
1 OLIGOPEPTIDES IN PARMIGIANO-REGGIANO CHEESE: MOLECULAR MARKERS OF TYPICALITY, TECHNOLOGY AND AGEING

1.1 Introduction

The conversion of fresh into mature cheese is largely influenced by proteolysis, although other factors, such as lipolysis and lactic and propionic acid fermentation are especially important for particular types of cheeses. Proteolysis is the most complex event in cheese ripening as it contributes to textural changes (breaking down the α S 1, α S 2, β and para-k casein network), it decreases the water activity through water binding by setting free carboxyl and amino groups, it increases the pH by producing NH_3 from deamination of free amino acids. Moreover, proteolysis directly contributes to flavour (release of peptides and amino acids) and off-flavours (bitter hydrophobic peptides), also liberating substrates for others reactions. Thus, for the development of an acceptable cheese flavor, a well-balanced breakdown of the protein (i.e., casein) into small peptides and amino acids is necessary.ⁱ

Proteolysis in cheese during ripening is catalyzed by the residual activity of the rennet enzymes (chymosin, pepsin or acid proteases), natural milk proteases (plasmin, cathepsins and somatic cells proteases), and proteolytic enzymes from starter lactic acid bacteria, secondary inoculum and non-starter microflora.ⁱⁱ Different enzymes with different specificities belong to the general class of “proteases”: enzymes specific towards proteins are called *proteinases*, enzymes specific towards oligopeptides are so called *peptidases*. A common feature of peptidases is their inability to hydrolyze intact casein but they have the ability to hydrolyze internal peptide bonds of casein-derived peptides. Peptidases are distinguished between *endo-* or *exo-peptidases* if they degrade internal bonds of the peptide or only terminal amino acids. Enzymes of rennet and milk are essentially proteinases and they are known to be highly specific towards caseins. LABs have a more complex enzymatic system which involves: a cell wall-associated caseinolytic proteinases which can hydrolyse intact caseins of high MW peptides formed by, for example, chymosin; extracellular peptidases, specific towards oligopeptides; amino acid and peptide transport systems; intracellular peptidases. All these enzymes together form the complex proteolytic system of lactococci (Figure 1.1). The progress of proteolysis in many ripened cheeses is first catalyzed by the residual chymosin (which has an hydrophilic character and has more affinity for the whey fraction, but it can also be partially trapped in the caseins network) and, to a lesser extent, by plasmin and milk proteinases, resulting in the formation of large and intermediate-sized peptides which are subsequently degraded by the coagulant and enzymes from the starter and non starter microflora of the cheese, resulting in the production of small peptides and free amino acids.

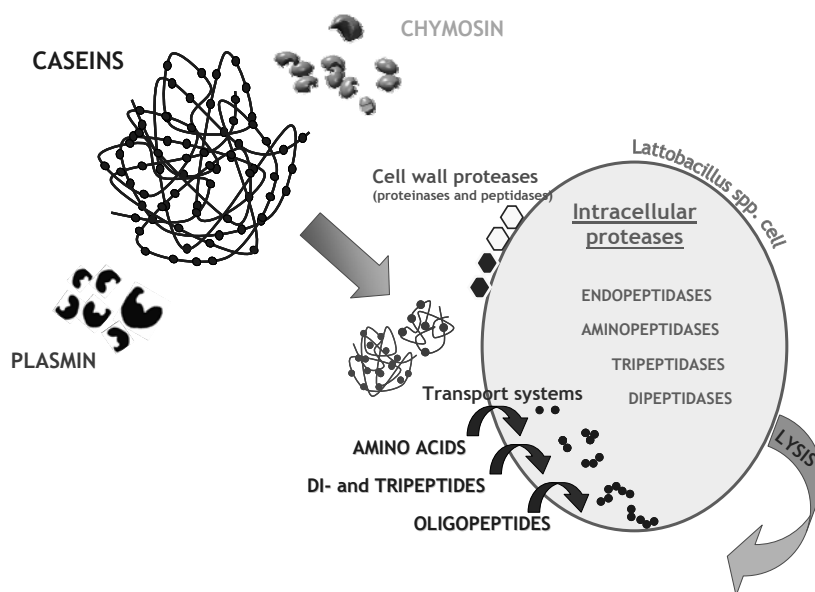


Figure 1.1. The main proteolytic enzymes involved in casein degradation during cheese ripening (adapted from ref.ii).

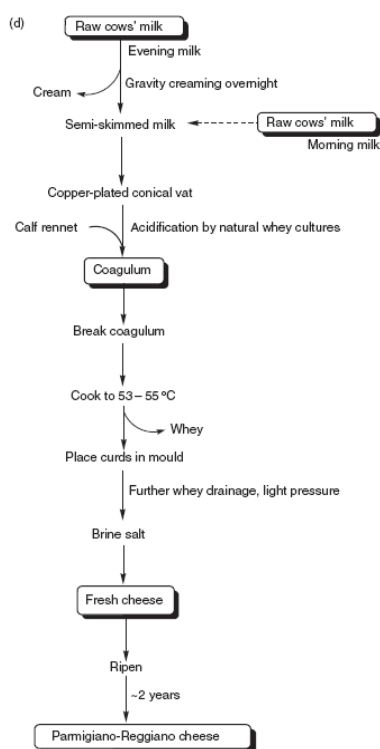


Figure 1.2. Flow diagram for the manufacture of Parmigiano-Reggiano cheese.

Parmigiano-Reggiano is a well-known Italian hard cheese, long ripened, made from raw and partly skimmed cow's milk. It is included in the list of Italian cheeses bearing the Protected Designation of Origin (PDO, EU regulation 2081/92). This definition includes technological characteristics and geographic restrictions. For example, the production of milk and its transformation into cheese can take place only in the provinces of Parma, Reggio Emilia, Modena (and in some parts of those of Bologna and Mantova). Cows have to be fed only on grass or hay and are reared following specific breeding techniques.

Obviously, also the production of cheese from raw milk must follow severe rules. The main stages of the production of Parmigiano-Reggiano cheese are as follows: the milk collected in the evening is partly skimmed by gravity all night long and it is then mixed with the whole milk of the morning after. Together with calf rennet, only a natural whey culture is allowed to be used as starter and it must be obtained from the previous day's cheesemaking whey incubated at a gradually decreasing temperature. The mixture of the raw partly skimmed cow's milk and natural whey starter is heated up to 54-56 °C in copper vats and rennet is added to start curding. The curd is then poured into wheel-shaped forms, salted in brine for up to one month and aged for at least one year. The entire caseification process is reported in Figure 1.2. It takes about 550 liters of milk to make one wheel of Parmigiano-Reggiano cheese, which weighs from 32 to 38 kg.

Several works have been published characterizing proteolytic peptides in cheeses (see ref. ii for a recent review).

Moreover, several data about the cleavage specificities of the main proteolytic enzymes are available (a summary is reported in Table 1.1), most of them a good part of them determined on model systems. Peptides are the primary results of these enzymatic activities, thus studying their presence in cheese, information on the enzymes involved can also be determined.

Table 1.1. The principal dairy proteases cleavage sites in cows' milk caseins.

	β casein	α S 1 casein	α S 2 casein	K casein
Milk Plasmin	Lys28-Lys29, Lys105-His106, Lys107-Glu108 secondary cleavages due to plasmin specificity to Lys-X and in a lesser extent Arg-X peptide bond.	Lys102-Lys103, Lys103-Tyr104, Lys105-Val106, Arg151-Gln152	Lys21-Gln22, Lys24-Asn25, Arg114-Asn115, Lys149-Lys150, Lys150-Thr151, Lys181-Thr182, Lys188-Ala189, Lys197-Thr198	
Curd Chymosin	Leu163-Ser164, Leu127-Thr128	Phe23-Phe24, Phe24-Val25, Phe32-Gly33, Leu40-Ser41, Leu149-Phe150, Phe153-Tyr154, Leu156-Asp157, Ala158-Tyr159, Phe179-Ser180, Trp164-Tyr165, Leu101-Lys102	Tyr89-Gln90, Thr120-Pro121, Asn159-Arg160, Phe88-Tyr89, Tyr95-Leu96, Gln97-Tyr98, Tyr98-Leu99, Phe163-Leu164, Phe174-Ala175Tyr179-Leu180	Phe105-Met106
LAB Proteinases CE	many possible cleavages depending on the strains			
Lab Endo Peptidases	PepO, PepF, PepE, PepG, acting especially on α S 1 CN f(1-23), α S 1 CN f(91-100), α S 1 CN f(165-199) and β CN f(193-209)			
LAB Esopeptidases*	Aminopeptidases (PepN, PepC, PepS, PepA, PepL) Proline-specific peptidases (PepQ, Pepl, PepR, PepX, PepP)			
Milk Elastase	Ile26-Asn27, Gln40-Thr41, Ile49-His50, Phe52-Ala53, Gln56-Ser57, Leu58-Val59, Asn68-Ser69, Val82-Val83, Val95-Ser96, Ser96-Lys97, Lys97-Val98, Ala101-Met102, Glu108-Met109, Phe119-Thr120, Glu131-Asn132, Leu163-Ser164, Ala189-Phe190, Phe190-Leu191 And Pro204-Phe205	Tyr159-Pro160, Phe179-Ser180, Phe24-Val25, Glu148-Leu149, Phe150-Arg151, Tyr154-Gln155, Tyr165-Tyr166, Ala163-Trp164, Tyr166-Val167, Val15-Leu16, Glu30-Val31, Tyr104-Lys105 And Ala143-Tyr144, Phe24-Val25, Tyr104-Lys105, Phe150-Arg151, Tyr154-Gln155, Val15-Leu16, Phe24-Val25, Glu148-Leu149, Ala163-Trp164, Thr171-Gln172, Pro197-Leu198		
Cathepsin D	Phe52-Ala53, Leu58-Val59, Pro81-Val82, Ser96-Lys97, Leu125-Thr126, Leu127-Thr128, Trp143-Met144, Phe157-Pro158, Ser161-Val162, Leu165-Ser166, Leu191-Leu192, Leu192-Tyr193, Phe205-Pro206	Phe23-Phe24, Phe24-Val25, Leu98-Leu99, And Leu149-Phe150	Leu99-Tyr100, Leu123-Asn124, Leu180-Lys181 And Thr182-Val183	
* Kirsj Savijoki . Hanne Ingmer . Pekka Varmanen, Proteolytic systems of lactic acid bacteria, Appl Microbiol Biotechnol (2006) 71: 394-406 § the peptidase database http://merops.sanger.ac.uk/index.htm				

