumin, α-lactalbumin, β-lactoglobulin, bovine immunoglobulin (Ig), and lactoferrin were from Sigma-Aldrich (Sigma-Aldrich, St. Quentin Fallavier, France).

Milk Samples. UHT milk used for the culture of *P. fluorescens* was of commercial origin. Industrial stable and unstable UHT milks were kindly provided by Arilait Recherches. Milks were considered unstable when manufacturers received client complaints about their gelation after storage or heating.

Peptide Synthesis for Rabbit and Mouse Immunization. The chosen peptide immunogen corresponded to the f99−115 sequence of κ-CN (PHPHLSFMAIPPKKNQD). This peptide was chemically synthesized by Epytop (Nîmes, France), with a purity of 90%. It was modified by C-terminal amidation and the addition of a cysteine residue at the N terminus. To increase their immunogenicity, fractions of the peptide were conjugated 2:1 w/w to keyhole limpet hemocyanin by the N-terminal sulfhydryl group.

Production of Polyclonal and Monoclonal Antibodies Specific for Peptide and κ-CN. Rabbit polyclonal anti-peptide antibodies were produced as previously described (*22*). All the blood samples collected were tested against κ-CN in antigen-coated on plate ELISA (ACP-ELISA), and the one showing the highest response was aliquoted, stored at −20 °C, and called poly-pep. Polyclonal antiserum against κ-CN was obtained from a rabbit immunized with 0.5 mg aliquots of κ-CN according to the same procedure as the one used for the synthetic peptide. All the blood samples collected were tested against κ-CN in ACP-ELISA, and the one showing the highest response was aliquoted, stored at −20 °C, and called poly-κ.

Mouse monoclonal anti-peptide antibodies were produced as described previously (*23*). Supernatants of hybrid clones were assayed by ACP-ELISA toward κ-CN. Ascites from the clone showing the highest response were obtained according to the procedure previously described (*24*). Monoclonal antibodies were purified from ascitic fluid by affinity chromatography using a HiTrap NHS-activated HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) as described previously (*25*). The purified antibodies were aliquoted, stored at −20 °C, and called mono-pep.

ACP-ELISA for Characterization of Antibody Specificity. This method was used to test the antibodies against the immunization peptide, κ-CN, para-κ-CN, and CMP and to check possible mono-pep cross-reactions with αs_1-CN, αs_2-CN, β-CN, α-lactalbumin, β-lactoglobulin, bovine serum albumin, Ig, and lactoferrin. Briefly, 100 μL of each antigen at 0.5 μg/mL in 0.1 M bicarbonate buffer, pH 9.6, was coated onto a microtiter plate (NUNC F96 Maxisorp, Dominique Dutscher, Brumath, France) and incubated for 1 h at 37 °C. The remaining binding sites were blocked by incubating 250 μL of gelatin (VWR International) at 10 g/L phosphate-buffered saline, 0.05% Tween 20 (PBS-T) for 1 h at 37 °C. Hybridoma culture supernatants and rabbit antibodies were diluted 1:2 and 1:500 in PBS-T, respectively, and incubated for 1 h at 37 °C. Bound mouse Ig was detected by incubating 100 μL of donkey anti-mouse Ig alkaline phosphatase conjugate (Jackson Immunoresearch, Interchim, Montluçon, France) diluted 1:3000 in PBS-T for 1 h at 37 °C. Bound rabbit Ig was detected by incubating 100 μL of goat anti-rabbit Ig alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1:3000 in PBS-T for 1 h at 37 °C. Wells were rinsed between incubation steps for 15 s with four changes of 250 μL of PBS-T. Following the last rinsing, 100 μL of *p*-nitrophenyl phosphate (Sigma-Aldrich) at 1 g/L in 1 M diethanolamine-HCl, 1 mM $MgCl_2$, and 0.1 mM zinc acetate was incubated in the wells. After 30 min at 37 °C, the absorbance at 405 nm was read against a blank and corrected according to the background signal in the absence of antigen.

Monoclonal Antibody Mono-pep Affinity Constant toward κ-CN. The monoclonal antibody mono-pep affinity constant toward κ-CN was determined on a Biacore 3000 (Biacore International AB, Uppsala, Sweden) as described previously (*26*).

