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Note

C-terminal end of κ-casein is hydrolysed before renneting during soft cheese manufacture

Rachel BOUTROU*, Daniel MOLLÉ, Joëlle LÉONIL

Laboratoire de Recherches de Technologie Laitière, INRA, 65, rue de Saint-Brieuc, 35042 Rennes Cedex, France

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Abstract – By studying the hydrolysis of κ-casein during soft cheese manufacture, we demonstrated that this casein was hydrolysed before renneting during milk maturation. The quantitative time course of the peptides κ-CN (f161-169) and κ-CN (f162-169) was determined throughout the drainage, and the CMP was sought out within the milk before inoculation and before renneting. In this way, we showed that before renneting, the lactococcal cell envelope proteinase produced the peptide κ-CN (f161-169) mainly from κ-casein, if not all, whereas the peptide κ-CN (f162-169) originated from CMP through the action of chymosin.

κ-casein / lactococcal starter / milk maturation / soft cheese

Résumé – L'extrémité C-terminale de la caséine κ est hydrolysée pendant la maturation du lait. Étudiant l'hydrolyse de la caséine κ au cours de la fabrication d'un fromage à pâte molle, nous avons montré que cette caséine était hydrolysée dès la maturation du lait par le levain lactique. Le suivi quantitatif des peptides κ-CN (f161-169) et κ-CN (f162-169) tout au long de l'égouttage, ainsi que la recherche de CMP dans le lait avant inoculation et avant emprésurage, ont été réalisés. Nous avons ainsi montré qu'avant l'emprésurage, la protéase de paroi du lactocoque produit le peptide κ-CN (f161-169) majoritairement à partir de la caséine κ, si ce n'est en totalité, tandis que le peptide κ-CN (f162-169) est issu du CMP sous l'action de la chymosine.

Caséine κ / levain lactique / maturation du lait / fromage à pâte molle

1. INTRODUCTION

Hydrolysis of bovine κ-casein by chymosin (EC 3.4.23.4) constitutes the first major proteolytic event of cheese-making. During this stage, the Phe_{105}-Met_{106} bond of κ-casein is rapidly hydrolysed, producing a N-terminal fragment (para-κ-casein, residue 1-105) and a C-terminal fragment (caseinomacropeptide (CMP), residue 106-169). Subsequent to the hydrolysis of this bond, CMP is released in the whey whereas para-κ-casein remains attached to the micelle. Although the hydrolysis of the bond

* Correspondence and reprints
E-mail: rboutrou@rennes.inra.fr

Phe_{105}-Met_{106} by milk-clotting enzymes has been extensively investigated [5, 6, 10–12, 17, 18], the action of these enzymes on further hydrolysis of κ-casein has been little studied. In fact, κ-casein has been shown to be relatively resistant to the action of chymosin at pH 6.6, 5.5 and 4.6 [5, 16, 17]. Chianese et al. [4] have identified in the acid whey some peptides hydrolysed from the κ-casein. Some peptides originating from this casein were also identified in the whey obtained during soft-type cheese-making [2].

In addition to coagulant enzymes added to milk, some enzymes are active during cheese manufacture. Among them, the cell envelope proteinase from lactococci, now named lactocepin (EC 3.4.21.96) is of special importance [13]. The occurrence of two main types of lactocepin (lactocepin I and lactocepin III) and of a number of proteinases of mixed-type specificity towards caseins has been established for lactococci [9]. The substrate specificity of several types of proteinase towards α_{s1}-, α_{s2}-, β- and κ-casein in a model system has been the subject of various reports [9, 13]. Little is known about the action of this enzyme in cheese.

The objective of the study was to determine κ-casein hydrolysis during soft-type cheese manufacture. With this aim, we quantitatively studied the time course of peptides released during cheese-making and the detection of CMP. The fragments κ-CN (f161-169) and κ-CN (f162-169) were chosen because, in a previous study [2], they were identified early in the expelled whey. In addition to the quantitative study of the release of both peptides, we determined which enzyme was responsible for their formation, as well as their origin, i.e. κ-casein or CMP.

2. MATERIALS AND METHODS

2.1. Milk and whey samples

Cheese milk was microfiltered before being inoculated with the single lactococcal strain *L. lactis* subsp. *lactis* AM2. After milk maturation, renneting was carried out using pure recombinant chymosin. Subsequent to milk coagulation, the curd was cut, gently stirred, and the whey expelled. The detailed making process of the soft-type cheese (three manufactures) has been previously described [3]. Analyses were performed on skim milk collected before lactic starter inoculation, on maturated milk collected before the renneting, and on the whey collected at each step of the process, i.e. at cutting, drawing, the three turns, and at demoulding.