Quite interestingly, LAB peptidases and proteinases specificities strongly depend on the bacterial species and strains. For example, *Lactobacillus helveticus* enzymatic pool includes aminopeptidase N (PepN), aminopeptidase C (PepC), X-prolyl dipeptidyl aminopeptidase (PepX), proline iminopeptidase (Pepl), prolinase (PepR), dipeptidase (PepD), cell envelope-associated proteinase (Prth) (which hydrolyzes Leu16-Asn17 and Asn17-Glu18 bonds of α S1-CN), oligopeptidases (PepO and PepE). *Streptococcus thermophilus* enzymatic pool includes Cell Envelope Proteinase (PrtS), glutamyl aminopeptidase (PepA), aminopeptidases (PepC and PepN), Oligoendopeptidase (PepO), aromatic and arginine aminopeptidase (PepS) and X-prolyl dipeptidyl aminopeptidase (PepX) and in its genes Oligoendopeptidase (PepF), Aminopeptidase (PepM, PepP), XAA-Pro aminopeptidase, Prolidase (PepQ), Tripeptidase (PepT) and Dipeptidase (PepV) are encoded^{iii,iv}. Information on carboxypeptidases from LAB is scarce, although carboxypeptidase activity of *L. helveticus* and *S.s thermophilus* has been observed on B-CN.^v

1.2 Aim of the work

In order to improve the knowledge of the action of the different proteolytic systems involved in Parmigiano-Reggiano cheese maturation and to identify oligopeptides to be used as markers of quality and ageing, we evaluated the oligopeptide fraction of samples of Parmigiano-Reggiano. These samples were taken from wheels of Parmigiano-Reggiano cheese, were produced on the same day, in the same factory, from the same milk, during the same caseification process, aged from 0 to 24 months. Also caseification milk was available. These homogeneous wheels offered a unique sampling plan to study and model the enzymatic events happening in Parmigiano-Reggiano during ageing.

1.3 Experimental part

1.3.1 Solvents and reagents

- ◆ HCl 0.1N (NORMEX)
- ◆ (L,L)-phenylalanylphenylalanine (Phe-Phe) (SIGMA-ALDRICH)
- ◆ Ethyl-ether (RIEDEL DE HAËN)
- ◆ Paper filters
- ◆ 0.45µm HVLP filters (MILLIPORE)
- ◆ PLGC filters, nominal molecular cut-off 10 000 Da (MILLIPORE)

- ◆ Milli Q H₂O
- ◆ Acetonitrile CH₃CN, HPLC grade (VWR)
- ◆ Methanol CH₃OH, HPLC grade (VWR)
- ◆ HCOOH, 99% (ACROS)

1.3.2 Instrumentation

- ◆ Ultra Turrax T50 basic (IKA-WERKE)
- ◆ ALC 4237R centrifuge
- ◆ 46 mm Sterifil Aseptic system (MILLIPORE)
- ◆ Alliance 2695 separation module (WATERS)
- ◆ Jupiter Phenomenex C18 column (5µm, 300 Å, 250x4.6 mm)
- ◆ Millipore PLGC filters (nominal molecular cut-off 10 000 Da)
- ◆ Amicon Micropartition system MPS-1 (Millipore).
- ◆ SQ mass spectrometer (Waters)
- ◆ Rotavapor Water bath 461, (BÜCHI)
- ◆ pH meter, 691 (METRÖHM)
- ◆ Bench scales (ORMA)

1.3.3 Samples

Cheese samples were obtained from the “Consorzio del Parmigiano-Reggiano”. They were taken from the curd, 12 and 48 hours after production, immediately after brining (1 month of ageing) and after 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 months, from November 2005 to November 2007. Each time, the wheels were sampled from a horizontal section in three different zones: internal (I) and external (E). At each sampling time the cheese was grated and kept at -20°C until the analyses were performed (usually a few days).

Figure 1.3. Sampling of the Parmigiano-Reggiano wheel.

1.3.4 Extraction and concentration of the oligopeptide fraction

10 g of finely grated cheese were suspended in 45 ml of 0.1 N HCl. (L,L)-phenylalanylphenylalanine (Phe-Phe) was added as an internal standard (2.5ml of a 1mM solution). The suspension was homogenized for 1 min and then centrifuged at 3400 g for 40 min at 4°C. The solution was filtered through paper filters and then extracted three times with 40 ml of ethyl ether. The aqueous solution was filtered through 0.45 µm filter. A total of 3 ml of the resulting solution was dried and redissolved in 900µl of a 0.1% formic acid solution (pH 3). The solution was diafiltered through filters with nominal molecular cut-off 10 000 Da at 2800 g. The filtrate was dried under nitrogen, redissolved in 500 µl of H₂O (0.1% HCOOH) and analyzed by HPLC/MS. Each sample was extracted at least twice.

1.3.5 LC/MS analysis of the oligopeptide fraction

HPLC-ESI-MS was carried out on the same HPLC system but two elution conditions were used. First, a long HPLC was utilized to allow a perfect separation and attribution of ions to each peptide. Then, a more efficient and shorter run was developed, perfectly fitting all kinds of samples. Eluents were in both cases as follows. Eluent A: H₂O (0.2 % CH₃CN and 0.1 % HCOOH); eluent B: CH₃CN (0.2 % H₂O and 0.1 % HCOOH). For the first type of analysis (“Long” running) elution was achieved in the following conditions: 0-15 min isocratic 100% A, 15-60 min linear gradient from 100% A to 67% A, 60-69 min isocratic 67% A, 69-70 min from 67% to 60% A, 70-80 min from 60%A to 0%A, 80-85 min isocratic 0% A, and reconditioning. In the second type of HPLC analysis (“Short” running), elution was achieved by: 0-10 min isocratic 95 % A; 10-50 min linear gradient from 95 % A to 60 % A; 50-56 min linear gradient from 60 % A to 0 % A; 56-62 isocratic 0 % A (100 % B); 62-64 min linear gradient from 0 % A to 95 %A, and reconditioning. Flow rate was

always 1 ml/min. MS conditions: ESI interface with 90% splitting of the column flow, positive ions, single quadrupole SQ Waters analyzer. Capillary voltage 3.2kV, cone voltage 30 V, source temperature 100 °C, desolvation temperature 150 °C, cone gas (N₂) 100 l/h, desolvation gas (N₂) 450 l/h. Acquisition in total ion mode (TIC, 100-1900 m/z); a scan time of 3 sec was used in the first type of analysis and of 1 sec in the second case.

1.3.6 Data analysis

LC/MS analysis of the extracted fractions was performed (full scan acquisition). Each HPLC-MS chromatogram was elaborated determining characteristic ions, molecular weights, retention times and in source CID fragments (if present) of the main peaks. MW determination was confirmed by Max Ent application of Mass Lynx Software in the case of MWs superior to 1000 Da. Identification of peptides was possible by applying a procedure previously developed in our research group^{vi}, exploiting in source CID fragments (as in the example reported in Figure 1.4).

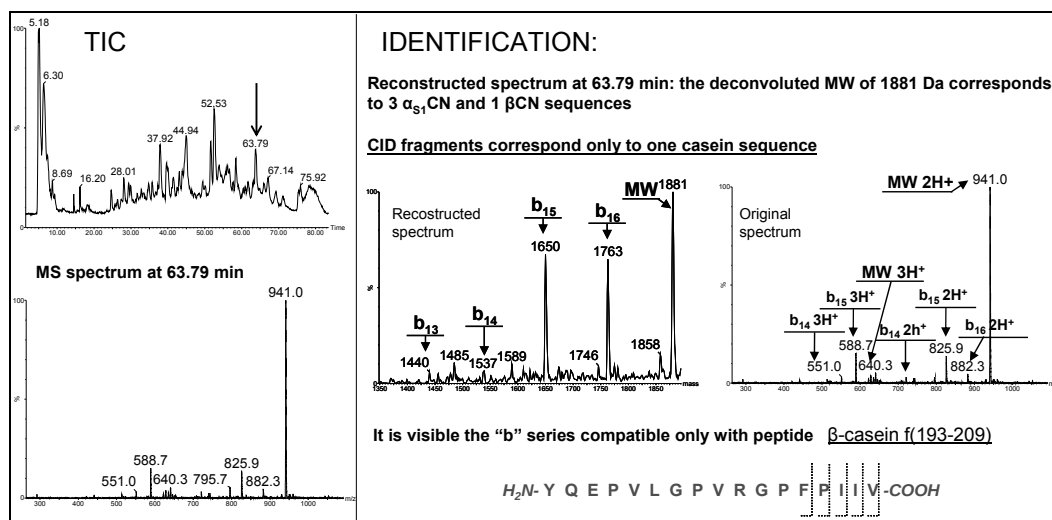


Figure 1.4. HPLC study and sequence identification.

Integration of the area of each peptide was performed in an automatic way by QuanLynx Software, after extraction of the peptide characteristic ions current (XIC technique). Integration parameters include: automatic ApexTrack Peak Integration and Noise measurement; smooth parameters: ± 1 scans and 1 smooth by “mean” method.

The integrated areas of each peptide in each sample were collected, and semiquantified by dividing for the Phe-Phe (Internal Standard) area in the same sample. A moisture correction was performed [corrected value = semiquantified value * (100/(100-W))], based on the moisture data of each sample (Table 1.2).

Table 1.2. Moisture (W) values for the cheese samples.

% W	extracted curd	curd, 12h	curd, 48h	1 month	2 months	3 months	4 months
external	46.20	42.60	40.10	37.14	36.45	35.72	35.43
internal	46.20	41.20	41.20	40.08	39.30	38.83	38.68

% W	6 months	8 months	10 months	12 months	16 months	20 months	24 months
external	35.32	34.84	34.31	34.06	32.93	31.45	30.14
internal	37.86	36.84	36.70	35.67	34.98	34.24	33.35

1.3.7 Statistical analysis

Semiquantitative data were entered into a Microsoft Excel spreadsheet and analyzed by using the SPSS 15.0 software. Principal Component Analysis was performed by factorial data reduction extracting 2 factors, with an unrotated factor solution in the space of the observed variables. The first and second PCA components were calculated as independent variables on data by linear regression analysis.

1.4 Results and discussion

First of all, a detailed analysis of the main peptides present in each LC-MS chromatogram was performed. Chromatograms were very different according to the different ageing times of the cheese, as it is evident in Figure 1.5.

