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Summary — A centripetal temperature gradient takes place in Grana Padano (GP) during moulding because of the slow heat transfer within the cheese and the fast cooling of the outer part. This gradient, in combination with the low pH value, induces a centripetal inactivation of alkaline phosphatase (ALP). Throughout the ripening process, the activity of the enzyme keeps > 3 10^5 mU/kg cheese in the peripheral zones and < 300 mU in the core. The objective of the present study was to determine whether the presence of heat load gradients and ALP inactivation improves the analytical distinction between GP and imitation hard cheeses and affects the ripening behaviour. Differently ripened samples of traditional GP, grana cheese of reduced size, or made from pasteurized milk following GP technology, or collected at the market were submitted to a stratigraphic study. For each cheese sample, subsequent portions collected from the rind to the core were analyzed for moisture and water activity, a_w, ALP activity, furosine, free amino acids (FAA), casein phosphopeptides (CPPs) and free fatty acids (FFA). Regarding cheese characterization, thermal treatments of cheese milk can be recognized in GP only by determining the enzyme activity in the most peripheral portions. When the size of the cheese is reduced, cooling in moulding is faster and ALP inactivation in the core is lower. When the moulded cheese is heated, inactivation of the enzyme also takes place in the outer parts. The furosine values, which describe the extent of heat load in dairy systems, increase from the outer part to the GP core (from 10 mg to 60 mg/100 g protein). This gradient is unaffected by prolonged ripening, but changes when modifications in technological parameters such as pasteurization of milk, forced heating in moulding and reduction of cheese size are introduced. The distribution of ALP and furosine values within the cheese constitutes a distinctive parameter between GP and its imitation products. Regarding cheese ripening, all the values of chemical indices adopted for describing the ripening of GP show a progressive increase from the inner to the outer portions of nine- or 15-month-old cheese samples. These patterns are more pronounced for most of the FAA, and are marked for free serine which increases in the outer zones up to 50% more than in the core. The amount of 'enzyme resistant' CPP β-CN(f16–22)3P, the accumulation of which requires the presence of active phosphatases and aminopeptidases, has the same stratigraphic behaviour as that of FAA. The close

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correlation ($r = -0.97$) between furosine and FAA values suggests a possible effect of the heat load occurring in moulding on the centripetal ripening of GP.

alkaline phosphatase / casein phosphopeptide / cheese / furosine / Grana Padano

Résumé — Effets du gradient de chaleur, pendant le moulage, sur la caractérisation et l'affinage du Grana Padano. Pendant le moulage du Grana Padano (GP), on constate un lent transfert de chaleur dans le fromage et un rapide refroidissement de la périphérie. Ces conditions, en combinaison avec la basse valeur du pH, déterminent un gradient centripète d'inactivation de la phosphatase alcaline (ALP) qui montre, pendant tout le temps de l'affinage, une activité supérieure à $3\ 10^5$ mU par kilo de fromage dans sa partie périphérique et qui est inférieure à 300 mU dans sa partie centrale. Le but de ce travail a été de vérifier si les gradients de chaleur et d'inactivation de l'ALP peuvent améliorer la distinction analytique entre le GP et les fromages d'imitation à pâte dure et en influencer l'affinage. Des échantillons de GP affinés durant des temps différents, d'autres de fromages similaires de dimension réduite, d'autres produits avec du lait pasteurisé ou achetés sur le marché, ont été soumis à des études stratigraphiques. Pour chaque exemplaire, des échantillons prélevés en différents points de la croûte jusqu'à la partie centrale ont été analysés pour en déterminer le degré d'humidité, l'a_w, l'activité de l'ALP, la furosine, les acides aminés libres (FAA), les caséinophosphopeptides (CPP) et les acides gras libres (FFA). Par rapport à la caractérisation du fromage, le gradient de l'ALP ne permet de reconnaître les traitements thermiques subis par le lait dans la fabrication du GP que si on détermine l'activité de cette enzyme dans les parties périphériques du fromage sous la croûte latérale. Si le fromage est de petite dimension, le refroidissement pendant le moulage est plus rapide, et l'inactivation de l'ALP est moins importante dans la partie centrale. Si, pendant le moulage, le fromage est soumis à un chauffage forcé, on observe aussi une inactivation significative de cette enzyme dans la partie périphérique. Si l'on examine les valeurs obtenues pour la furosine, on constate qu'elles varient avec l'intensité du chauffage naturel et qu'elles vont en augmentant de la partie périphérique vers la partie centrale du GP (de 10 mg à 60 mg/100 g de protéines). Ce gradient n'est pas influencé par un affinage prolongé, mais il change quand on apporte des modifications à la fabrication traditionnelle du GP, comme, par exemple, la pasteurisation du lait, le chauffage forcé pendant le moulage ou la diminution des dimensions de la meule. La distribution des valeurs de la furosine et de l'ALP dans les fromages constitue un paramètre pour distinguer le GP de ses produits d'imitation. Quand on examine l'affinage du fromage, les valeurs de tous les indicateurs chimiques adoptés pour évaluer le degré d'affinage du GP augmentent progressivement de la partie centrale vers la partie périphérique dans des échantillons ayant 9 ou 15 mois d'affinage. Cette distribution est plus prononcée pour la plupart des FAA et, en particulier, pour la sérine libre qui montre, dans la partie périphérique, des valeurs qui sont jusqu'à 50 % plus élevées que dans la partie centrale. La quantité du CPP β-CN(f16–22)3P « enzyme-resistant », lié à la présence de phosphatase et d'aminopeptidase actives, montre la même distribution stratigraphique que les FAA. La corrélation inverse entre les valeurs de furosine et des FAA ($r = -0,97$), permet l'hypothèse que le chauffage, pendant le moulage, peut avoir une action sur l'affinage centripète du GP.