2.2. Quantification of the peptides κ-CN (f161-169) and κ-CN (f162-169)

The C-terminal fragment of κ-casein (f161-169) was targeted in milk and whey samples. It corresponds to the peptide TVQVTSTAV, with a 905.0 $g \cdot mol^{-1}$ theoretical molecular mass. The calibration was performed using the corresponding synthetic peptide as a standard (Neosystem, Strasbourg, France), solubilised at 2 $mg \cdot mL^{-1}$ in distilled water and diluted in the range from 0 to 50 pmol using 20% acetonitrile and 0.1% TFA buffer, i.e. the conditions used for the elution of the relevant peptide. Calibration was performed from total ion count (TIC). The peptide κ-CN (f162-169) was also quantified. Because its ionisation yield did not differ from that of the peptide κ-CN (f161-169), the same curve of calibration was obtained for both peptides.

Fixed volumes of 6 mL samples were used in order to calculate the concentration of both peptides. Before analysis of the samples, whey proteins were precipitated in 6% TCA for 30 min at 30 °C and centrifuged (2500 *g*, 10 min at 20 °C). The supernatant was filtered through a 0.45 μm filter (Minisart, Sartorius, Göttingen, Germany). The samples were desalted by solid phase extraction (Chromabond® C_{18}ec polypropylene column; Macherey-Nagel, Hoerd, France) using acetonitrile as eluent, and concentrated by drying (Speed-Vac

concentrator, Bioblock, Paris, France). The whey powder was solubilised in 1 mL 0.2% trifluoroacetic acid (TFA; Pierce, Touzart et Matignon, Vitry-sur-Seine, France) and filtered through a 0.45 μm filter before analysis.

The samples were analysed on an Agilent HP1100 LC System (Bios Analytique, Courtabœuf, France) directly interfaced with the mass spectrometer API III Plus (ESI-MS/MS) (Sciex, Thornill, Canada). 40 μL samples were injected on a C_{18} symmetry column (2.1 × 150 mm, Waters, Milford, MA, USA) using solvent A (0.106% TFA (v/v) in MilliQ water) and solvent B (0.1% TFA (v/v) and 80% acetonitrile (v/v) in MilliQ water). A linear gradient from 2 to 53% solvent B for 70 min was applied for the elution at a flow rate of 0.25 mL·min^{-1} at 40 °C and detection was performed at 214 nm. The mass analyses were determined by on-line coupling between reverse-phase HPLC and mass spectrometry using an electrospray tandem mass spectrometer. The ion source voltage was set at 4.8 kV and the nozzle voltage at 80 V. The quadrupole mass analyser (in the Q3 quadrupole mode) was scanned over a mass-to-charge range of 600–2200 g·mol^{-1} with a step size of 0.3 g·mol^{-1} and a dwell time of 0.5 ms per step. The molecular masses were determined from these data using an Apple Macintosh computer power PC 80/8100 and the software package Biomultiview 1.3.1 supplied by Sciex (Thornill, Canada). After extraction of a mass-to-charge of 905 g·mol^{-1} and verification of the elution time of the peak (32 min), TIC was measured over 20 scans for the samples. According to the calibration table obtained by direct injection in ESI/MS, the concentration of each peptide was determined by using the package software MacQuant 1.4 supplied by Sciex (Thornill, Canada).

2.3. Detection of CMP

After pH 4.6 precipitation of caseins by addition of 1N HCl, centrifugation and filtration of the supernatant through a 0.45 μm filter, the presence of CMP in milks was sought out. Milk before inoculation and milk before renneting were analysed using LC/MS as described above, except with a mass-to-charge range of 800–2400. In bulk milk from Holstein cows, i.e. milk we used to manufacture soft-type cheeses, CMP_A represents more than 70% of total CMP. Thus, to seek out CMP in milk samples, the molecular mass 6787 g·mol^{-1} for the aglyco-CMP_A was expected by the detection of their specific ions 1359^{5+}, 1698^{4+} and 2264^{3+}.

2.4. Bacterial enumeration

1 mL milk or 1 g curd was homogenised with an Ultra-Turrax disperser (Janke and Kunkel GmbH, Staufen, Germany) twice for 30 s in 10 mL peptone water. The starter lactococci were enumerated on lactose M17 plates (Difco) after 48 h incubation at 30 °C.

3. RESULTS

First, the presence of the peptide κ-CN (f161-169) in milk and whey samples was detected using LC-MS. Except in the milk before starter inoculation, this peptide was found in all samples, i.e. from the milk collected before the renneting to the whey collected at the demoulding. Second, the release time course of this peptide was followed (Fig. 1). The quantification was achieved according to the calibration curve (r^2 = 0.9884). The concentration of κ-CN (f161-169) increased from the inoculation time to the cutting of the curd and reached 224 pmol·mL^{-1} whey. Thereafter, the concentration of κ-CN (f161-169) remained almost constant until the end of drainage.