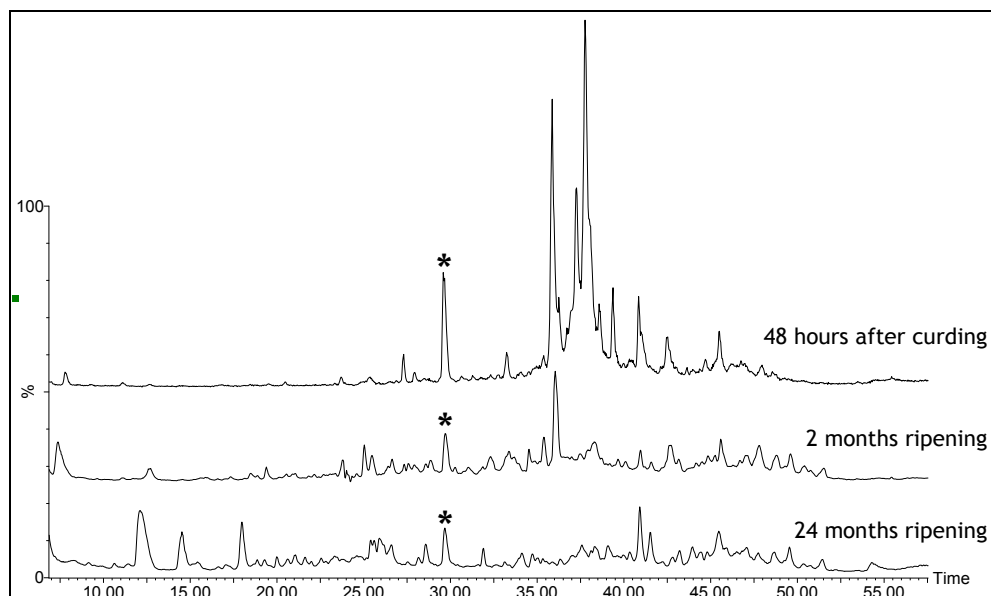


Figure 1.5. Overlaid TIC chromatograms (step = 25%) of three different cheese samples: 48 h after curding, after 2 months and 24 months of ripening. (*) Internal standard, Phe-Phe. “Short” running conditions.

MWs and sequence information from the fragment ions combined with database searching on casein sequences, led to the identifications of 82 peptides, most of them being present in the various samples (reported in Table 1.3).

Table 1.3. Identified peptides in Parmigiano-Reggiano cheese.

CODE	identification	MW	Characteristic ions
p1	Lac-Val •	189	144.1 (i_1) + 190.2 (MH^+)
p2	Lac-Ile •	203	158.2 (i_1) + 204.2 (MH^+)
p3	Lac-Leu •	203	158.2 (i_1) + 204.2 (MH^+)
p4	Lac-Met •	221	222.1 (MH^+) + 176.1 (i_1)
p5	Lac-Phe •	237	238.1 (MH^+) + 192.1 (i_1) + 120 (i_2)
p6	Pyr-Ile •	242	243.2 (MH^+) + 197.1 (i_1) + 132 (y_1)
p7	Pyr-Leu •	242	243.2 (MH^+) + 197.1 (i_1) + 132 (y_1)
p8	γ Glu-Val •	246	247.2 (MH^+) + 118.1 (y_1)
p9	Lac-Tyr •	253	254.2 (MH^+) + 208.2 (i_1) + 136.1 (i_2)
p10	γ -Glu-Ile •	260	261.2 (MH^+) + 132.1 (y_1)
p11	γ -Glu-Leu •	260	261.2 (MH^+) + 132.1 (y_1)
p12	Pyr-Phe •	276	277.2 (MH^+) + 231.1 (i_1) + 166(y_1) + 120 (i_2)
p13	γ -Glu-Met •	278	279.3 (MH^+) + 150.1 (y_1)
p14	γ -Glu-Phe •	294	295.2 (MH^+) + 166 (y_1)+ 120 (i_2)
p15	γ -Glu-Tyr •	310	311.0 (MH^+) + 182.0 (y_1)
p16	α S1 CN f (10-13)	414	414.3 (MH^+) + 244.1 (y_2)
p17	B CN f (1-3)	416	417.3 (MH^+)
p18	α S1 CN f (1-4)	536	537.3 (MH^+) + 383.3 (b_3)
p19	α S1 CN f (10-14)	542	543.3 (MH^+) + 373.3 (y_3)
p20	B CN f (1-4)	545	546.3 (MH^+)
p21	α S1 CN f (16-20)	601	602.4 (MH^+)

CODE	identification	MW	Characteristic ions
p22	α S1 CN f (30-35)	707	708.4 (MH^+)
p23	α S1 CN f (186-192)	734	734.4 (MH^+)
p24	α S1 CN f (1-6)	745	746.4 (MH^+) + 373.8 (MH_2^{2+})
p25	α S1 CN f (10-16)	754	755.3 (MH^+)
p26	B CN f (47-52)	755	756.3 (MH^+) + 378.7 (MH_2^{2+})
p27	B CN f (1-6)	787	788.3 (MH^+) + 657.3 (b_5) + 528.8 (b_4)
p28	α S1 CN f (18-23)	791	791.3 (MH^+) + 322.3 (y_2)
p29	α S1 CN f (24-30)	805	806.2 (MH^+)
p30	α S1 CN f (1-7)	874	438.3 (MH_2^{2+}) + 875.4 (MH^+)
p31	α S1 CN f (17-23)	905	905.4 (MH^+) + 453.3 (MH_2^{2+}) + 791.5 (y_6)
p32	B CN f (60-68)	1001	501.4 (MH_2^{2+}) + 334.8 (MH_3^{3+})
p34	B CN f (111-119)	1122	1123.2 (MH^+) + 562.3 (MH_2^{2+})
p35	α S2 CN f (154-162)	1132	566.8 (MH_2^{2+})
p36	α S1 CN f (115-123)1P	1138	1139.1 (MH^+) + 570.3 (MH_2^{2+})
p37	α S1 CN f (1-9)	1140	570.8 (MH_2^{2+}) + 381.0 (MH_3^{3+})
p38	B CN f (199-209)	1151	576.4 (MH_2^{2+}) + 1151.6 (MH^+)
p39	B CN f(17-25)2P	1154	578.5 (MH_2^{2+}) + 1155.3 (MH^+)
p40	α S1 CN f (169-179)	1198	300.2 (MH_4^{4+}) + 400.3 (MH_3^{3+}) + 600.3 (MH_2^{2+})
p41	α S1 CN f (24-34)	1237	1237.3 (MH^+) + 619.3 (MH_2^{2+})
p42	α S1 CN f (14-23)	1246	1246.3 (MH^+) + 623.8 (MH_2^{2+})
p43	B CN f(16-25)3P	1348	1348.7 (MH^+) + 674.9 (MH_2^{2+})
p44	B CN f (71-83)	1403	702.9 (MH_2^{2+}) + 1404.1 (MH^+)
p45	α S1 CN f (24-36)	1495	748.5 (MH_2^{2+}) + 499.3 (MH_3^{3+}) + 430.4 (MH_3^{3+})
p46	B CN f (82-95)	1510	755.9 (MH_2^{2+}) + 504.2 (MH_3^{3+})
p47	α S1 CN f (1-13)	1535	768.4 (MH_2^{2+}) + 512.7 (MH_3^{3+})
p48	B CN f (195-209)	1589	795.5 (MH_2^{2+}) + 1589.6 (MH^+)
p49	B CN f(17-28) 3P	1590	795.9 (MH_2^{2+}) + 465.7 (MH_3^{3+})
p50	α S1 CN f (1-14)	1664	832.9 (MH_2^{2+}) + 555.8 (MH_3^{3+}) + 431.7 (MH_4^{4+})
p51	B CN f (16-28)3P	1703	852.4 (MH_2^{2+}) + 568.9 (MH_3^{3+}) + 1703.6 (MH^+)
p52	α S1 CN f(24-38)	1707	854.4 (MH_2^{2+}) + 570.3 (MH_3^{3+})
p53	B CN f (194-209)	1717	1718.5 (MH^+) + 859.5 (MH_2^{2+})
p54	B CN f (15-28)3P	1790	895.9 (MH_2^{2+}) + 597.8 (MH_3^{3+})
p55	B CN f(15-28) 4P	1870	936.0 (MH_2^{2+}) + 624.5 (MH_3^{3+})
p56	α S1 CN f (1-16)	1877	939.1 (MH_2^{2+}) + 626.6 (MH_3^{3+}) + 549.9 (MH_4^{4+})
p57	B CN f(193-209)	1881	941.2 (MH_2^{2+}) + 628.0 (MH_3^{3+}) + 1881.0 (MH^+)
p58	α S1 CN f (1-17)	1991	996.0 (MH_2^{2+}) + 664.6 (MH_3^{3+}) + 582.8 (MH_4^{4+})
p59	B CN f(14-28) 4P	1999	1000.5 (MH_2^{2+}) + 667.5 (MH_3^{3+})
p60	B CN f (103-119)	2042	681.6 (MH_3^{3+}) + 594.2 (MH_4^{4+})
p61	B CN f(13-28) 4P	2098	1050.0 (MH_2^{2+}) + 700.4 (MH_3^{3+})
p62	B CN f(12-28) 4P	2212	1106.6 (MH_2^{2+}) + 738.3 (MH_3^{3+})
p63	B CN f(11-28) 4P	2340	1171.0 (MH_2^{2+}) + 781.0 (MH_3^{3+}) + 586.0 (MH_4^{4+})
p64	B CN f(142-161)	2340	781.3 (MH_3^{3+}) + 1171.3 (MH_2^{2+})
p65	B CN f (159-183)	2746	1374.0 (MH_2^{2+}) + 916.6 (MH_3^{3+})
p66	α S1 CN f (1-23)	2764	1382.6 (MH_2^{2+}) + 922.1 (MH_3^{3+}) + 691.8 (MH_4^{4+}) + 553.7 (MH_5^{5+})
p67	α S1 CN f (174-199)	2836	1418.8 (MH_2^{2+}) + 946.3 (MH_3^{3+}) + 710.2 (MH_4^{4+})
p68	B CN f (98-124)	3132	1567.3 (MH_2^{2+}) + 1044.9 (MH_3^{3+}) + 784.3 (MH_4^{4+})
p69	α S1 CN f (157-188)	3452	1151.2 (MH_3^{3+}) + 863.9 (MH_4^{4+}) + 691.3 (MH_5^{5+}) + 576.2 (MH_6^{6+})
p70	α S1 CN f (156-187)	3477	1739.6 (MH_2^{2+}) + 1160.0 (MH_3^{3+}) + 870.2 (MH_4^{4+})