Quantitative Inhibition ELISA. An inhibition ELISA was performed for the quantification of κ-CN intact at the $Phe_{105}-Met_{106}$ bond. ELISA plates were coated with 0.5 μg/mL purified κ-CN in 0.1 M bicarbonate buffer, pH 9.6 (100 μL per well), and incubated for 1 h at 37 °C. Blocking of the remaining binding sites was performed with 250 μL of gelatin at 10 g/L in PBS-T for 1 h at 37 °C. Serial dilutions of a milk powder of known κ-CN concentration (*23*) in 0.4 M trisodium citrate, 0.05% Tween 20 (TCS-T) were used as standards (κ-CN concentrations ranging from 0 to 20 μg/mL). Milk samples diluted in TCS-T (four dilutions from 1:1000 to 1:10 000, 75 μL) were incubated in test tubes with 75 μL of a 1:250 dilution of purified monoclonal antibody mono-pep for 10 min at 37 °C. One hundred microliters of the mixture was then added to each ELISA plate well and further incubated for 30 min at 37 °C. The reaction was revealed by incubating 100 μL of donkey anti-mouse Ig alkaline phosphatase conjugate (Jackson Immunoresearch) diluted 1:3000 in PBS-T for 1 h at 37 °C. Finally, 100 μL of *p*-nitrophenyl phosphate (Sigma-Aldrich) at 1 g/L 1 M diethanolamine-HCl, 1 mM $MgCl_2$, and 0.1 mM zinc acetate was incubated in the wells. After 30 min at 37 °C, the absorbance at 405 nm was read against a blank.

Analytical Characteristics of the ELISA. The detection limit of the assay was calculated from the mean of the measured content of a representative blank sample ($n = 20$) plus 3 times the standard deviation of the mean (mean + 3SD). Assay precision was defined by determining intra-assay variation that was obtained by 10 successive analyses of the same sample at four different dilutions (1:1000, 1:2500, 1:5000, and 1:10 000).

To determine accuracy, the recovery of exogenous amounts of κ-CN added to raw milk was calculated. Eight different quantities of milk powder corresponding to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 mg/mL κ-CN were added to the raw milk sample. Each sample was analyzed in duplicate at four dilutions (1:1000, 1:2500, 1:5000, and 1:10 000).

Strains of Proteolytic Psychrotrophic *P. fluorescens*. Two strains (CNRZ 793 and CNRZ 795) were taken from the INRA collection.

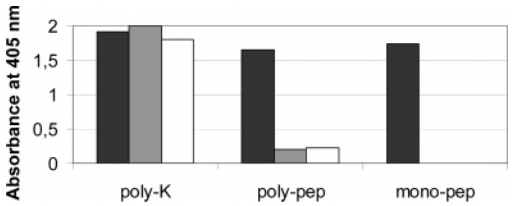

Figure 2. ELISA reactivity of antibodies poly-κ, poly-pep, and mono-pep toward κ-CN (black bars), para-κ (gray bars), and CMP (white bars).

They were stored at −20 °C in a glycerol solution (300 mL/L) and culture broth and were reactivated by two successive 24 h cultures at 30 °C in a brain−heart infusion (Biokar Diagnostics, Pantin, France) at 30 °C before inoculation to milk samples.

Microbiological Analyses. Microbial counts were made by enumerating the colonies on the surface of a nutritive agar inoculated according to the spiral plate count method (*27*) after homogenizing the milk sample for 30 s with an Ultraturrax (IKA, Staufen, Germany) as described previously (*28*). All these analyses were run in duplicate, and results were expressed as cfu/mL milk. The psychrotrophic *Pseudomonas* were determined by identification of lactose-negative colonies on violet red bile agar after 24 h of incubation at 30 °C, followed by 24 h of incubation at room temperature.

Inoculation Procedure of a Milk Sample with Strains CNRZ 793 and 795. The culture broth of each pure strain was homogenized for 30 s using an Ultraturrax (IKA) at 20000 revolutions/min, then diluted 1:10 in peptone water (1 g/L). Samples of this dilution were inoculated at 4.5 10^5 cfu/mL into 500 mL of UHT milk: 0.1 mL for strain CNRZ 793 and 0.5 mL for strain CNRZ 795. A third flask containing 500 mL of UHT milk was not inoculated and was used as a negative control. The three flasks were incubated at 37 °C for 5 days, and 10 mL aliquots were collected aseptically every 12 h for *P. fluorescens*, κ-CN, and pH determination.