The peptide κ-CN (f162-169) was also quantified in milk and in whey collected during drainage (Fig. 1). No peptide was found in milk either before starter inoculation or before renneting. This peptide appeared in the whey at the cutting step,

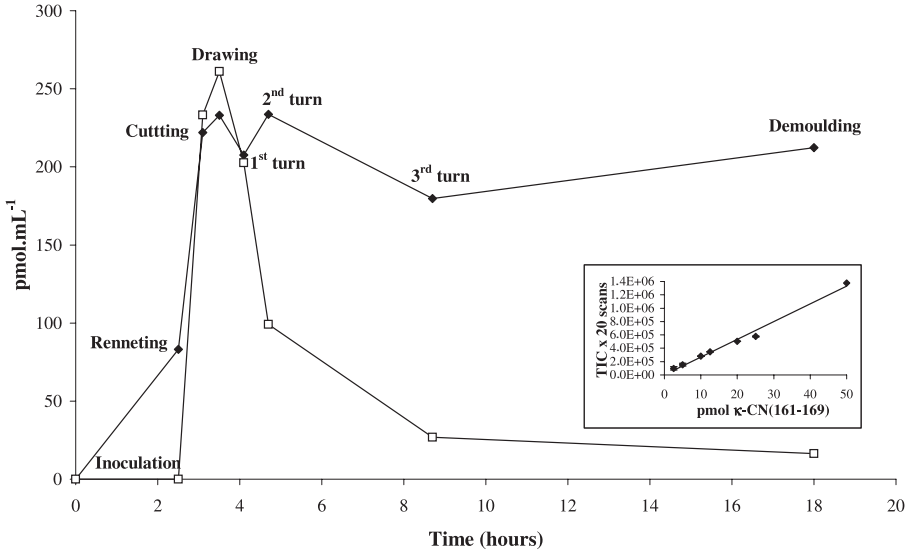

Figure 1. Quantification of the peptides κ-CN (f161-169) (♦) and κ-CN (f162-169) (□) in the milk before starter inoculation and before renneting, and in the whey samples at each step of the drainage. The standards used for the calibration are visualised in the insert.

and was then in equal concentration to that of the κ-CN (f161-169). Both peptides remained at the same concentration until the first turn. Thereafter, the concentration of κ-CN (f162-169) decreased drastically and only a small quantity was found at the end of drainage.

No specific ions of aglyco-CMP_A were detected in milk either before inoculation or before renneting. Consequently, if there was CMP in these milk samples, its quantity was under the detection threshold of the method used. The threshold, maximum 0.01 µg, corresponds to 0.25 µg·injected mL^{-1}, i.e. 37 pmol·mL^{-1} milk. This concentration is 2.2 times lower than the concentration of the peptide κ-CN (f161-169) measured in the milk before renneting. Thus, we can say that almost half the quantity of the peptide originated from κ-casein, and little, if any, from CMP.

The growth curve of the lactococcal starter is presented in Figure 2. The exponential growth phase began at renneting and was nearly ended at the first turn. From the second turn, the starter was in the stationary growth phase.

4. DISCUSSION

4.1. The peptide 161-169 comes from κ-casein through lactocepin activity

In a previous study, 17 peptides originating from κ-casein were identified at the end of soft-type cheese-making [2]. Among them, the peptide κ-CN (f161-169) was identified as soon as the first turn, i.e. 1.5 h after renneting. Thus, we wanted to determine the earliest step of cheese-making at which this peptide was released.

The identification of the peptide κ-CN (f161-169) in milk before addition of chymosin was of great importance. It showed that κ-casein was already cleaved before renneting during soft cheese manufacture. Since at this stage of the cheese-making,

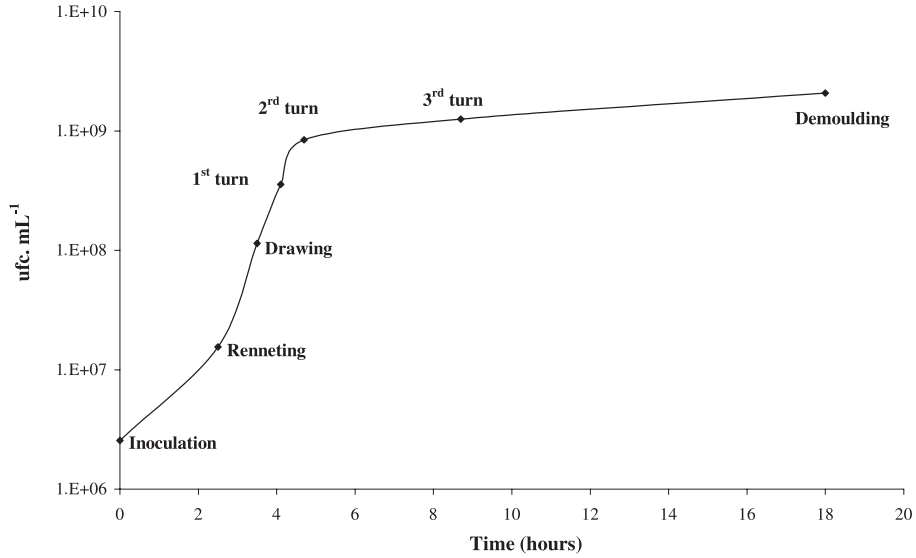

Figure 2. Enumeration of the lactococcal starter AM2 throughout the drainage period.

