FREE AND TOTAL GMP (GLYCOMACROPEPTIDE) CONTENTS OF MILK DURING BOVINE LACTATION[1]

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SUMMARY

Individual milk samples taken every two weeks from parturition to the end of lactation from 34 animals of three different herds and breeds were analyzed for free-GMP. A milk pool of each herd was analyzed for free and total GMP (released from k-casein by the action of rennin) and the data were correlated with sanitary conditions of animal and udder, phase of lactation and milk production. Most udder problems were concentrated near parturition, with few and spaced occurrences of clinical mastitis. The Californian Mastitis Test (CMT) results showed oscillations compatible with the phases of lactation period and environmental conditions. The widest variations in free-GMP occurred as a function of lactation period and as a consequence of clinical or subclinical mastitis. Higher levels were observed at the beginning of lactation (5.87mg L^{-1} of sialic acid), becoming normal with mean values of about 3.30mg L^{-1} at the end of the second month, and increasing again during the final third of lactation. On average, the same trends were observed for total GMP released by commercial rennet, beginning with slightly high values (35.59mg L^{-1}), becoming normal by the sixth month with values close to 27.15mg L^{-1}, and rising gradually up to the end of lactation, with 58.35mg L^{-1} of sialic acid. These results prove to be useful for the correct interpretation of tests applied to milk selection with respect to proteolytic status or even to restrain frauds by the addition of whey to milk.

Keywords: glycomacropeptide (GMP); sialic acid; milk; lactation; bovine.

RESUMO

VARIAÇÃO DOS TEORES DE GMP (GLICOMACROPEPTÍDEO) LIVRE E TOTAL NO LEITE BOVINO DURANTE A LACTAÇÃO. Amostras quinzenais, desde o parto até o final do período de lactação, obtidas de 34 vacas de três diferentes raças e propriedades, foram analisadas quanto à presença de GMP livre. Um "pool" das amostras quinzenais de cada rebanho foi analisada tanto para o conteúdo de GMP livre quanto para o GMP total (liberado da κ-caseína pela ação da renina), correlacionando-os com as condições sanitárias do animal e do úbere, à fase da lactação e à produção de leite. A maioria dos problemas sanitários concentrou-se próximo ao parto, com poucas e espaçadas ocorrências de mastites clínicas. Os resultados do teste de CMT mostraram reações compatíveis às fases da lactação. Para o GMP livre as maiores variações ocorreram em função do período de lactação e em conseqüência de mastites clínicas e subclínicas. Valores elevados foram observados no início da lactação (5,87mg de ácido siálico/L de leite), normalizando para valores próximos de 3,30mg/L já ao final do segundo mês e voltando a elevar-se no terço final da lactação. Em média, as mesmas tendências foram observadas para o teor de GMP total liberado pela ação de coalho comercial, iniciando com valores ligeiramente elevados (35,59mg/L), tornando-se normal e assim se mantendo até o sexto mês com valores próximos a 27,15mg/L, e novamente elevando-se gradualmente até o final da lactação, com 58,35mg de ácido siálico/L de leite. Esses resultados mostram-se úteis para a correta interpretação de métodos aplicados à seleção do leite, seja em relação ao "status" proteolítico da matéria-prima ou mesmo para coibição de fraudes por adição de soro ao leite.

Palavras-chave: glicomacropeptídeo (GMP); acido siálico; leite; lactação; bovinos.

1 – INTRODUCTION

According to JOLLES & FIAT [14], κ-casein is the main glycoprotein of cow's milk. Its polysaccharide part is O-glycosidically linked to threonine residue 133, containing only three different sugars: GaL, GaLNAc and NeuNAc. Human κ-casein contains at last three times more carboydrate than bovine casein.

Glycomacropeptide (GMP) released from κ-casein by the action of chymosin (rennet) during cheese making is a C-terminal peptide (residues 106-169) containing varying amounts of sugar including N-acetylneuraminic acid (sialic acid) [5]. GMP is a single peptide of 6,755 Da. containing 64 amino acid residues (4 Asp, 11Thr, 6 Ser, 10 Glu, 8 Pro, 1 Gly, 6 Ala, 6 Val, 1 Met, 7 Ile, 1 Leu and 3 Lys), and lacking sulphur and aromatic amino acids [15, 23, 26].

The κ-casein GMP is an important proteolytic action marker, being useful to ensure quality control of raw milk as well as to control industrialization processes, mainly in the production of cheese and sweet whey. Determination of sialic acid in sweet whey is useful since the concentration of sialic acid reflects the amount of GMP present [1].