phosphatase alcaline / caséinophosphopeptide / fromage / furosine / Grana Padano

INTRODUCTION

Grana Padano (GP) and Parmigiano Reggiano (PR), the two well known Italian PDO (protected designation of origin) long-ripened hard cheeses (35–40 kg), are made from raw milk. The traditional cheesemaking technology also includes natural creaming, addition of natural starter, milk clotting at 32 °C, curd cooking up to 55 °C, and cheese moulding at room temperature for 48 h (Parisi, 1971).

We have recently demonstrated (Pellegrino et al, 1995) that alkaline phosphatase

(ALP) is totally inactivated in the core of the cheese (< 300 mU/kg cheese), while the activity in the outer part is 3 10^5 mU/kg or more. No relevant inactivation is observed during cheesemaking until the end of curd cooking. The pH of cheese in moulding drops to 5.1–5.2 and the temperature decreases to 35–40 °C at the surface, but because of the slow heat-transfer within the cheese, the core remains at 52–56 °C for 8–10 h. Such a combination of time/temperature/pH conditions promotes the formation of a centripetal gradient of ALP inactivation which remains unmodified from moulding throughout ripening. This ALP gradient can affect the final characteristics of cheese, in which phosphatases play a significant role in ripening. This role has been reviewed by Fox et al (1993) and pointed out in GP by Ferranti et al (1997).

Moreover, the significant heat load on the inner zones of the cheese together with the low pH value may reduce the activity of other indigenous and heat-sensitive enzymes, like acid protease (Kaminogawa et al, 1972) and lipases (Castberg, 1992). Severe heating in cheesemaking enhances the plasmin activity of curd (Delacroix-Buchet and Fournier, 1992), probably by an increased conversion of plasminogen into active enzyme, as observed in milk by Richardson (1983). Moreover, heating largely destroys the activity of residual milk-clotting enzyme (Garnot and Mollé, 1987). In addition, growth rate and activity of microorganisms can be limited in the core of moulded cheese by thermal stress (Heap and Lawrence, 1988). Because all these factors are reported to assume key roles in ripening, a stratigraphic approach seems to be necessary in view of achieving a more reliable analytical characterization of GP and PR.

The aim of this work was to study the significance of the heat load gradient occurring in moulding for the analytical characterization of GP and its ripening behaviour.

MATERIALS AND METHODS

Cheese samples

Twelve traditional GP cheeses 24-, 48-h, 9-, 15- and 24-month old, were produced at six dairies in the PDO area under the surveillance of the 'Consorzio di Tutela del Formaggio Grana Padano'.

Two 12-month-old grana cheeses, one produced from pasteurized milk (72 °C/15 s) and one from the corresponding raw milk, were obtained under controlled conditions at the 'Istituto Superiore Lattiero Caseario' (Mantova, Italy) following the cheesemaking technology for GP.

One grana cheese of smaller size (Ø, 11 cm) made to experimental scale with raw milk following the cheesemaking technology for PR was provided by the Department of Protection and Improvement of Agricultural Food Production (Reggio Emilia, Italy). This cheese was moulded in a thermostatic cell from 55 to 20 °C, as described by Ferri et al (1992).

Eleven samples of grana cheese, including one of reduced size (Ø, 16 cm), all produced in countries other than Italy, were collected at the Italian market.