only the starter was added into the milk, the release of this peptide results from the action of the lactocepin of *L. lactis* AM2. Indeed, lactococcal proteinase was active during milk maturation to ensure the growth of the strain. Furthermore, the growth curve of the lactococcal starter fits in with the production of the peptide κ-CN (f161-169) throughout the draining period. The bond Asn_{160}-Thr_{161} of κ-casein was shown to be rapidly cleaved by the P_{III}-type proteinase of *L. lactis* AM1 [19] and *L. lactis* SK11 [15], and the proteinase of AM2 had been classified into a "deviating P_{III}-type group" according to its specificity towards α_{s1}-CN (f1-23) [9]. Furthermore, we demonstrated that most, if not all, of the peptide was produced directly from κ-casein. This finding is against the currently accepted idea that the site Phe_{105}-Met_{106} is the first to be hydrolysed of the κ-casein. The peptide κ-CN (f161-169) was also one of the most identified oligopeptides of acid whey from Quarg cheese [4], but since this peptide was identified in a whey representative of the whole drainage, no information about the beginning of the release of this peptide was available.

In addition to its specificity in situ, the hydrolysis of the 160-161 bond of κ-casein by the lactocepin of *L. lactis* allows us to gain knowledge of the role of the proteolytic system of *L. lactis* used as a starter during the manufacture of soft cheese. Indeed, our results demonstrated that the proteolytic system (proteolytic enzymes and transport systems) used among others some peptides, which directly originated from κ-casein to ensure the initial growth of the lactococcal starter on milk. Further release of κ-CN (f161-169) from κ-casein by the lactocepin occurred as *L. lactis* was in the exponential growth phase.

4.2. The peptide 162-169 comes from CMP through chymosin activity

The peptide κ-CN (f162-169) was first identified at the cutting of curd. This peptide might have several origins: κ-casein, CMP or κ-CN (f161-169). The latter case implies the action of an aminopeptidase.

This excludes the action of the chymosin that does not have such an activity. Aminopeptidases are liberated consecutive to the lysis of lactococcal cells. But the strain AM2 did not lyse after two days of ripening [1] and a fortiori the cells did not lyse only a few hours after the beginning of the cheese-making. Thus the fragment 162-169 is most probably released from κ-casein or CMP, through the action of lactocepin or chymosin. The bond 161-162 has never been reported to be cleaved by purified lactocepin, i.e. free enzyme, from either κ-casein or CMP [15, 19], even in various humectant systems in which the water activity and the salt concentration were equivalent to those measured in Cheddar cheese [14]. In our study, the bond 161-162 might have been cleaved by the anchored lactocepin, whose specificity differs from that of the free one [8]. However, if the anchored lactocepin had such an activity, the peptide should have already been identified in milk at renneting. As it was not, the peptide κ-CN (f162-169) may originate from the action of chymosin on κ-casein or CMP. Few peptides result from the action of chymosin incubated with κ-casein, and none originate from its C-terminal region. But some data were derived from incubations of chymosin with purified CMP [16] under neutral to mild acid conditions, i.e. conditions at the renneting of soft cheese curd. They allowed us to identify peptides originating from the C-terminal end of CMP, among them the fragments (f161-169) and (f162-169). Thus the peptide κ-CN (f162-169) most probably originates from CMP, liberated through the action of chymosin from the renneting. This is reinforced by the observed decrease in the κ-CN (f162-169) concentration after the drawing. Indeed, because 76% of both the chymosin and the CMP were thrown away at moulding [3], chymosin could no longer produce from CMP the peptide κ-CN (f162-169), whose quantity largely decreased thereafter.

The amount of CMP within the whey collected throughout the drainage was determined using the cation exchange FPLC method as described by Léonil and Mollé [10] (data not shown). The concentration of CMP measured in whey at the cutting ($0.91\ g{\cdot}L^{-1}$) was in agreement with those obtained from renneted raw milk by Ferron-Baumy et al. [7]. The concentration of CMP in whey decreased thereafter, showing that the CMP was degraded from the drawing step. This result is in opposition to the common accepted idea that the whole CMP is eliminated since it is within the whey.

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