CODE	identification	MW	Characteristic ions
p71	B CN f(37-67)	3580	512.5 (MH ₇ ⁷⁺) + 597.6 (MH ₆ ⁶⁺) + 717.0 (MH ₅ ⁵⁺) + 896.1 (MH ₄ ⁴⁺)
p72	αS1 CN f(85-114)	3601	901.2 (MH ₄ ⁴⁺) + 721.2 (MH ₅ ⁵⁺)
p73	αS1 CN f (155-187)	3605	1803.4 (MH ₂ ²⁺) + 1202.7 (MH ₃ ³⁺) + 902.3 (MH ₄ ⁴⁺)
p74	αS1 CN f (83-114)	3860	965.6 (MH ₄ ⁴⁺) + 773.0 (MH ₅ ⁵⁺) + 644.4 (MH ₆ ⁶⁺)
p75	B CN f(59-96)	4024	1007.1 (MH ₄ ⁴⁺) + 1342.6 (MH ₃ ³⁺)
p76	B CN f(57-93)	4065	1355.7 (MH ₃ ³⁺) + 1016.9 (MH ₄ ⁴⁺)
p77	αS1 CN f(80-114)	4238	1060.2 (MH ₄ ⁴⁺) + 848.5 (MH ₅ ⁵⁺) + 707.4 (MH ₆ ⁶⁺) + 606.4 (MH ₇ ⁷⁺)
p78	B CN f (55-93)	4253	1064.3 (MH ₄ ⁴⁺) + 1418.6 (MH ₅ ⁵⁺)
p79	B CN f (53-93)	4454	1114.2 (MH ₄ ⁴⁺) + 1485.1 (MH ₅ ⁵⁺)
p80	B CN f (51-93)	4696	1175.0 (MH ₄ ⁴⁺) + 1566.0 (MH ₅ ⁵⁺)
p81	B CN f (99-159)	7054	1008.6 (MH ₇ ⁷⁺) + 882.6 (MH ₈ ⁸⁺) + 784.8 (MH ₉ ⁹⁺) + 706.4 (MH ₁₀ ¹⁰⁺) + 642.3 (MH ₁₁ ¹¹⁺) + 588.9 (MH ₁₂ ¹²⁺)
p82	B CN f (99-160)	7182	1026.9 (MH ₇ ⁷⁺) + 898.8 (MH ₈ ⁸⁺) + 799.0 (MH ₉ ⁹⁺) + 719.3 (MH ₁₀ ¹⁰⁺) + 654 (MH ₁₁ ¹¹⁺) + 599.6 (MH ₁₂ ¹²⁺)
	Phe-Phe	312	313.2 (MH ⁺) + 166.0 (y ₁) + 120.0 (i ₂)
• are Non-Proteolytic peptides, which will be characterized in the following chapter.			

Of these peptides, 15 were non proteolytic peptide-like structures (NPPs), which will be discussed in the following chapter, only one has been found to take its origin from αS2 casein, and the other 66 identified peptides originate all from αS1 and B casein. 34 peptides were already identified in other works ^{ii,vii,viii,ix,x,xi}, 48 have not been previously reported.

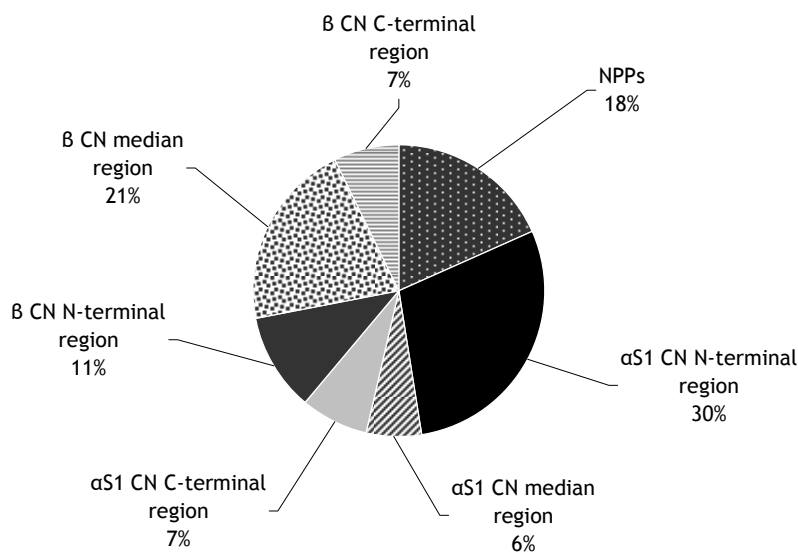


Figure 1.6. Percent distribution of peptides in function of their sequence of origin.

Principal Components Analysis (PCA) was performed in order to have a general overview of the factors affecting the data variability. PCA extracts from a set of p variables a reduced set of m components (or factors) that account for most of the variance in the p variables: m can be considered as super-ordinate dimensions.^{xii}

PCA was performed on data derived from the semiquantification values (corrected for the moisture content) of every peptide in every sample. The score plot including all the analyzed samples derived from the calculation of the factor score of every sample for each component is reported in Figure 1.7. Samples have been labeled according to their ageing times (in days)

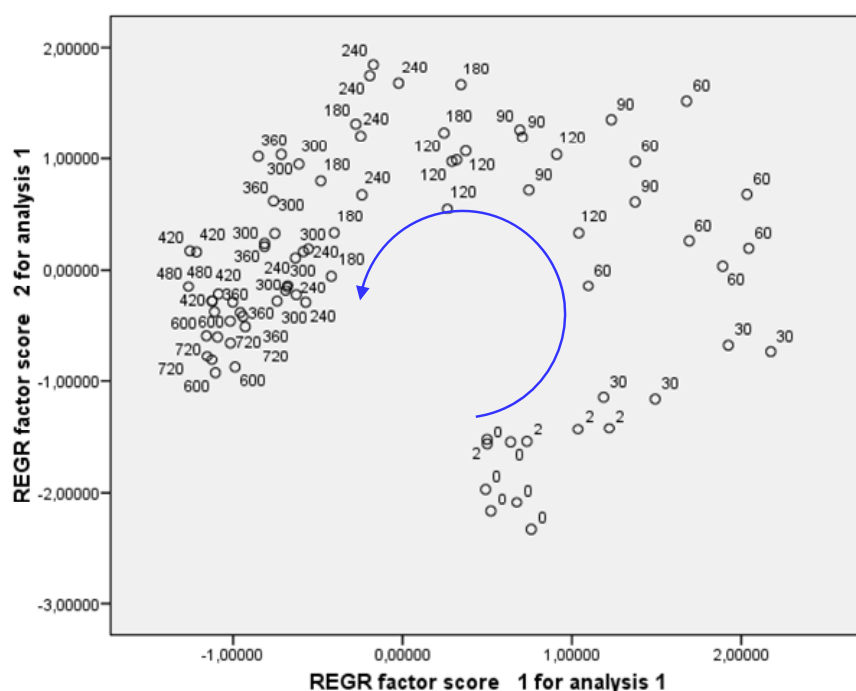


Figure 1.7. Score plot of the collected data: samples are indicated with their ageing time (expressed in days).

It is evident that the distribution of the samples is mainly affected by the ageing time; it could also be noticed that among “younger” samples there was a higher variability than in “older” samples, i.e. great changes occurred in the first months of ripening, and at long ageing time (from 10-12 to 24 months) samples seemed to be progressively more similar.

The trend of the single peptides during the ageing time was studied and peptides were grouped according to the ageing period in which they reached the maximum amount: peptides reaching the maximum in the curd and progressively disappearing (group 1), peptides reaching the maximum during the first three months (Group 2), peptides reaching the maximum around the 4th-6th month (Group 3), peptides having the maximum at the 10th-12th month (Group 4), peptides whose quantity was always increasing during ageing (Group 5).

The Loading plot of the PCA is reported in Figure 1.8: peptides (from P1 to P82 as in Table 1.3) are represented in a 2-dimensional plan generated by the two calculated main factors (component 1 and component 2) which explain, respectively, 40.8 and 18.3% of the variance. Peptides have been grouped according to their trend during the ageing time, as indicated above.

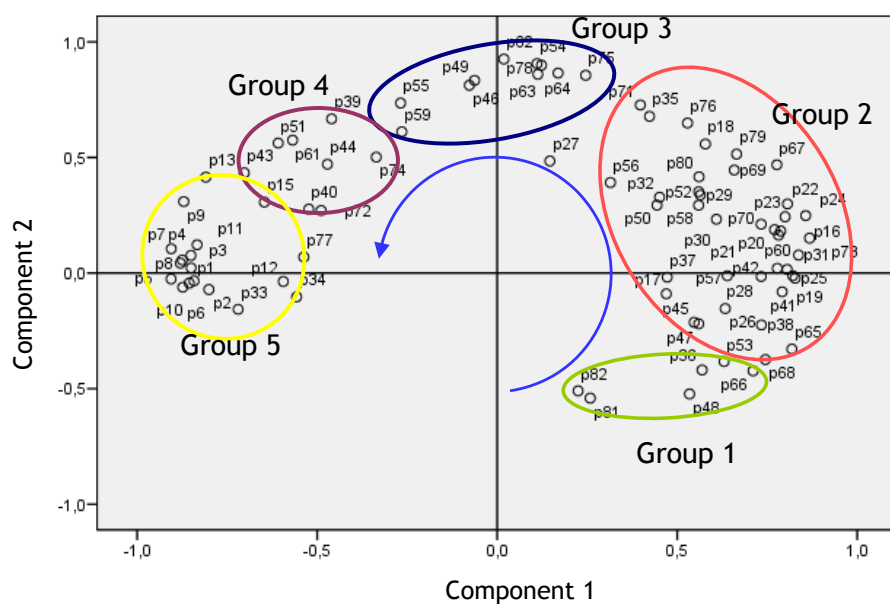


Figure 1.8. Loading plot of Parmigiano-Reggiano peptides.

A comparison of the Score Plot with the Loading Plot confirmed that the major source of the variability was the ageing time and that the oligopeptide fraction accordingly changed during the ageing: peptides in group 1 were the most abundant in curd samples, whereas group 5 peptides characterized the long ripened cheeses.

The composition of the different peptide groups will be reported in tables and discussed in detail.

1.4.1 Changes of the peptide pattern during the ageing

1.4.1.1 Group 1 peptides (maximum amount in the curd)

The average amounts of these peptides at the different ageing times is summarized in Figure 1.9.