Stratigraphic sampling

When the whole cheese was available (experimental or known origin samples) a vertical 4 cm thick section was cut from the round side of the cheese to the centre. After removing 3 mm rind, the section was divided into ten portions from the round side and four portions from the flat side, according to the scheme in figure 1. For

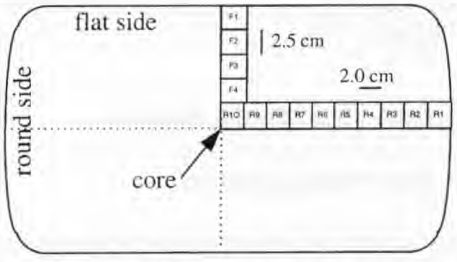

Fig 1. Scheme of sampling from a quarter of a 4-cm thick vertical section of cheese.
Schéma d'échantillonnage au quart d'une section verticale de 4 cm d'épaisseur d'un fromage.

the smaller-sized cheese, a 2 cm thick vertical section was sampled. For the commercial samples, a pyramidal portion from the round side to the core of the cheese was collected when possible; otherwise the outer part containing the round side was sampled. In all cases, subsequent 2-cm deep portions were cut from the round side after removing the rind. All the portions were finely ground before analysis.

Physico-chemical and chemical methods

Water activity (a_w) was measured by a Novasina apparatus (Zurich, Switzerland) operating at 25 °C. Moisture and protein content were determined according the International Dairy Federation (IDF) standard 4A:1982 and IDF standard 20B:1993 respectively. ALP activity was measured by the fluorometric method using Fluorophos (Advanced Instruments Inc, Needham Heights, MA, USA) as substrate and was expressed in mU/kg cheese according to the IDF standard 155:1992.

Furosine level was determined by reverse phase (RP) – high-pressure liquid chromatography (HPLC) according to the procedure of Resmini et al (1990), and quantification was performed using the Neosystem standard molecule (Strasbourg, France). Free amino acids (FAA) were extracted and quantified by ion-exchange chromatography, as previously reported (Resmini et al, 1993).

Casein phosphopeptides (CPPs) were determined by HPLC according to Ferranti et al (1997), and identified by fast atom bombardment (FAB) – MS, as previously described (Addeo et al, 1992, 1994).

Free fatty acids (FFA) were determined by GC according to the procedure of De Jong and Badings (1990).

RESULTS AND DISCUSSION

Cheese characterization

Utilization of raw or pasteurized milk in cheesemaking can be established by determining the ALP on a sample representative of the whole cheese (IDF, 1995). Due to the presence of the ALP inactivation gradient within the cheese as described in the introduction, this analytical approach can lead to incorrect interpretations when GP and PR are analyzed. If a traditional GP is compared with a similar grana cheese made from pasteurized milk, ALP activity is different only in the outer layers ($> 2\ 10^5$ mU/kg versus < 100 mU/kg) (fig 2). Therefore, thermal treatments of milk can be evaluated in GP and PR only by determining ALP activity just below the rind, preferably on the round side. This evaluation can be performed on the flat side as well, but with a lower discriminating power (fig 2; box). The thick wooden board traditionally placed on top of the fresh cheese in mould slows down the cooling of the flat sides, giving rise to significant ALP inactivation.

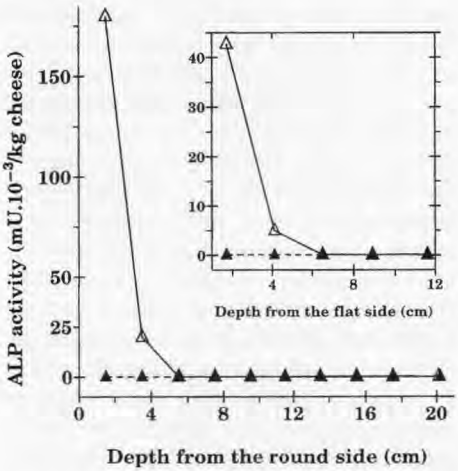

Fig 2. ALP activity determined from the round or the flat side (box) in a 12-month old grana cheese made from pasteurized milk (– –▲– –) or from the corresponding raw milk (—Δ—) Grana Padano technology.
Activité de la phosphatase alcaline (ALP) déterminée à partir du côté rond ou du côté plat d'un fromage de type grana affiné pendant 12 mois, et fabriqué avec du lait pasteurisé (– –▲– –) ou avec le lait cru correspondant (—Δ—) suivant la technologie de fabrication du Grana Padano.

The size of the cheese also affects the ALP gradient, as shown in figure 3, where the core of a commercial 16-cm diameter grana cheese has an ALP value which is higher than that of the traditional GP (Ø, 40 cm). The experimental 11-cm diameter grana cheese was moulded in a thermostatic cell from 55 to 20 °C for 24 h, to allow the growth of thermophilic bacteria and lactic acid fermentation (Ferri et al, 1992). The ALP activity of this small cheese in comparison with GP was lower at the surface and higher in the core (fig 3). When the size of GP was reduced, the cooling of moulded cheese was faster, and the ALP inactivation in the core was limited. When the cheese was kept warm in moulding, significant ALP inactivation was observed also in the outer parts, due to the additional heat load.