Determination of Protease Optimum Temperature. Proteolysis caused by a thermoresistant proteinase in a UHT milk sample could be exacerbated by incubation of the milk sample at the proteinase optimum temperature. This temperature was therefore determined for the two strains (CNRZ 793 and 795) that were available. A 500 mL flask of UHT milk was inoculated with strains CNRZ 793 and 795 at 4.5 10^5 cfu/mL and incubated for 5 days at 37 °C. This resulted in the destabilization of milk. Then, the milk samples were centrifuged at 50 000*g* for 1 h at 20 °C. The supernatants were collected, stored at −20 °C, and were considered as a source of *P. fluorescens* protease. Ten milliliters of UHT milk samples was inoculated with 10 μL of the protease preparations and incubated for 2 h at 30, 35, 40, 45, 50, and 55 °C. Since the optimum temperatures determined from this first experiment were equivalent for both strains and found to be around 45 °C, the experiment was carried out again, the inoculated milks this time being incubated at 40, 42.5, 45, 47.5, and 50 °C.

Application of the Inhibition ELISA to Stable and Unstable UHT Milks. Thirteen stable and nine unstable UHT milks were kindly provided by Arilait Recherches. κ-CN intact at the Phe_{105}−Met_{106} bond was quantified using the ELISA inhibition assay as described previously. All the samples were quantified at their reception and after a 24 h incubation at 47.5 °C. Significance of the differences observed between stable and unstable milk κ-CN concentration was assessed by a Student's *t* test.

RESULTS

Production of Monoclonal Antibodies. One fusion experiment yielded 131 hybridomas. Among those, 27 recognized the immunization peptide, but only one (mono-pep) cross-reacted with purified κ-CN. Its affinity constant (K_D) toward κ-CN, as determined on Biacore, was 6.09×10^{-9} M.

Characterization of Antibody Specificity. Poly-κ, poly-pep, and mono-pep antibodies were tested in ACP-ELISA toward κ-CN, para-κ-CN, and CMP (**Figure 2**). Poly-κ reacted equally with κ-CN, para-κ-CN, and CMP. Poly-pep was able to recognize κ-CN but showed only low reactivity toward para-κ-CN and CMP. Finally, mono-pep reacted only against κ-CN. The sharp specificity of mono-pep is in complete accordance with what was expected and demonstrates that this antibody is directed against the Phe_{105}−Met_{106} peptide bond. Finally, mono-pep was tested in ACP-ELISA against αs_1-CN, αs_2-CN, β-CN, α-lactalbumin, β-lactoglobulin, bovine serumalbumin, Ig, and lactoferrin and did not show any cross-reaction with these proteins. From these results, mono-pep was selected to be used in the inhibition ELISA.

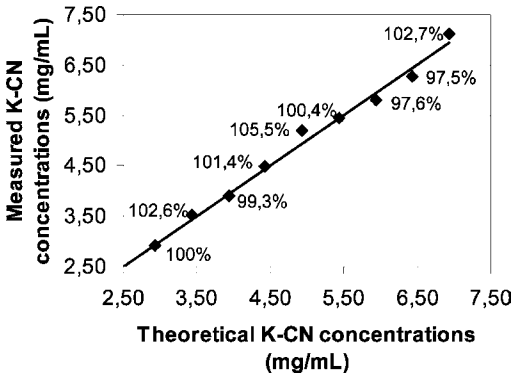

Figure 3. Determination of recovery (in %), after exogenous addition of κ-CN, in a milk sample. Eight different quantities of milk powder corresponding to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 mg/mL κ-CN were added to a raw milk sample that was analyzed, in duplicate at four dilutions.

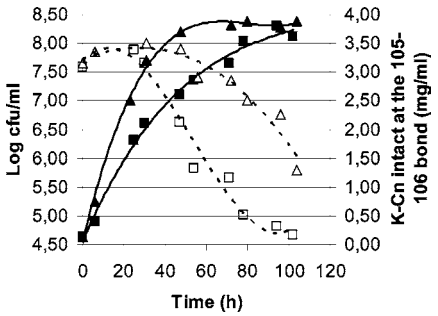

Figure 4. Growth of *P. fluorescens* strains CNRZ 793 (▲) and 795 (■) and the evolution of κ-CN intact at the Phe_{105}–Met_{106} peptide bond hydrolyzed by strain 793 (△) and 795 (□) when inoculated to a UHT milk stored for 5 days at 37 °C.