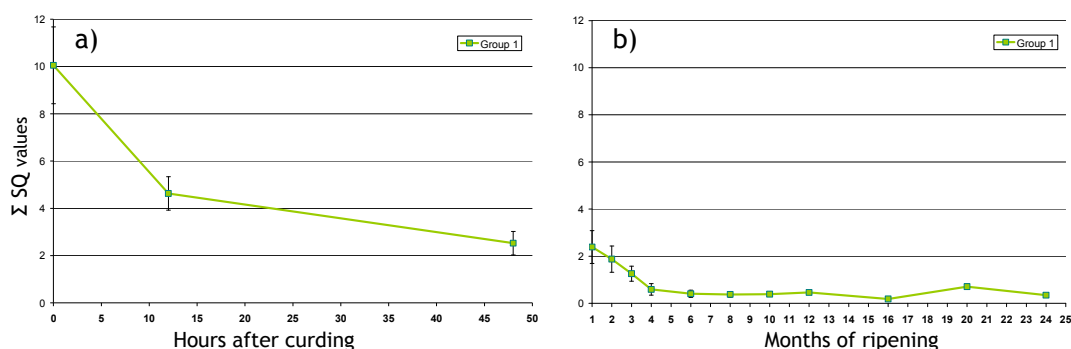


Figure 1.9. Amounts of group 1 peptides in function of the ageing time: a) hours after curding and b) months of ageing. The amount is obtained from the sum of semiquantified values of the peptides of the group¹.

The peptides belonging to this group are reported in Table 1.4.

Table 1.4. List of group 1 peptides.

Group 1	Group 1 (identity)
P66	αS1 CN f (1-23)
P81	B CN f (99-159)
P82	B CN f (99-160)

αS1 CN f (1-23) is a very well known, ubiquitous peptide in dairy products. It is derived from the chymosin action, the main rennet enzyme thus rapidly increasing in the curd at the start of the process. Since this peptide presents many preferential cleavage sites of bacterial proteolytic enzymes, it is rapidly degraded when chymosin activity decreases, although a small amount can be found up to the end of ageing. Peptides B CN f (99-159) and B CN f (99-160) were the only peptides also found in the milk used for cheese preparation, so they probably derive from milk endogenous enzymes and they are also rapidly degraded in the first hours of curding.

1.4.1.2 Group 2 peptides (maximum during the first three months of ageing)

This group is the most rich in peptides. Their relative amount is quite low in the first hours of the cheese-making process, then it reaches a maximum after 1-3 months. Then, they definitively decrease during the subsequent months, without disappearing (Figure 1.10).

¹ SQ value= (Integrated area of the peptide/Integrated area of the internal standard), corrected by moisture content, as described in the experimental section.

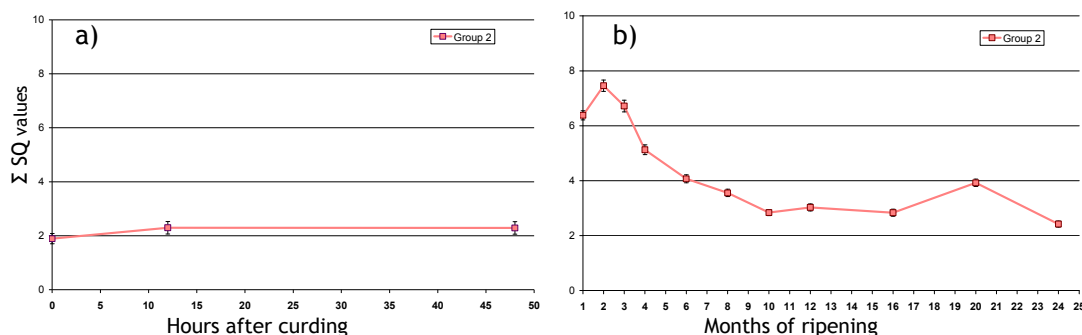


Figure 1.10. Amounts of group 2 peptides in function of the ageing time: a) hours after curding and b) months of ageing. The amount is obtained from the sum of semiquantified values of the peptides of the group.

The identification of these peptides (reported in Table 1.5) allowed some light to be shed on the enzymatic activities occurring during the first three months.

Table 1.5. List of group 2 peptides . In BOLD letters, peptides whose origin by a particular enzymatic activity can be inferred (by the N-terminal or C-terminal (or both) end).

max at 1 months	max at 2 months	max at 3 months
B CN f (1-3)	B CN f (1-6)	αS1 CN f (18-23)
B CN f (1-4)	αS1 CN f (1-4)	αS1 CN f (1-9)
αS1 CN f (186-192)	αS1 CN f (10-14)	αS1 CN f (174-199)
B CN f (47-52)	αS1 CN f (16-20)	αS1 CN f (156-187)
B CN f (199-209)	αS1 CN f (10-16)	αS1 CN f (155-187)
B CN f (195-209)	αS1 CN f (10-13)	B CN f (57-93)
B CN f (194-209)	αS1 CN f (1-7)	B CN f (53-93)
B CN f (193-209)	αS1 CN f (17-23)	αS1 CN f (24-34)
B CN f (103-119)	αS1 CN f (14-23)	αS1 CN f (24-36)
B CN f (159-183)	αS1 CN f (1-14)	αS1 CN f (24-38)
B CN f (51-93)	αS1 CN f (1-16)	
B CN f (98-124)	αS1 CN f (1-17)	

After one month of ripening, all the peptides deriving from the C-terminal part of B casein, starting from **B CN f (193-209)**, which is known to be a chymosin-^{xiii} but also a cell envelope proteinase-derived peptideⁱⁱ, reach their maximum. In particular, in Parmigiano-Reggiano cheese, the cleavage between the positions 192-193 might be attributed mainly to LAB proteinases: in fact its trend showed a delayed increase in comparison with **αS1 CN f (1-23)**, it was not consumed as **αS1 CN f (1-23)** and showed global similarity with other group 2 peptides. The related peptides (Figure 1.11), are probably produced by an aminopeptidasic degradation.

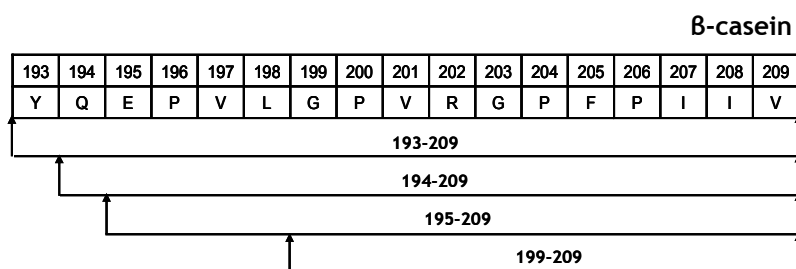


Figure 1.11. Observed peptides derived from the C-terminal end of B casein.

According to their trend during ageing (reported in detail in Figure 1.12) it seems that, although B CN f(193-209) is present up to the end of the ageing period, the aminopeptidasic activity which gives rise to the smaller peptides decreases, since these peptides disappear after 4-6 months of ageing.

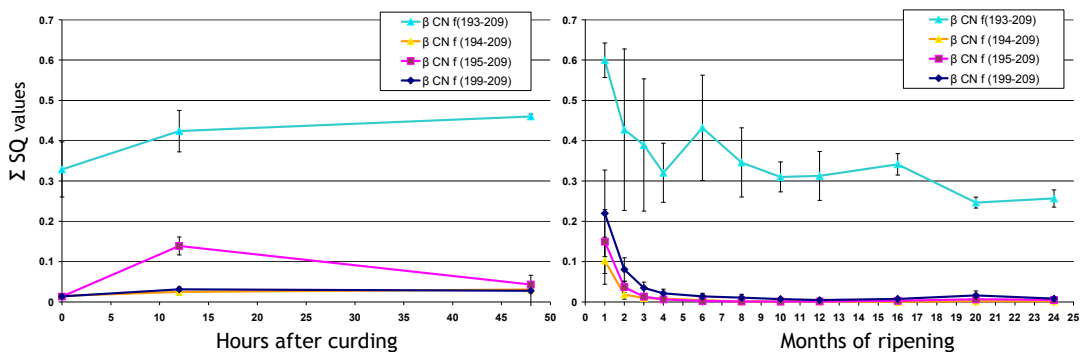


Figure 1.12. Amounts of peptides derived from the C-terminal end of B casein in the course of the time.

Peptides B CN f (1-3) and B CN f (1-4) (maximum at 1 month) and peptide B CN f (1-6) (maximum at 2 months) derive from LAB cell wall associated proteinases.

Peptides deriving from the αS1 N-terminal region reach a maximum at 2 months of ageing, with the simultaneous decrease of the peptide αS1 CN f (1-23). Their N- and C-terminal ends actually correspond to well-known cleavage sites of LAB cell-wall associated proteinases (in red in Figure 1.13) and LAB intracellular endopeptidases (in blue, in Figure 1.13).^{xiv}

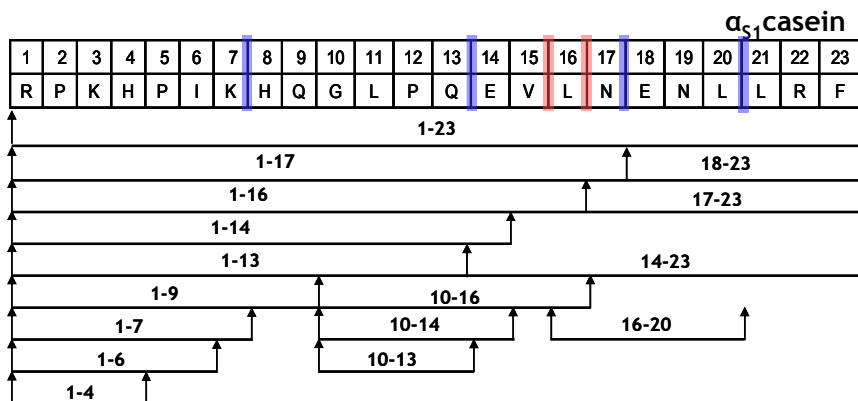


Figure 1.13. Observed peptides derived from the N-terminal part of αS1 casein.

In general, peptides released from casein by proteinases are subsequently hydrolyzed by peptidases inside the cells or by peptidases released in the matrix after cell lysis (Figure 1.1 and ref. i). In particular, LAB cell-wall associated proteinases are known to cleave the bonds Val₁₅-Lys₁₆ Lys₁₆-Asn₁₇, thus forming the observed peptides αS1 CN f(1-16) and f(17-23) starting from the αS1 CN f(1-23) peptide.

The observed trends of other peptides belonging to this group confirmed the action of the LAB enzymes (both proteinases and peptidases) directly on the free substrate αS1 CN f (1-23), indicating that these peptides probably derive from the internal cleavages of the peptide originated by chymosin. The decrease of these peptides after the first months is due to their degradation to amino acids by the action of exopeptidases, i.e. aminopeptidases.

Peptides αS1 CN f (1-4), αS1 CN f (1-6), αS1 CN f (1-14) and αS1 CN f (10-14) do not correspond to known cleavage sites of endoproteases or endopeptidases: this could be due to a typical enzymatic activity characteristic of the microflora of Parmigiano-Reggiano or possibly to carboxypeptidase activities.