The sialoconjugate content of human milk has been extensively documented; however, little attention has been paid to the changes occurring in these compounds in cows during lactation [17]. Beneficial biological roles attributed to GMP or peptides derived from it include stimulation of cholecystokinin (a hormone regulating energy and food intake) released from intestinal cells, inhibition of platelet aggregation and support of beneficial intestinal bacteria (Bifidobacteria). In an in vitro study, GMP prevented adhesion of cariogenic bacteria to tooth surfaces, leading researchers to speculate that GMP may reduce dental caries. Because GMP lacks the amino acid phenylalanine, it has potential use as an ingredient in foods for patients with phenylketonuria [10,13].

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Glycomacropeptide is known to be a biologically active compound and is considered to be a potential ingredient for functional foods and pharmaceuticals [1,21]. The European Economic Community (ECC) adopted the determination of sialic acid level as the most appropriate procedure to reveal and inhibit frauds by adding whey to milk and milk powders. In this feature, HPLC has been the choice method for quantitative GMP determination by the Brazilian Standard Methodology [3]. Despite its advantages, HPLC is expensive and time-consuming, factors that impair its widespread utilization.

FUKUDA, RÖIG & PRATA [6, 7] described a new spectrophotometric method for the quantitative determination of sialic acid in milk and milk products using acidic ninhydrin (GAITONDE'S reagent). Due to the qualities of the method, they proposed its use as a screening test for selection of milk. However, while GMP is released from κ-casein by the action of chymosin, it is now well documented that normal milk has naturally variable amounts of free-GMP, varying according to lactation stage and sanitary conditions of the udder.

Recently, FUKUDA, RÖIG & PRATA [8] (unpublished data) showed an excellent correlation between the quantitative sialic acid method (acid ninhydrin) and HPLC for GMP (r = 0.996), permitting the widespread use of the spectrophotometric method, a simple, inexpensive and rapid method.

Considering the variability of milk glycosylation and the need for this information for the correct interpretation of methods for GMP analysis, the objective of the present study was to verify the variations of free-GMP and GMP released from κ-casein by rennin in bovine milk in terms of lactation stage, milk production and sanitary udder conditions throughout lactation for three groups of different cattle herds.

2 – MATERIAL AND METHODS

Thirty-four cows were selected at the beginning of the lactation period from three different farms and breeds (april/98 to june/99, west region of Sao Paulo state, Brazil). Animals from farm A (10) were 7/8 Holstein cows producing an average of 11kg of milk in the first of two milkings. Animals from farm B were 11 pure Holstein cows producing an average of 23kg of milk in the main milking and, animals from farm C, were 13 half-bred cows (Holstein x Nelore) producing an average of 9kg of milk in the first of two milkings.

Samples were collected at 15 day intervals from the colostral period to the end of lactation. Before sample collection the sanitary conditions of the animals were evaluated at the first milking time, with special attention to the udder. The CMT tests were applied and individual milk production was recorded.

Samples were carried to the laboratory in an ice-bath. Individual samples were analyzed for free-GMP. A pool of samples from all animals on each farm was analyzed for free-GMP and, after clotting milk with commercial rennet simulating the usual practices of cheese making, for rennin-released GMP (or total GMP).

Free-GMP: each milk sample was mixed with the same amount of a 24% TCA solution, left to stand for 30 minutes, and filtered (Whatman). Duplicates of 10mL of the filtrates were mixed with 1mL of 20% PTA solution and centrifuged at 3,500rpm/10 minutes. The supernatant was discarded and the pellet remaining in each tube was washed with 8mL of ethanol, and centrifuged again, and the supernatant was discarded. Two (2)mL of glacial acetic acid were added to each test tube (pellet) and the preparation was mixed well in a vortex mixer to re-dissolve the pellet. One (1)mL of ninhydrin acid solution (GAITONDE'S reagent) was added and the preparation was placed in a strong water-bath for exactly 10 minutes, followed by an ice-water bath to cool the samples to room temperature. Absorbance readings of each test tube were taken in the visible range of the spectrophotometer at 470nm. The results were recorded and average absorbance, standard deviations and coefficients of variation were calculated. Sialic acid was quantitated using a standard curve constructed with pure sialic acid (95% sialic acid from SIGMA Co.)[27, 28].

Total-GMP: after clotting with proportional amounts of commercial rennet solutions, each pool sample was cut for separation of sweet whey. Samples of whey were mixed with the same amount of 24% TCA solution and then submitted to the same procedures as used for free-GMP, yielding the amount of GMP released from κ-casein by the action of rennin.