Analytical Characteristics of the ELISA. Several attempts have been made to reduce the duration of the assay (data not shown). The incubation step between mono-pep and κ-CN was reduced to 10 min, whereas incubation of the antigen−antibody mixture on the coated κ-CN was optimal at 30 min. The detection limit, determined from the mean of the measured content of a representative blank sample ($n = 20$; mean + 3SD), was established at 15.3 ng/mL. The reproducibility established for the intra-assay was 8.9%. Finally, exogenous additions of eight different quantities of milk powder corresponding to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 mg/mL κ-CN were recovered between 97.5 and 105.5% (**Figure 3**).

Milk Proteolysis Caused by Strains CNRZ 793 and 795. *P. fluorescens* CNRZ 793 and 795 strains showed different behaviors when inoculated at 4×10^5 cfu/mL to UHT milk for 5 days at 37 °C (**Figure 4**). Strain 793 grew rapidly to reach the stationary phase after 48 h. In contrast, it took almost 5 days for strain 795 to reach the stationary phase. Proteolytic activity of strain 795 toward the Phe_{105}−Met_{106} κ-CN bond began after 32 h of incubation and led to the hydrolysis of all of the κ-CN present in UHT milk after a 5 day incubation. Strain

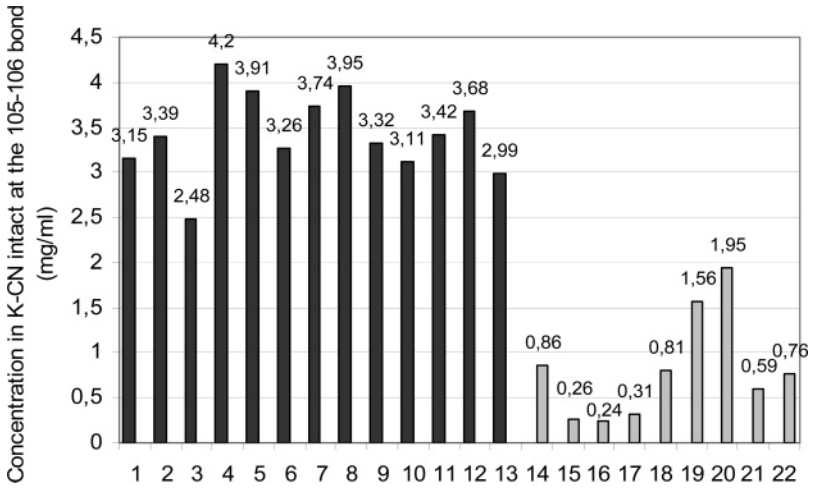

Figure 5. Concentrations in κ-CN intact at the Phe_{105}–Met_{106} peptide bond found by ELISA in 13 stable (black bars) and nine unstable (gray bars) commercial UHT milk samples.

793 showed weaker proteolytic activity toward the Phe_{105}−Met_{106} κ-CN bond, and less than 50% of the κ-CN was hydrolyzed after 5 days of incubation.

Determination of Protease Optimum Temperature. In a first experiment, UHT milk aliquots were incubated for 5 days at 30, 35, 40, 45, 50, and 55 °C with a protease preparation of strains CNRZ 793 and 795. The protease optimum temperatures determined from this first experiment were equivalent for both strains and found to be around 45 °C. Then, a second experiment was carried out, the inoculated milks being this time incubated at 40, 42.5, 45, 47.5, and 50 °C for 5 days. Results obtained were again identical for both strains, and the optimum temperature was found to be 47.5 °C.

Application of the Inhibition ELISA to Stable and Unstable UHT Milks. The concentration of κ-CN intact at the Phe_{105}−Met_{106} bond was determined by ELISA in 13 stable and nine unstable UHT milks with and without 24 h incubation at 47.5 °C. Incubation of the samples at 47.5 °C did not change the κ-CN concentrations measured on both stable and unstable UHT milks, showing that even unstable milks lose their bacterial proteolytic activity after a certain time of storage. Stable UHT milks showed κ-CN intact at the Phe_{105}−Met_{106} bond concentrations ranging from 2.48 to 4.20 mg/mL. In contrast, those obtained for unstable UHT milks were significantly lower ($p < 0.001$) and ranged from 0.24 to 1.95 mg/mL (**Figure 5**).

DISCUSSION

The development of an ELISA allowing the quantification of κ-CN intact at the Phe_{105}−Met_{106} bond in UHT milk is presented in this study. This technique is based on the use of a monoclonal antibody specifically directed against this peptide bond. Applied to a limited number of commercial stable and unstable UHT milks, the techniques showed that destabilization of UHT milk was accompanied by an extensive proteolysis of κ-CN at the 105−106 peptide bond. This technique could therefore constitute a powerful tool to quickly detect UHT milk destabilization upon storage.