After three months of ripening, peptides apparently derived from the action of *Lactococcus* cell wall proteinases (CEP) acting on B casein (as desumed by their N-terminal or C-terminal ends) also reached a maximum, : B CN f (53-93), B

CN f (55-93), B CN f(57-93) and B CN f(59-96) (N-terminal ends correspond to cleavage sites of cell proteinases); B CN f (60-68) (both ends); B CN f (1-6) (C-terminal end).^{xiv}

Also several peptides derived from the **C-terminal region of α S1 casein** can be attributed to the activity of *Lactococcus* cell wall proteinases or endopeptidases: α S1 CN f (157-188), α S1 CN f (156-187), α S1 CN f (155-187), α S1 CN f (174-199).

Cell envelope proteinases of *Lactococcus* sppⁱ also extensively degrade the **α S1 casein region included between Phe₂₄ and Asn₃₈**, giving rise to peptides α S1 CN f (24-34), α S1 CN f (24-36), α S1 CN f(24-38), α S1 CN f (24-30) and probably also to α S1 CN f (30-35).

Finally, B CN f (103-119), B CN f (159-183) and B CN f (60-68) showed a C-terminal ending compatible with cathepsin cleavages^{xv,xvi}. Cathepsins are proteinases associated with somatic cells in milk, with high activities although present in low quantities.

To summarize, the second group of peptides is mainly produced by peptidase and proteinase activities of LAB, generating a large number of peptides during the first months of cheese ripening, which will be subsequently largely degraded during ageing, very likely by exoprotease activity. In any case, a low but significant amount of these peptides can also be found in the ripened product (Figure 1.10). It is quite evident that LAB enzymes strongly influence the proteolytic modifications in cheese. As has been said before, LAB in Parmigiano-Reggiano mainly derive from the natural whey starter; the dominant microflora of the natural whey starter are the lactic acid bacteria selected by the process of curd heating and, therefore, these LAB are thermophilic. Among these, *L. helveticus* is the dominant one^{xvii}, followed by *S. thermophilus*. Since LAB can be found as different strains, the use of a natural whey starter implies a strong link between the product and its original geographic area of production. Thus, some of the unusual cleavage sites here observed could be typical of the strains present in the starter.

1.4.1.3 Group 3 peptides (maximum at 4-6 months of ageing).

Group 3 include peptides with a maximum at 4-6 months of ageing, which strongly decrease after the 10th -12th month (Figure 1.14).

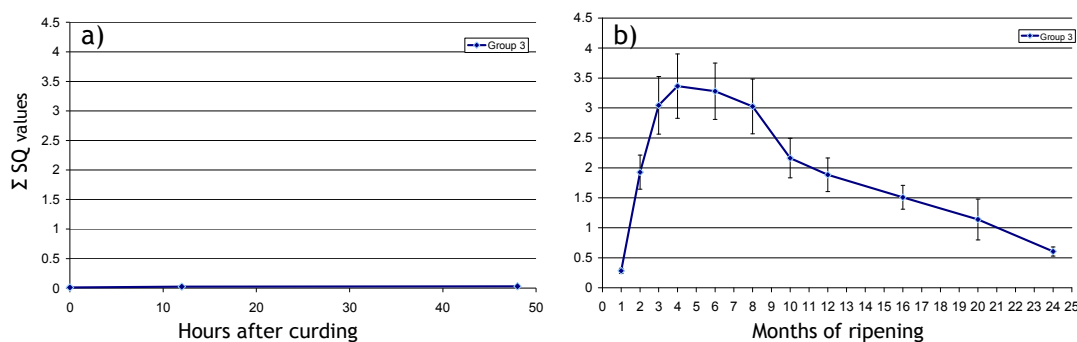


Figure 1.14. Amounts of group 3 peptides in function of the ageing time: a) hours after curding and b) months of ageing. The amount is obtained from the sum of semiquantified values of the peptides of the group.

Peptides given in Table 1.6 belong to this 3rd group.

Table 1.6. List of group 3 peptides.

Group 3	Group 3 (identity)
P49	B CN f(17-28) 3P
P54	B CN f (15-28)3P
P55	B CN f(15-28) 4P
P59	B CN f(14-28) 4P
P62	B CN f(12-28) 4P
P63	B CN f(11-28) 4P
P40	α S1 CN f (169-179)
P46	B CN f (82-95)
P64	B CN f(142-161)

These peptides are unrelated to group 1 and group 2 peptides: this suggests that the enzymes involved in the formation of this group act, starting after the first month of ageing, on the intact proteins. Another characteristic feature is that this group includes mainly phosphopeptides of β -casein ending at position 28: the cleavage between the positions 28-29 is known to derive from the action of plasmin^x. All these phosphopeptides differ at the N-terminal end and therefore seem to derive from the action of aminopeptidases. Since these peptides originate from plasmin, an endogenous milk enzyme easily denatured by heat treatments, they can be considered as good markers of the technology (raw milk is used for Parmigiano-Reggiano cheese production).

Several hypothesis can be also made as to the origin of the other peptides belonging to this group. α S1 CN f (169-179) had only one recognizable cleavage site (Tyr₁₆₈-Pro₁₆₉) already ascribed to *Lactococcus* endopeptidasic activity, which can be assumed to act on a longer precursor, such as the α S1 CN f (157-188) peptide observed in group 2. The B CN f (82-95) peptide was consistent with the elastase activity^{xviii}, which cleaves Val₈₂-Val₈₃ and Val₉₅-Ser₉₆ bonds, whereas the B CN f(142-161) peptide derives from a Cathepsin D activity^{xix}. Elastase and cathepsins are milk enzymes which derive from somatic cells, very specific towards caseins.

1.4.1.4 Group 4 peptides, with a maximum at 10-12 months of ageing

This group includes peptides which reach a maximum between the 8th and the 12th month, and then slowly decrease and are still present in good amount in the 24-months old cheese (Figure 1.15).

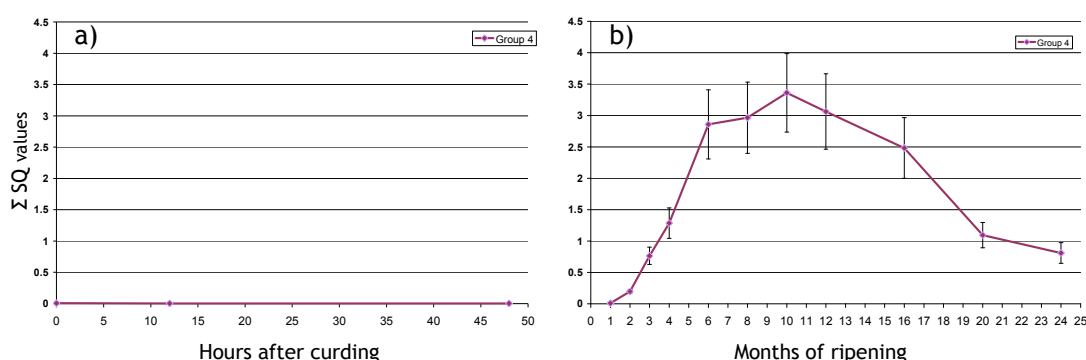


Figure 1.15. Amounts of group 4 peptides in function of the ageing time: a) hours after curding and b) months of ageing. The amount is obtained from the sum of semiquantified values of the peptides of the group.

Group 4 peptides are reported in Table 1.7.

Table 1.7. List of group 4 peptides.

Group 4	Group 4 (identity)
---------	--------------------

P39	B CN f(17-25)2P
P43	B CN f(16-25)3P
P51	B CN f (16-28)3P
P61	B CN f(13-28) 4P
P44	B CN f (71-83)

This group mainly includes other phospho-peptides of β casein, probably derived from the combined action of plasmin and aminopeptidases.

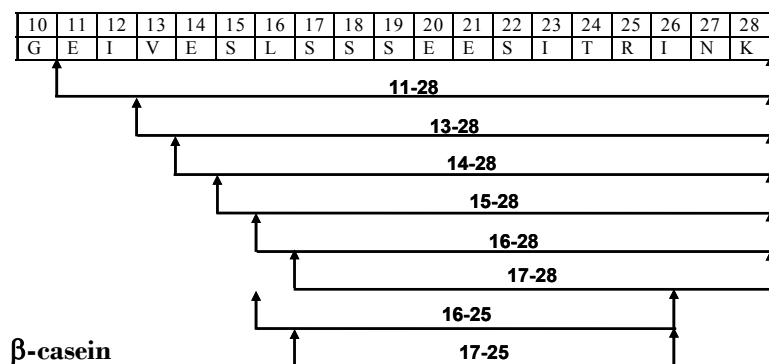


Figure 1.16. Observed phospho peptides from β -casein in Parmigiano-Reggiano cheese.

1.4.1.5 Group 5 peptides (increasing during the ageing time).

The amount of these peptides increased during time (Figure 1.17).

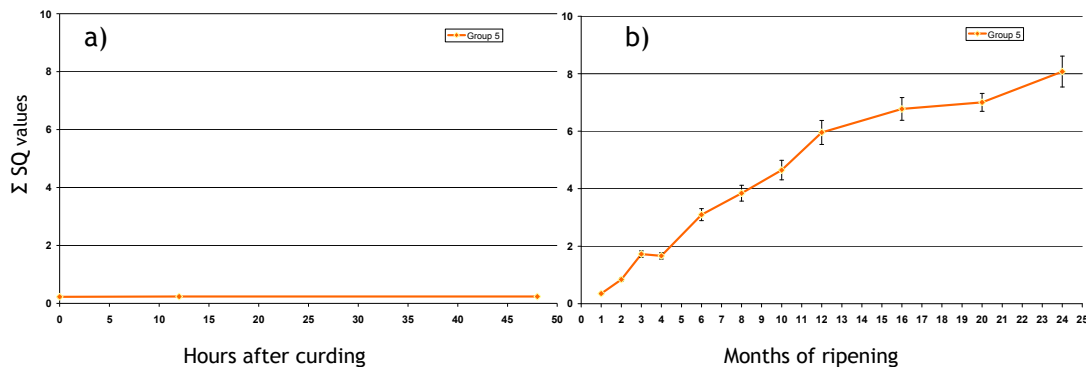


Figure 1.17. Amounts of group 5 peptides in function of the ageing time: a) hours after curding and b) months of ageing. The amount is obtained from the sum of semiquantified values of the peptides of the group.

Peptides of this group are reported in Table 1.8: this group includes two “families”: all non-proteolytic peptide-like molecules (NPP, discussed in the following chapter), and other peptides arising from the direct proteolysis of caseins.

Table 1.8. List of group 5 peptides.