The presence of reducing sugars like lactose, glucose and galactose promotes the early Maillard reaction (MR) in cheese provided sufficient heating occurs (Resmini and Pellegrino, 1991). The extent of early MR evaluated by determination of furosine, a molecule obtained from acid hydrolysis of the lysine–Amadori compound (Finot et al, 1968), confirms that a heat load gradient takes place from the outside to the inside of GP during moulding (fig 4). No appreciable MR is promoted by cooking of the curd, since the furosine value of the extracted curd (6 mg/100 g protein) is close to that found in raw milk (Resmini et al, 1992). On the contrary, furosine formation takes place in the core of the moulded cheese to an unexpectedly high extent, which

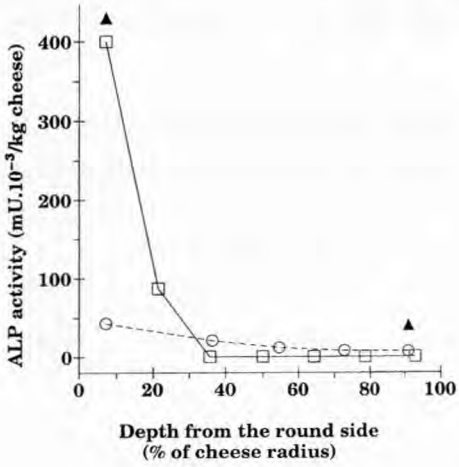

Fig 3. ALP activity in ripened cheeses of different size: (❑) traditional 15-month old GP (Ø, 40 cm); (○) experimental grana cheese (Ø, 11 cm) moulded in a thermostatic cell from 55 to 20°C; (▲) grana cheese of reduced size (Ø, 16 cm) collected at the market.
Activité de la phosphatase alcaline (ALP) dans des fromages affinés de tailles différentes : (❑) GP traditionnel de 15 mois d'affinage (Ø 40 cm) ; (○) fromage expérimental de type grana (Ø 11 cm) moulé dans une cellule thermostatée entre 55 et 20 °C ; (▲) fromage de type grana de taille réduite (Ø 16 cm) prélevé sur le marché.

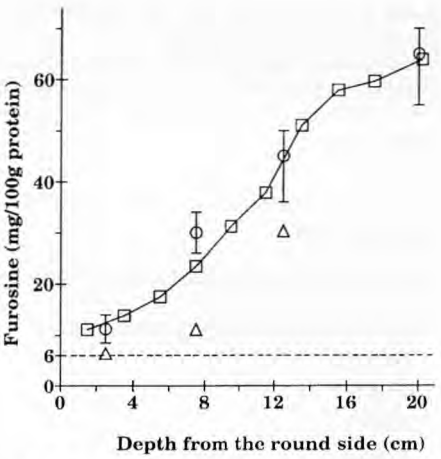

Fig 4. Furosine values in GP determined at various times of manufacture: at extraction from the vat (– – –), after 24 h (Δ) and 48 h (Φ, range of values for six samples) moulding; and after 15 month ripening (❑).
Valeurs de la furosine dans du Grana Padano à différents temps de la fabrication : en sortie de cuve (– –), après 24 heures (Δ) et 48 heures (Φ étendue de valeurs sur six échantillons) de moulage et après 15 mois d'affinage (❑).

approaches that found in UHT milk (Resmini and Pellegrino, 1991). Changes in reducing sugar type and pH values which parallel the growth of lactic acid bacteria probably also play an important role in this regard. Since reducing sugars disappear within a few hours (Pellegrino et al, 1995) and the Amadori compound is relatively stable in the absence of further heating (Ledl, 1990), the furosine gradient established in GP moulding does not change in ripening, even after 15 months.

The stratigraphic values of furosine appear to be characteristic of GP and are related to the cheesemaking process. Grana cheeses produced outside the PDO area under conditions not strictly comparable to the traditional ones show rather different profiles (table I). A reduction in cheese size allows faster cooling in moulding, hence limits the furosine level of the core. On the contrary, the heating of moulded cheese increases MR more in the outer than in the inner zones. Abnormal levels and distribution of furosine found in cheese samples from the market may be due to modifications in cheesemaking technology which can affect the heat load occurring in moulding, such as utilization of starter cultures other than the natural ones (Heap and Lawrence, 1988).