The ELISA is based on the use of a monoclonal antibody specifically directed against the 105−106 peptide. Immunizing rabbits with the whole κ-CN molecule raised an antiserum that reacted equally with κ-CN and its two fragments generated by chymosin (i.e., para-κ-CN and CMP). When rabbits were immunized with the synthetic peptide κ(f99−115) covering the 105−106 cleavage site, the antiserum obtained reacted strongly with κ-CN and showed little cross-reaction with either para-κ-CN or CMP. Use of such a synthetic peptide immunogen allowed for the orientation of the host immune response toward the area of interest, most of the Igs of the serum being directed against the 105−106 bond. However, slight cross-reactions still observed with para-κ-CN and CMP proved that among the antiserum raised, some Igs were directed against the 99−105 and the 106−115 region of the immunization peptide. It is generally accepted that the minimal length of a continuous B-cell epitope is around six amino acids, whereas T-helper cell epitopes have been shown to be −nine to 11 amino acids (*29*). The 17 amino acid immunization peptide we used may therefore contain overlapping B- and T-helper cell epitopes. Lower cross-reactivities with para-κ-CN and CMP could have been obtained by using a shorter peptide. However, we have already shown that the length of a synthetic immunization peptide was a key parameter for causing the immune response of a host, the optimal length being around 20 amino acid residues (*22*). The absence of cross-reactions between mono-pep and para-κ-CN and CMP shows that this monoclonal antibody is perfectly directed against an epitope that comprises the 105−106 peptide bond. It seems that we were quite lucky to obtain this monoclonal antibody since among the 27 hybridomas that were producing anti-peptide antibodies, mono-pep was the only one to cross-react with κ-CN. It is therefore possible that the 99−115 peptide, when synthesized, adopts a conformation different from the one it has in κ-CN.

The inhibition ELISA conditions were optimized to make the assay as rapid as possible. The contact time between mono-pep and κ-CN was only 10 min without generating a significant decrease of the signal. This reduction of time was probably made possible because of the high affinity existing between mono-pep and κ-CN ($K_D = 6.09 \times 10^{-9}$ M).

The ELISA developed allowed us to study the proteolysis caused by two proteolytic strains of *P. fluorescens* on κ-CN at the 105−106 bond. Strains CNRZ 793 and 795 showed different behaviors in terms of growth and proteolytic activity (**Figure 4**). Indeed, strain CNRZ 793 showed a rapid growth, but its proteolytic activity began only at the beginning of the stationary phase and was limited. In contrast, strain CNRZ 795 showed a slower growth, but its proteolytic activity began at the beginning of the exponential phase of growth. There is no general agreement on this point in the literature. Whereas some authors found that the protease synthesis occurred mainly at the end of

the exponential phase and the beginning of the stationary phase (*9*), some observed maximal proteolytic activity during the stationary phase (*30*) and others during the exponential phase of growth (*31*).

The activity optimum temperature that we observed for both strains was 47.5 °C. This is in perfect accordance with the results of other researchers that found the optimal temperature to be around 45 °C (*32*). However, no residual proteolytic activity was found on commercial unstable milks by incubating them at 47.5 °C. This tends to demonstrate that after a certain time of storage, the bacterial proteolytic activity responsible for the milk instability decreases until total disappearance, this probably being the result of protease autodegradation, although subsequent work is needed to demonstrate this hypothesis.

Application of the assay to stable and unstable UHT milks showed that values obtained for unstable milks were lower than the ones obtained for stable milks. It looks as if the assay allows proteolysis at the $Phe_{105}-Met_{106}$ bond of κ-CN to be detected. These are only preliminary results obtained on a very limited number of samples. We will now have to determine whether unstable milks give statistically significant lower concentrations of κ-CN intact at the $Phe_{105}-Met_{106}$, by analyzing many samples. This work is currently in progress, but as destabilization phenomena are quite rare, it will take a few years to obtain a sufficient number of data.

In conclusion, we have developed an ELISA method allowing for the detection of instability in UHT milk. However, this technique could be even more useful if it could have a predictive value and could be applied directly to raw milk, before heat treatment. To reach this goal, we need to determine the optimal conditions for incubating the milk to exacerbate the proteolysis phenomenon and be able to detect it by ELISA. Such work is also currently in progress.

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