3 – RESULTS AND DISCUSSION

The levels of free-GMP for all individual samples showed high average values near the colostral period at the beginning of lactation, corresponding to 5.87mg L^{-1} of sialic acid, with a fast decrease to normal milk levels, with mean values of about 3.30mg L^{-1}, showing little variation from the second month to the final third of lactation, when a trend to a permanent increase of free levels was observed, but with wide variation among animals and groups.

TABLE 1. Free and total GMP levels detected in individual and pooled milk samples obtained from animals from three farms.

Farm	Free-GMP (individual samples) mg/L						Total GMP (pool samples) mg/L					
	n	X^1	Sd^2	Md^3	Min.	Max.	n	X^1	Sd^2	Md^3	Min.	Max.
A	190	4.69	1.25	4.16	3.26	9.94	22	39.45	14.86	33.94	22.02	77.06
B	220	3.69	0.22	3.47	3.01	5.25	22	33.21	11.01	30.82	20.73	71.19
C	260	4.77	1.28	4.29	2.92	8.35	22	42.93	14.68	37.98	25.69	80.18
Total	670	4.33	0.62	3.96	-	-	22	38.80	12.11	34.55	-	-

n = samples; x^1 = Average mean; Sd^2 = Standard deviation; Md^3 = Median value; Min = Minimum value; Max = Maximum value.

MORRISSEY [19] showed that bovine milk contains 177mg L^{-1} of sialic acid with 142mg L^{-1} (80.2%) attached to the casein fraction. Thus, 19.8% or 35mg L^{-1} is free

sialic acid or other sialoconjugates. HOFI et al [12] found 169mg L^{-1} of sialic acid in bovine milk, 74% attached to the casein fraction. Of this amount, only 50-55% was released by the action of chymosin. For buffalo milk, GANGULI [9] showed contents varying from 1.57 to 2.70mg of sialic acid/g of casein micelle. Rennin action was found to release only 47% of the submicelle content of and about 85% of the casein micelle content.

The differences observed in the present work may be due to the experimental conditions. While MORRISSEY [19] and HOFI et al. [12] analyzed milk for total sialic acid content, here the values represent only the sialic acid attached to free-GMP peptides. Free sialic acid or other low molecular weight sialoconjugates remain soluble in the supernatant phase after centrifugation and then are discarded. The average values of free-GMP for each animal group (farms A, B and C) are shown in *Figure 1*, with distinction for the average values for all animals and the entire lactation period. It could be observed that trends remained the same until the 11th sample (180 days) with little variations among results for different farms and breeds. While two farms showed a permanent increasing trend in free GMP content, the other showed unchanged values until the 17th sample (270 days).

For normal milk, although individual variations have occurred, the average values of free-GMP remained practically constant, near 3.30mg L^{-1}. Strong individual variations in this phase were correlated with clinical and subclinical mastitis as recorded by sanitary udder evaluation at each sample collection, confirmed by literature data [2, 24, 25]. In these cases, values of about 19.8mg L^{-1} of sialic acid were seen, always associated with sanitary conditions of udder. At the end of the lactation period a permanent trend to increasing free GMP values was observed. Lower data on the curves were due to the exclusion of experimental animals from milking.

For all farms, free GMP levels of pooled milk samples changed from 3.10 to 6.60mg L^{-1} throughout the lactation period, with an average of 4.30mg L^{-1} of sialic acid and a median of 4.00mg L^{-1}.

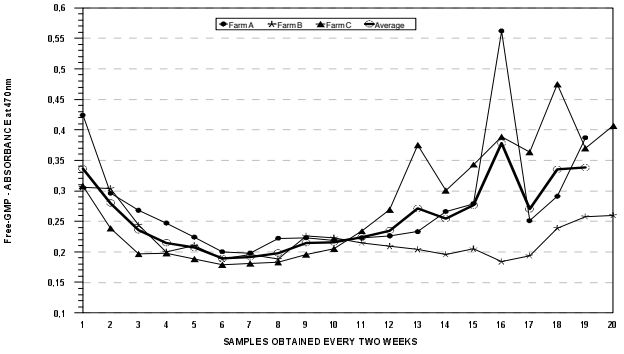

FIGURE 1. Evolution of free-GMP in milk for each farm group cows with distinction for average results for all animals during the entire lactation period.

Variations in total GMP, or GMP released from κ-casein by the action of rennin, were similar to variations in free GMP. High values were observed at the beginning of the lactation period, followed by a fast decrease to normal values which continued unchanged until the 12th sample (195 days) and then by strong variations and a trend to increasing values until the end of lactation (*Figure 2*). Total GMP changed from 24.2 to 64.4mg L^{-1} of sialic acid, with an average value of 38.8 and a median of 34.2mg L^{-1}. On average, these values were 9 to 10 times greater than those observed for free-GMP. Sweet whey obtained during cheese making shows GMP values that are 9 to 15 times greater than values observed for free GMP content of normal milks.