Cheese ripening

The stratigraphic values of moisture content after 9- or 15-month ripening show a rather uniform distribution within cheese, apart from the first 2-cm layer below the rind where they are significantly lower

Table I. Stratigraphic values of furosine (mg/100 g protein) in ripened cheese samples of known origin or collected at the market.
Valeurs stratigraphiques de la furosine (mg/100 g de protéine) dans des échantillons de fromages affinés d'origine connue ou sélectionnés sur le marché.

Cheese type	*Depth from round side (cm)*			
	1–2	*8–10*	*14–16*	*18–20*
Traditional GP	9–12	26–34	39–51	56–67
Experimental grana cheese of reduced size **	10.9	15.0 (core)		
Market grana cheese produced abroad:				
Denmark	15.9	24.8	70.0	95.1
France	9.8	10.0	16.0	16.9
France	55.5		29.3	
France	36.3	30.7	20.0	
Germany	19.9	18.1	16.0	
Germany	8.5		11.0	
Germany	13.5	25.0		
Germany	230.6		43.6	
United Kingdom	15.5	14.7	7.9	
Argentine	30.9	24.5		

* Range of values for six samples ripened for 9–24 months. ** Diameter: 16 cm.
Étendue de valeurs sur six échantillons affinés pendant 9–24 mois.

(fig 5). Small variations are also found in values of a_w. Moreover, the NaCl content, absorbed from the brine by the outer part, is reported to be homogeneously distributed within the cheese after 6-month ripening (Resmini et al, 1974) with the pH values fully comparable between the outer part and the core in the ripened cheese (Pecorari et al, 1995). Therefore, the possible stratigraphic behaviour as regards enzymatic activity taking place during the ripening of GP may be caused by factors other than gross composition.

In previous studies (Resmini et al, 1985; Ferranti et al, 1997) we indicated considerable zonal variations in the release of FAA in GP and PR. The amount is minimum, in the 1–2 cm below the rind, due to low moisture content which causes delay in or even absence of proteolysis (van den Berg and Exterkate, 1993). It reaches a maximum value in the layers just below the rind, then decreases regularly towards the core. A similar distribution was reported by Lavanchy and Sieber (1993) in Sbrinz and Emmental cheeses, even if the gradient decreases progressively during ripening for most of the amino acids. The behaviour found in the present study for GP (fig 6) fully agrees with our previous observations. These typical patterns look comparable in 9- or 15-month old GP samples, despite the significant increase in the total amount of FAA observed during this ripening period (from 13.5–15.5 g to 19.5–21.7 g/100 g protein). Pasteurization of milk does not affect this distribution in a 12-month grana cheese (fig 6), but depresses its FAA content in comparison to control raw-milk cheese (14.5–16.8 g versus 17.8–21.2 g/100 g protein).

A negative close ($r = -0.97$) linear correlation exists between the FAA accumulation gradient and the furosine level gradient which describes the heat load in moulding. This correlation may derive from the partial inactivation of proteolytic

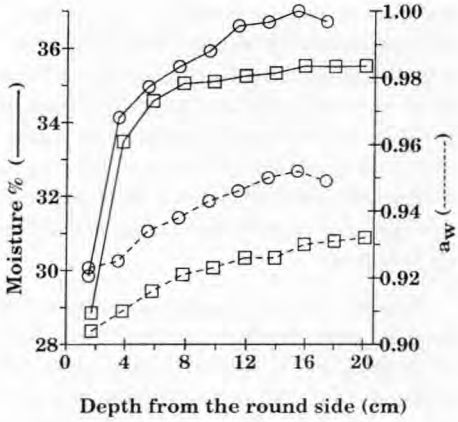

Fig 5. Moisture and a_w values in GP cheese after 9-month (○) and 15-month (❑) ripening.
Humidité et valeurs de a_w *dans du GP après 9 mois (○) et 15 mois (❑) d'affinage.*

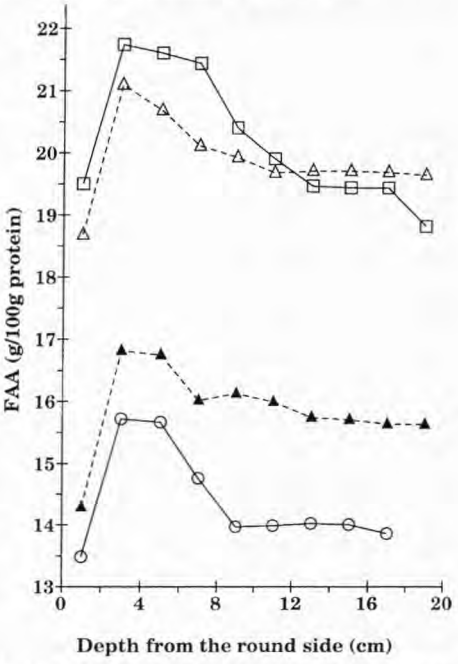

Fig 6. Free amino acids (FAA) in GP cheese after 9- (○) and 15-month (❑) ripening and 12-month old grana cheese made from pasteurized milk (▲) or from the corresponding raw milk (Δ).
Acides aminés libres (FAA) dans du GP après 9 mois (○) et 15 mois (❑) d'affinage et du fromage de type grana de 12 mois fabriqué à partir de lait pasteurisé (▲) ou de lait cru correspondant (Δ).