Group 5	Group 5 (identity)
P1	Lac-Val
P2	Lac-Leu
P3	Pyr-Ile
P4	γ Glu-Val
P5	γ-Glu-Ile
P6	γ-Glu-Leu
P7	γ-Glu-Phe
P8	γ-Glu-Tyr
P9	Lac-Ile
P10	Lac-Met
P11	Lac-Phe
P12	Pyr-Leu
P13	Lac-Tyr
P14	Pyr-Phe
P15	γ-Glu-Met
P33	αS1 CN f(75-82)1P
P34	β CN f (111-119)
P72	αS1 CN f(85-114)
P74	αS1 CN f (83-114)
P77	αS1 CN f(80-114)

Since their origin was very different, their trend was analyzed separately (Figure 1.18).

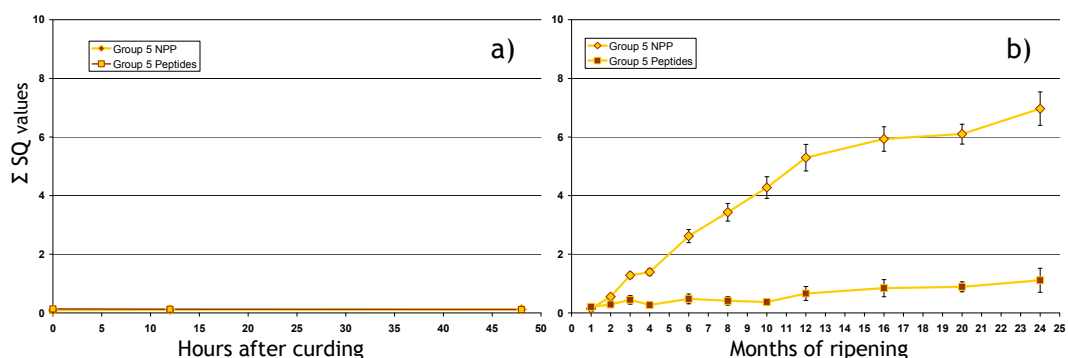


Figure 1.18. Amounts of proteolytic peptides and non-proteolytic peptide-like molecules in function of the ageing time.

Peptides arising from casein proteolysis increase very slowly, whereas the amount of NPPs increase at a much faster rate. This is consistent with the non standard structures of NPPs, which are not recognized and are not cleaved by proteinases. For this reason, they are ideal markers of the ageing of the cheese. The structural characterization of NPPs and the discussion about their formation is reported in chapter **Errore. L'origine riferimento non è stata trovata.**

Peptides α S1 CN f(85-114), α S1 CN f(83-114) and α S1 CN f(80-114) are closely related; they probably derive from the known cleavage sites for plasmin^{xx} followed by aminopeptidasic, or dipeptidil-peptidasic^{iv}, degradation.

As far as the other peptides are concerned, peptide α S1 CN f(75-82)1P has already been reported^{xi} and discussed as consistent with lactococcal cell envelope proteinases: this indicate that these activities, as already evidenced by group 2 peptides, are partially present also at long ageing times. Peptide β CN f(111-119) was formed by the cleavage between Phe₁₁₉-Thr₁₂₀, known to be typical of elastase^{xviii}.

1.4.2 Kinetics of proteolysis in the external and internal parts of the wheels

Data of the trends during ageing in the internal and in the external part of the wheel are given in the figures below, always considering the grouping above presented.

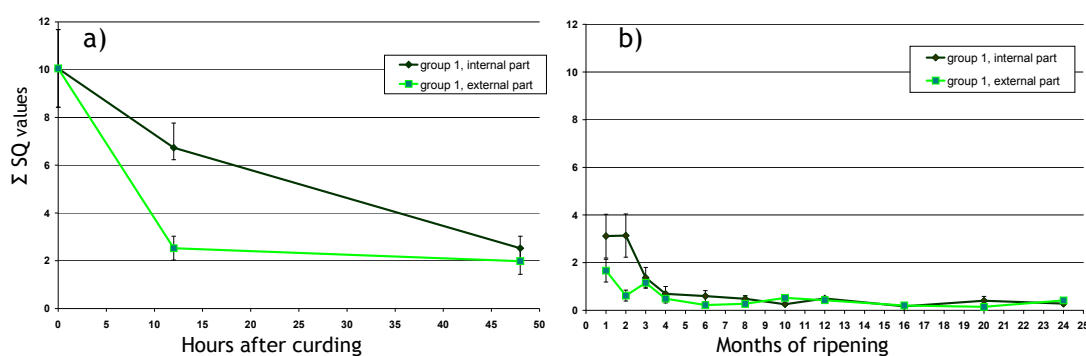


Figure 1.19. Amounts of group 1 peptides, in the external and internal parts of the wheel, during ageing: a) hours after curding and b) months of ageing.

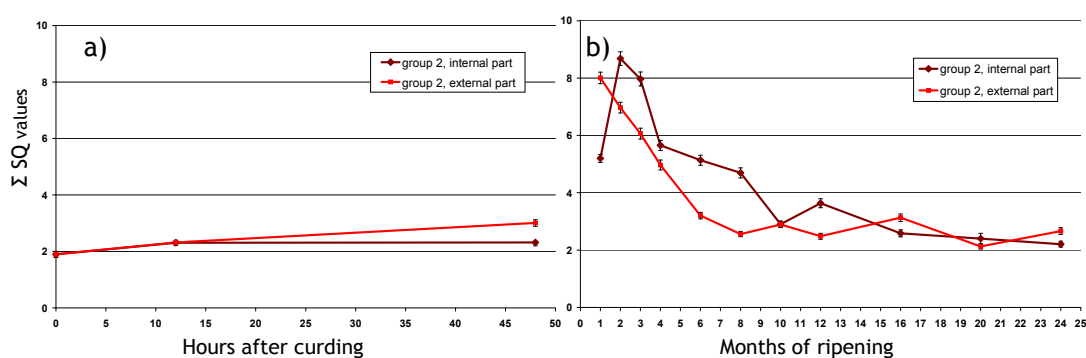


Figure 1.20. Amounts of group 2 peptides, in the external and internal parts of the wheel, during ageing: a) hours after curding and b) months of ageing.

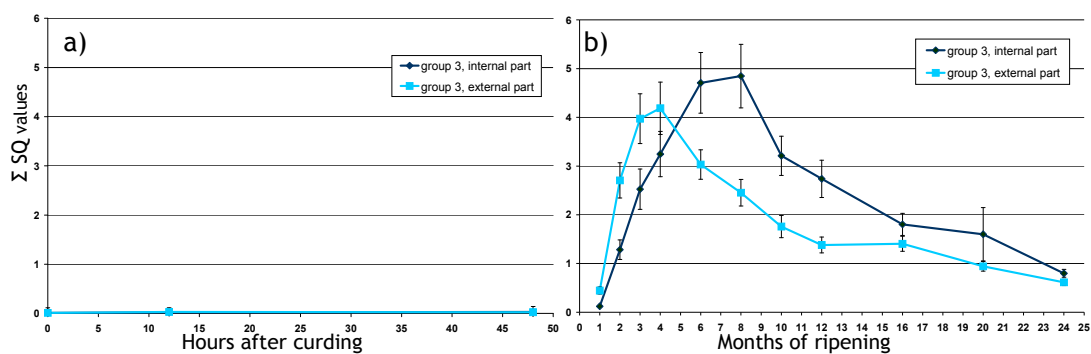


Figure 1.21. Amounts of group 3 peptides, in the external and internal parts of the wheel, during ageing: a) hours after curding and b) months of ageing.

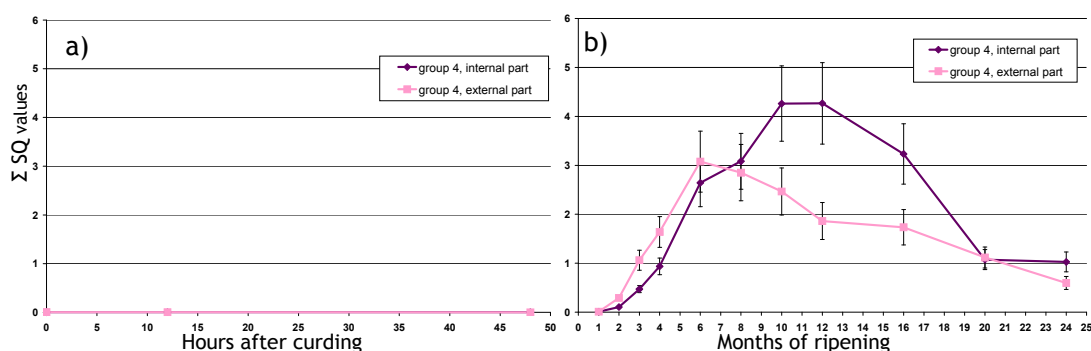


Figure 1.22. Amounts of group 4 peptides, in the external and internal parts of the wheel, during ageing: a) hours after curding and b) months of ageing.

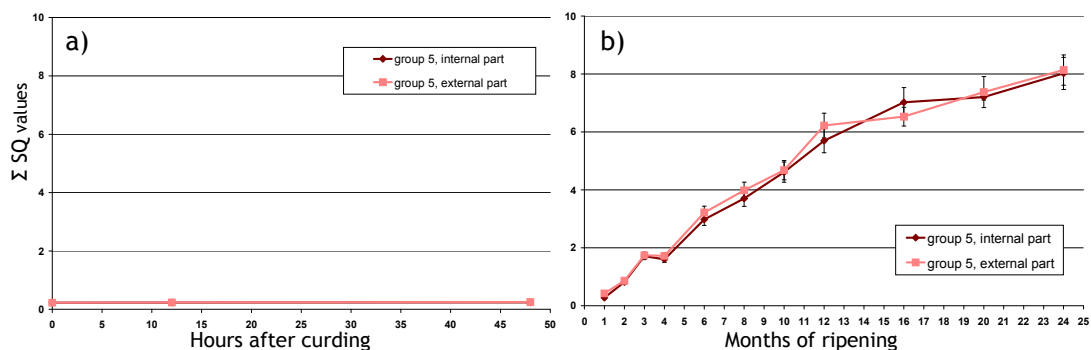


Figure 1.23. Amounts of group 5 peptides, in the external and internal parts of the wheel, during ageing: a) hours after curding and b) months of ageing.

1.4.2.1 Group 1 peptides

From the graphs reported in Figure 1.19 it is evident that group 1 peptides started at the same concentrations and decreased more rapidly in the external part of the wheels, thus indicating that they were hydrolyzed faster in the external part.

1.4.2.2 Group 2 peptides

The graphs reported in Figure 1.20 show that, during the first hours of curding, group 2 peptides increased more rapidly in the external part of the wheels than in the internal part. This confirmed once again that group 2 peptides derived mainly from group 1.

Moreover, in the first months, group 2 peptides of the external part were also degraded more rapidly.