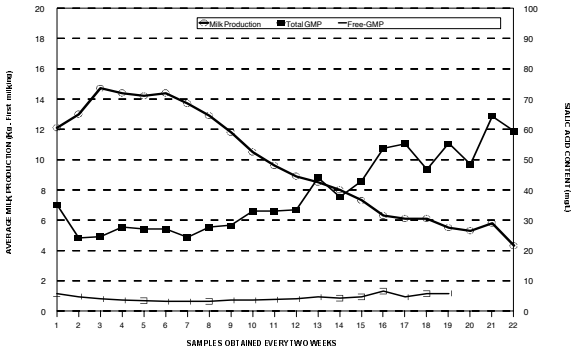

FIGURE 2. Evolution of Free and Total GMP throughout the lactation period with respect to average milk production in the sampled milking.

The trends observed here both for free and total GMP are similar to those observed by MARTIN et al. [17]. They showed the highest values in the colostrum, decreasing in transitional and mature milks and increasing again in late-lactation bovine milk. Similar trends were described by MARIER et al. [16], MORRISSEY [19], ROBITAILLE et al. [22] and SHING & GANGULI [24] for bovine milk, and by CARLSON [4], HEINE, WUTZKE & RADKE [11], MILLER et al. [18] and NAKANO, SUGAWARA & KAWAKAMI [20] for human milk. Total sialic acid content of human milk is very high when compared to bovine milk, with reported values varying from 25682mg L^{-1} to 1,300 ± 322mg L^{-1}. MILLER et al. [18] showed that sialic acid content of human milk decreases 71% from the first week to the third month of lactation. They suggest that the changes observed may simply reflect the aging of the cells responsible for milk secretion, but are also consistent with a programmed adaptation of the milk composition to the needs of the infant.

While the second statement seems to be true, the first does not. For bovine milk, the highest values and widest variations are seem at the end of lactation period, just at moment that the udder undergoes remodeling, with increased desquamation of epithelial cells, decreased on milk production and increased levels of proteolytic enzymes in milk, such as plasmin.

Figure 3 summarizes the relationship between free and total GMP with the evolution of CMT tests applied

to all experimental cows throughout lactation, with 680 CMT results. It could be observed that both for free and total GMP the trend to increasing values while lactation progressed is similar to the trend of evolution of somatic cellular content of milk, expressed by the results of the CMT tests. The drops in curves for both GMP and CMT observed at the end of lactation (end of the third period) were artificially produced by the exclusion of experimental cows from the milking line, with lactation ending at different times.

Another interesting result is illustrated in *Figure 2*, which shows the evolution of free and total GMP in relation to milk production. Although working with different breeds with different capabilities for milk production, on average it could be observed that the curves showed inverse trends. While during phases of high production free and total GMP remained practically invariable with lower values, during the third period both free and total GMP tended to show a permanent increase with decreasing milk production. The decrease in milk production during the third period reflects a normal physiological feature, with a decreased synthesis rate and an increase in epithelial cell desquamation, with reflexes on the CMT tests. With inverse trends for free and total GMP we may speculate that the rate of glycosylation of the casein fraction of milk remains practically constant throughout lactation, with κ-casein being more glycosylated while lactation progresses.

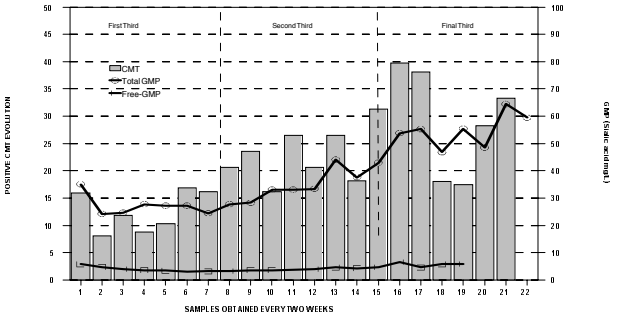

FIGURE 3. Evolution of free and total GMP throughout the lactation period with reference to the evolution of CMT positive results.

4 – CONCLUSIONS

Milk free and total (released by rennet) GMP varies according to lactation period, sanitary conditions of udder (clinical and subclinical mastitis) and as an inverse function of milk production.

Free GMP shows mean values of 3.30mg of sialic acid/L in mature milk, being high near the colostral period and during the third phase of lactation. GMP released from κ-casein by rennet shows a similar pattern of evolution while lactation progresses, with an average of 38.80mg/L and a median of 34.40mg/L. These values are, on average, at least 10 times higher than free GMP in mature milk.

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