enzymes or limited growth of microorganisms promoted by heating. The FAA accumulation gradient occurring in cheese from the core to the peripheral zones specifically involves several amino acids, more particularly ser (table II). The level of free ser in a 15-month-old GP shows a 50% increase from the core (R10 portion) to the outer zone (R2 portion).

Recently we have pointed out that in GP ripening, dephosphorylation of serP parallels the release of FAA from soluble peptides (Ferranti et al, 1997). The peptide degradation involves firstly dephosphorylation of the serP N-terminal and then release of ser by aminopeptidases. This mechanism was also confirmed by the extremely low levels of serP found in the FAA fraction of GP and PR, whereas free ser accumulated (Resmini, unpublished data) as a consequence of the simultaneous presence of both active phosphatase and aminopeptidase. In addition, some short-chain 'enzyme-resistant' CPPs mainly represented by β-, α_{s1}-, and α_{s2}-CN-derived peptides containing three contiguous phosphate residues accumulated in GP ripening.

Table II. Stratigraphic values of free amino acids ((FAA; g/kg protein) in 15-month ripened Grana Padano.
Valeurs stratigraphiques des acides aminés libres (g/kg de protéine) dans un fromage Grana Padano affiné pendant 15 mois.

FAA	*Portions*													
	F1	*F2*	*F3*	*F4*	*R1*	*R2*	*R3*	*R4*	*R5*	*R6*	*R7*	*R8*	*R9*	*R10*
Asp	5.7	6.0	5.7	5.6	6.6	6.8	6.2	5.9	5.3	5.0	5.0	4.8	5.1	5.0
Thr	6.8	7.3	7.2	7.1	6.1	7.4	7.7	8.0	7.3	7.1	6.8	6.2	6.4	6.2
Ser	8.5	9.2	9.1	8.9	8.9	9.9	9.4	9.3	8.2	7.7	7.3	6.7	6.8	6.6
Asn	7.8	8.9	8.2	8.1	6.6	7.8	8.0	9.0	8.2	8.0	8.0	7.4	7.7	7.4
Glu	32.9	36.6	35.1	34.5	36.2	41.0	40.0	38.7	34.8	34.1	32.7	30.6	32.5	31.5
Gln	1.2	1.4	1.4	1.3	0.8	1.2	1.2	1.5	1.5	1.0	1.2	1.1	1.2	1.2
Gly	3.7	4.3	4.3	4.2	4.4	5.0	4.8	4.9	4.4	4.3	4.2	3.9	4.1	4.0
Ala	5.3	5.9	5.9	5.8	5.0	5.8	5.9	6.3	5.7	5.6	5.6	5.2	5.6	5.4
Cit	8.8	9.6	8.9	8.8	7.5	8.5	8.4	9.2	8.7	8.2	8.4	8.0	8.6	8.2
Val	13.7	15.2	15.1	14.8	13.9	15.8	15.9	16.7	15.6	15.1	14.8	13.8	14.3	13.8
Met	4.7	4.6	4.6	4.6	5.0	5.0	5.0	5.0	4.5	4.5	4.4	4.1	4.4	4.4
Ile	10.8	10.9	11.0	10.8	11.7	12.2	11.8	12.4	11.3	10.9	10.3	9.7	10.1	9.7
Leu	17.6	17.8	18.0	17.7	17.1	17.8	17.6	19.1	18.4	17.7	17.6	17.0	18.4	17.2
Tyr	6.4	5.4	4.9	4.8	6.5	5.3	5.7	5.7	4.7	4.8	4.7	4.8	4.8	4.8
Phe	10.4	10.7	1.0	1.0	9.5	10.5	10.9	11.4	10.4	10.3	10.2	9.6	10.2	10.7
Orn	1.2	1.4	1.5	1.5	1.5	1.7	1.6	1.8	1.5	1.6	1.6	1.5	1.6	1.6
Lys	22.6	26.4	25.4	24.9	22.5	26.5	26.4	27.6	25.3	24.3	24.0	23.0	24.4	23.5
His	6.3	7.2	7.1	7.0	5.7	6.6	6.8	7.3	6.9	6.7	6.9	6.7	7.3	7.1
Arg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
Pro	17.4	21.7	21.2	20.8	19.6	22.6	22.8	23.7	21.6	21.6	21.2	18.9	20.7	19.8
Total	190.6	209.3	193.2	189.8	195.0	217.4	216.1	223.4	204.0	199.1	194.6	183.0	194.3	188.1

For portion symbols see figure 1.
Pour les symboles des portions : voir fig 1.