During the production of Parmigiano-Reggiano, the external part is more exposed to temperature decrease after extraction of the cooked curd from vats, and is more rapidly affected by salt during brining. Thus, the internal part reaches the conditions of the external part later. The data here presented are consistent with the fact that the internal part of the cheese reaches the conditions of the external part more slowly. Thus, the peptide content is the expression of the enzymatic activity modulated by the medium conditions.

Moreover, these data seem to indicate that LAB enzymes were more active in the external part, probably indicating that LAB typical of Parmigiano-Reggiano natural whey starter grew more rapidly in the external part.

1.4.2.3 Group 3 peptides

Also for group 3 peptides (in Figure 1.21), the delay of proteolysis between the external and the internal part of the wheel is evident. The amount of peptides increased and were hydrolyzed more rapidly in the external part; in correspondence with the 4th month in the external part and with the 8th month in the internal part. The salt concentration at 8-10 months of ageing in the internal part becomes as it was at 4 months in the external part (data collected by “Consorzio del Parmigiano-Reggiano”, not shown). The enzymes involved seem to be directly modulated by salt and by the dimension of the wheels, i.e. the technology of production.

1.4.2.4 Group 4 peptides

For the 4th group of peptides, the same conclusion as for group 3 peptides can be drawn, except for the fact that, in this case, the amounts of peptides reach the maximum, both in the external and in the internal parts of the cheese, later on (6 months and 10-12 months respectively).

1.4.2.5 Group 5 peptides

Peptides belonging to group 5 are once again very particular: they are fairly homogeneous between the external and the internal part of the wheel, meaning that activities which generated them are less salt-dependent than all the other activities involved in cheese ageing.

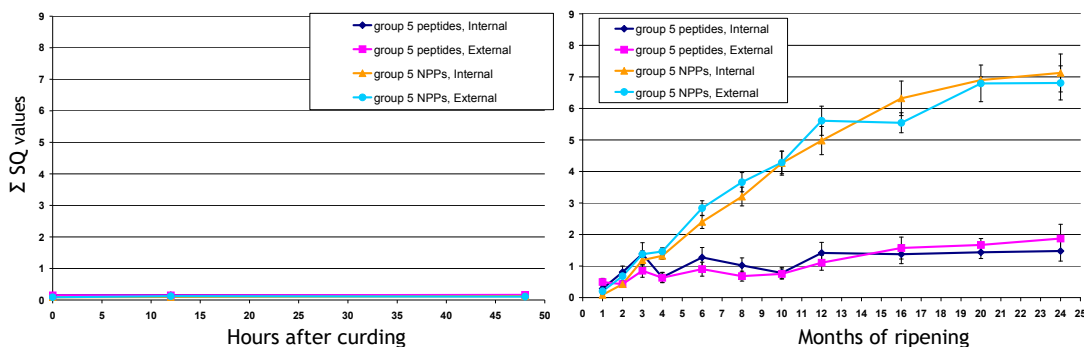


Figure 1.24. Amounts of group 5 proteolytic peptides and non-proteolytic peptide-like molecules in function of the ageing time and of the part of the wheel.

1.5 Conclusions and perspectives

In this work an extensive and detailed study of the oligopeptide fraction of Parmigiano-Reggiano cheese, mapped for the first time completely from the caseification process to 24 months of ageing, has been performed by original LC/MS methodologies.

This fraction has been shown to strongly evolve during ageing, leading to dynamic changes of the peptide pattern during ageing. Proteolysis occurs with different kinetics in the internal and external part of the wheels, and only at long ageing times the differences disappear.

The peptidic fraction studied can be considered a mirror of the enzymatic activities which take place in cheese. Typical cleavage sites of enzymes coming from lactic acid bacteria, rennet and milk have been found in many

identified peptides, allowing the most characterizing enzymatic activities which formed the observed peptides at the different ageing times to be assessed (Figure 1.25).

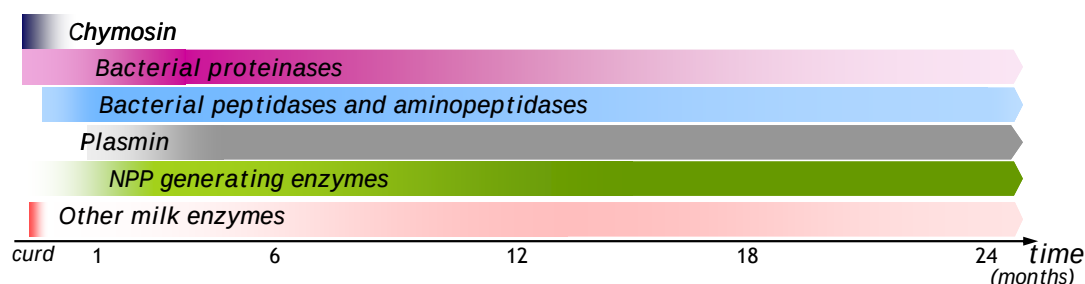


Figure 1.25. Enzymatic activities during Parmigiano-Reggiano ageing mapped by their specific peptides released in cheese. A more intense color corresponds to a more intense activity (presumed information based on our data).

The oligopeptide fraction dynamically evolves during the ageing time according to an evolving enzymatic activity determined by the combination of typical starter bacteria and typical technology. These activities are strictly related to the technology (temperature of cooking, amount of salt, wheel dimensions, etc.) and to the area of production (since the starter bacteria directly come from the previous day whey culture), thus the evolving proteolytic pattern constitutes a dynamic fingerprint of the Parmigiano-Reggiano typicality. A parallel study on the microorganism development and on the enzymatic activities has been performed by the Unit of Food Microbiology headed by Prof. E. Neviani on the same samples.

1.6 Acknowledgements

This work was performed thank to a grant from the Emilia-Romagna Region, in collaboration with the Consorzio del Parmigiano Reggiano.

ⁱ P.L.H. McSweeney Biochemistry of cheese ripening *International Journal of Dairy Technology* **2004** 57 (2-3), 127–144.

ⁱⁱ M.J. Sousa, Y. Ardo, P.L.H. McSweeney, Advances in the study of proteolysis during cheese ripening *International Dairy Journal* **2001** 11, 327–345

ⁱⁱⁱ <http://merops.sanger.ac.uk/index.htm>

^{iv} Kirsi Savijoki, Hanne Ingmer, Pekka Varmanen Proteolytic systems of lactic acid bacteria, *Appl Microbiol Biotechnol* **2006** 71: 394–406

^v Deutsch S M, Molle D, Gagnaire V, Piot M, Atlan D and Lortal S Hydrolysis of sequenced β -casein peptides provides new insight into peptidase activity from thermophilic lactic acid bacteria and highlights intrinsic resistance of phosphopeptides. *Applied and Environmental Microbiology* **2006** 66 5360–5367.

^{vi} Stefano Sforza, Linda Ferroni, Gianni Galaverna, Arnaldo Dossena, And Rosangela Marchelli, Extraction, Semi-Quantification, and Fast On-line Identification of Oligopeptides in Grana Padano Cheese by HPLC-MS *J. Agric. Food Chem.* **2003**, 51, 2130–2135,

^{vii} Sforza, S.; Galaverna, G.; Neviani, E.; Pinelli, C.; Dossena, A.; Marchelli, R. Study of the oligopeptide fraction in Grana Padano and Parmigiano-Reggiano cheeses by liquid chromatography-electrospray ionization mass spectrometry. *Eur. J. Mass Spec.*, **2004**, 10, 421–427.

^{viii} Paolo Piraino, Vivek K. Upadhyay, Rocco Rossano, Paolo Riccio, Eugenio Parente, Alan L. Kelly, Paul L.H. McSweeney, Use of mass spectrometry to characterize proteolysis in cheese, *Food Chemistry* **2007** 101; 964–972

^{ix} Addeo F, Chianese L, Sacchi R, Musso SS, Ferranti P, Malorni A., Characterization of the oligopeptides of Parmigiano-Reggiano cheese soluble in 120 g trichloroacetic acid/l. *J Dairy Res.* **1994** 61, 3:365–74.

^x Ferranti P, Barone F, Chianese L, Addeo F, Scaloni A, Pellegrino L, Resmini P. Phosphopeptides from Grana Padano cheese: nature, origin and changes during ripening. *J Dairy Res.* **1997** 64,4:601–15.

^{xi} Martin Lund And Ylva Ardö Purification and Identification of Water-Soluble Phosphopeptides from Cheese Using Fe(III) Affinity Chromatography and Mass Spectrometry, *J. Agric. Food Chem.* **2004**, 52, 6616–6622,

^{xii} C.J. Coker, R.A. Crawford, K.A. Johnston, H. Singh, L.K. Creamer, Towards the classification of cheese variety and maturity on the basis of statistical analysis of proteolysis data—a review, *International Dairy Journal*, **2005**, 15 631–643

^{xiii} Visser S, Slagter A J, On the specificity of chymosin (rennin) in its action on bovine β casein, *Meth. Milk Dairy J*, **1977**, 31, 1162–1166

-
- ^{xiv} P.F.Fox, P.L.H. McSweeney, Proteolysis in cheese during ripening, *Food Rev. Int.*, **1996**, 12(4), 457-509 ,
- ^{xv} T. Considine, S. Geary, A.L. Kelly, P.L.H. McSweeney Proteolytic specificity of cathepsin G on bovine as1- and b-caseins, *Food Chemistry* **2002**, 76, 1, 59-67,
- ^{xvi} T. Considine, A. Healy, A.L. Kelly, P.L.H. McSweeney, Hydrolysis of bovine caseins by cathepsin B, a cysteine proteinase indigenous to milk, *International Dairy Journal* **2004** 14: 117–124,
- ^{xvii} M. Gatti , C. Lazzi , L. Rossetti , G. Mucchetti And E. Neviani. Biodiversity in *Lactobacillus helveticus* strains present in natural whey starter used for Parmigiano Reggiano cheese, *Journal of Applied Microbiology* **2003**, 95, 463–470,
- ^{xviii} T. Considine, A. Healy, A.L. Kelly, P.L.H. McSweeney, Proteolytic specificity of elastase on bovine b-casein, *Food Chemistry*, **1999**, 66; 463-470
- ^{xix} M.J. Hurley, L.B. Larsen, A.L. Kelly, P.L.H. McSweeney, The milk acid proteinase cathepsin D: a review, *International Dairy Journal* **2000** 10: 673-681
- ^{xx} Addeo, F., Chianese, L., Salzano, A., Sacchi, F, Cappuccio, U., Ferranti, P. and Malorni, A. "Characterization of the 12 % trichloroacetic acid-insoluble oligopeptides of Parmigiano-Reggiano cheese," *J. Dairy Res.*, **1992**.59: 401-411.