A free ser gradient parallel to that of ALP activity is observed in 9- and 15-month ripened GP samples as well as in 12-month ripened grana cheese made from pasteurized milk. Since indigenous ALP is fully inactivated in pasteurized milk, no gradient in free ser depending on the unequal activity of the enzyme within the cheese should be observed. However, the stratigraphic accumulation of 'enzyme-resistant' CPP β-CN(f16–22)3P (Ferranti et al, 1997) in 15-month-old GP (fig 7) follows the behaviour of the heat-load gradient. Moreover, the HPLC patterns of CPPs occurring in the outer zone (fig 8A) and in the core (fig 8B) of GP differ both in the number of peaks and in their relative amount. The identification of the single CPP components is reported in table III. CPPs such as β-CN(f16–22)3P, α_{s1}-CN(f65–74)3P, and α_{s2}-CN(f7–18)3P are common to either portions. Two sets of peptides, one from β-CN, ie, 14/15/16–25 and one from α_{s1}-CN, ie, 61/62/63/64/65–79, are specific of the inner portion R10. The four CPPs β-CN(f13–25)3P, α_{s1}-CN(f61–74)4P, α_{s2}-CN(f7–18)4P and α_{s1}-CN(f67–74)2P are specific to the outer portion R1. These results suggest that CPPs are formed through a similar mechanism involving formation of components with a higher number of residues in the core of cheese than in the

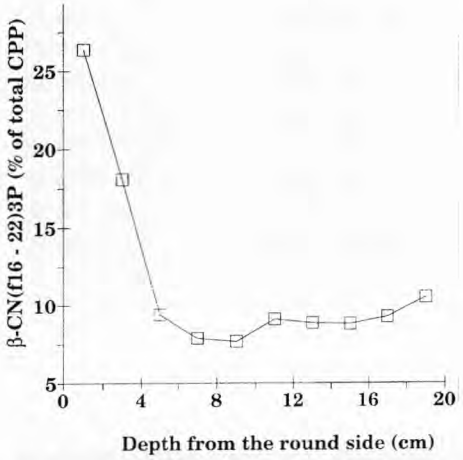

Fig 7. Amount of the β-CN(f16–22)3P phosphopeptide as a relative percent of total casein-phosphopeptides (CPP) in 15-month ripened GP cheese.
Teneur en phosphopeptide β-CN (f16–22)3 P exprimé en % relatif des caséinophosphopeptides totaux (CPP) dans du GP de 15 mois.

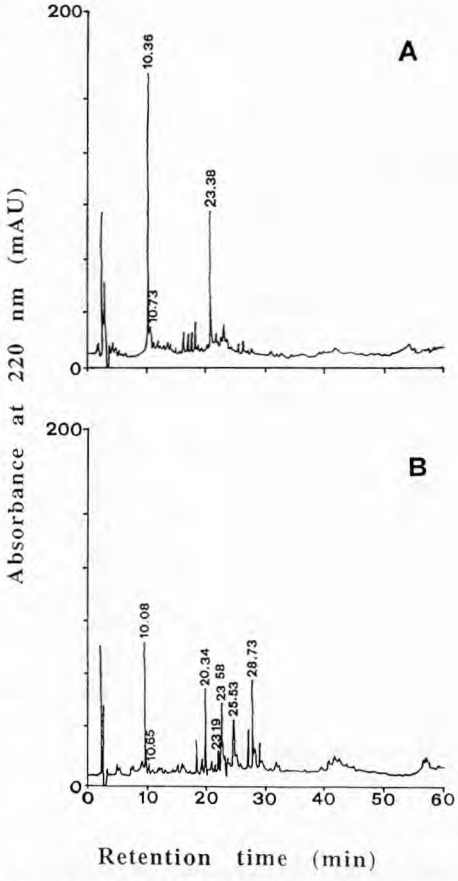

Fig 8. HPLC patterns of casein phosphopeptides determined (**A**) in the outer portion and (**B**) in the inner portion of 15-month ripened GP cheese.
*Profils HPLC des caséinophosphopeptides déterminés : dans la portion extérieure (**A**) et la portion intérieure (**B**) d'un GP de 15 mois.*

Table III. Identification by FAB/MS of casein phosphopeptides isolated by HPLC from the outer (fig 8A) and inner (fig 8B) portion of a 15-month-old cheese.
Identification par FAB/MS des caséinophosphopeptides isolés par HPLC de la portion externe (fig 8A) et la portion interne (fig 8B) d'un fromage GP de 15 mois.

Retention time (min)	*MH+*	*N-terminal sequence*	*Peptide*
R1 portion			
10.36	978	Leu-SerP-SerP-	β(f16–22) 3P
10.73	1065	Ser-Leu-SerP	β(f15–22) 3P
23.38	1034	SerP-SerP-Glu	α_{s1}(f67–74) 2P
	1314	Ile-SerP-SerP	α_{s1}(f65–74) 3P
	1481	SerP-Ile-SerP	α_{s1}(f64–74) 4P
	1534	Val-Ser-SerP	α_{s2}(f7–18) 3P
	1614	Val-SerP-SerP	α_{s2}(f7–18) 4P
	1743	Val-Glu-SerP	β(f13–25) 4P
	1810	Glu-Ala-Glu	α_{s1}(f61–74) 4P
R10 portion			
10.08	978	Leu-SerP-SerP	β(f16–22) 3P
10.65	1065	Ser-Leu-SerP	β(f15–22) 3P
20.34	1348	Leu-SerP-SerP	β(f16–25) 3P
21.95	1644	Glu-SerP-Leu	β(f14–25) 4P
23.58	1314	Ile-SerP-SerP	α_{s1}(f65–74) 3P
	1435	Ser-Leu-SerP	β(f15–25) 3P
25.53	1401	Ser-Ile-SerP	α_{s1}(f64–74) 3P
	1534	Val-Ser-SerP	α_{s2}(f7–18) 3P
	1715	Val-SerP-SerP	α_{s2}(f7–19) 4P
	1966	Ile-SerP-SerP	α_{s1}(f65–79) 4P*
	2132	SerP-Ile-SerP	α_{s1}(f64–79) 5P
	2261	Glu-SerP-Ile	α_{s1}(f63–79) 5P
	2332	Ala-Glu-SerP	α_{s1}(f62–79) 5P
	2461	Glu-Ala-Glu	α_{s1}(f61–79) 5P
	2873	SerP-Leu-SerP	β(f15–28) 4P
	1999	Glu-SerP-Leu	β(f14–28)

* Glutamic acid in position 78.

outer zones. The imbalance of production of peptides with different molecular weight may be the result of a different exopeptidase activity. The CPPs in the portions R1 and R10 of GP cheese made from pasteurized milk were examined by HPLC (data not shown). The patterns are close to the homologous ones in the control grana cheese made from raw milk, but in the portion R1 of pasteurized milk cheese the amount of β-CN(f15–22)3P is similar to that of β-CN(f16–22)3P. These results indicate that the nature of milk, raw or pasteurized, is without any effect on the extent of dephos-

phorylation. Hence, size and quantity of CPPs within GP may be determined by a gradient of aminopeptidase activity, which is higher in the peripheral zones than in the core. Finally, the heat-load gradient may induce differences in the extent of cell lysis which is sensitive to heat shock (Thiboutot et al, 1995), therefore affecting the accumulation of CPPs and FAA within the cheese.

The lipolytic activity within GP was evaluated by computing the amount of FFA from C_6 to C_{18}. The behaviour of FFA in 9- and 15-month ripened samples (data not reported) follows that of FAA, while the concentration is rather constant within grana cheese made from pasteurized milk. These results suggest that a gradient of lipase activity may also exist within the cheese. The effect of microflora living on the cheese surface cannot be excluded, but it should be limited in GP because of the thick (3–5 cm) and compact rind, which is regularly cleaned every 15 days throughout ripening (Parisi, 1971).

CONCLUSIONS

During spontaneous cooling of moulded GP, the core but not the outer part remains at 52–56 °C for 8–10 h. This restricted heating, in combination with the low pH value produces some established effects on the cheese. Other effects can at present only be hypothesized, and require further studies.

The well-defined gradients of ALP activity and furosine values, both related to the heat load gradient, provide precise indications on possible heat treatments of raw material or manipulation of the traditional cheesemaking technology. These chemical indices are useful for a better characterization of this PDO cheese.

Moreover, the present study clearly demonstrates that the ripening which occurs in GP has a centripetal behaviour. Direct dependence of this behaviour upon the heat load gradient occurring in GP moulding can be hypothesized, especially as far as accumulation of CPPs and FAA is concerned. In this regard, further investigations are necessary.

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