UNIVERSITY OF ALBERTA

Goat's Glycomacropeptide and Glycomacropeptide Depleted-Whey: Isolation and Characterization

by

Eryck Romeo Silva Hernández

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall, 2004

Your file Votre référence
ISBN: 0-612-96018-8
Our file Notre référence
ISBN: 0-612-96018-8

Acknowledgments

I greatly acknowledge the financial support from the doctoral scholarship awarded by the Programa de Formación de Profesores de la Universidad Veracruzana (UV-PROMEP), México, as well as from the partial scholarship awarded by the Dirección de Enseñanza Media Superior y Superior del Estado de Veracruz, México. In addition, I greatly acknowledge the financial support from the University of Alberta, Parmalat Canada, the Alberta Agricultural Research Institute, and the Natural Sciences and Engineering Research Council of Canada.

Many thanks to my supervisory committee, but especially to my direct supervisor, Dr. Lech Ozimek, for his unconditional support related and non-related to the university affairs.

I would like to express my special acknowledgement to Dr. Takuo Nakano, who deserves all my respect and appreciation. Without Dr. Nakano's help, my studies would not have been the same.

Thank you very much to Dr. Iñigo Verdalet, who has always encouraged me to keep growing intellectually, as well as spiritually. Thanks also to Dr. Mario Miguel Ojeda and Dr. Wendy Wismer for their unconditional support and trust.

I would like to say thanks to my wife, Marisol, for supporting me during our good and hard times in Canada, as well as to my sons Kevin and Eryck, who have always made my life worth it. In addition, I would like to show my appreciation to my mother, Lucia, and to my brothers, Dyck and Chuck, as well as to their families for their emotional support.

I also would like to express my gratitude to all those who in one or another way, encouraged or influenced me to study this PhD, especially to my father, Ricardo(†); my grandmother, Chonita(†); Doña Maria and her family; my uncles, aunts and their families; Rosy, Rafa, Arturo Morales and Rubén D. Mendiola.

Thanks are extended to all the people who made my PhD studies more comfortable, especially to Bob and Betty Voelker, Nathalie, Juan, Mireya, Jorge, Gina, Francisco, Melissa, Jonathan, Norma and Jacqueline, as well as to Linda and Dave Hut, and Kathy and Gary Short. Thanks are also extended to my colleagues and friends Sylvia, Sirinda, Hisham, Judy, Mei, Nuch, Gordon, Anchelee, Jessy, Annika, Vivian, Teri, Singh, Carlos, Paul, Abdul and Arun, and to the rest of my friends at the University of Alberta and at the Edmonton Mennonite Centre for Newcomers.

Finally, I would like to say thanks to my former colleagues at the World Food Program, especially to Yutong Li, whose friendship crossed the time-barrier.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Introduction[1]

1.1 INTRODUCTION

Functional food is a term used to designate all those foods that have beneficial physiological functionality beyond adequate nutritional effects (1, 2). It is now known that there are some food components (added or not) that help to improve the state of health or reduce the risk of disease. Lactoval in milk, phytosterols in margarine, folic acid in bread, soluble fiber on fruit juice and β-carotene in carrots are some examples. Therefore, functional foods have become an important area in food science and nutrition.

According to Horton (3), Hilliam (1) and Roberfroid (2), there is evidence that the European, Japanese and American markets are becoming increasingly interested for functional foods. The increase in life expectancy, the belief that it is possible to influence one's own health and the awareness of the importance of prevention are the main factors that influence the demand for these kinds of products. Consequently, there is a strong intention by some governments to make functional foods an integral part of nutritional promotion in order to stimulate and develop this field for the benefit of consumers' health (1-3).

Milk, the first food for mammalian neonates, contains diverse constituents with physiological functionality (4). Some of these bio-active milk compounds (or their derived products) are already being used in several commercial products, such as lactoperoxidase in toothpaste (anti-cavities), lactoferrin in infants' formulas (anti-bacterial) and lactulose as a bifidogenic product (3, 4). However, more dairy products could be used as functional foods, but they have yet to be exploited.

---

[1] Part of of this chapter has been published. Silva-Hernandez, E.R.; Nakano, T.; Ikawa, N.; Ozimek, L. Goat kappa-casein Glyco-macropeptide In: *Food Science and Product Technology. Research* Signpost. India. 2002, Pp. 104-110.

Nowadays, many conventional dairy products can be considered foods with physiological functionality (4). This characteristic of extending beyond ordinary nutritional effects is attributed to many milk constituents, such as proteins, lipids, carbohydrates and even hydrolyzed milk compounds. In addition, some dairy byproducts have become an important source of nutrients. Whey proteins, for example, are well known because of their high nutritional value in addition to their versatile functional properties in food products (5). Furthermore, value-added fractionated milk ingredients are rapidly becoming economically important (6).

Use of sweet whey proteins has not only helped to prevent the pollution caused by the disposal of this byproduct of cheese-making but also has made this byproduct economically significant. However, there are other uses that might add even more value to sweet whey, such as the isolation of glycomacropeptide.

Glycomacropeptide (GMP), found in sweet whey (or cheese whey), is a C-terminal hydrophilic glycopeptide released from κ-casein after chymosin hydrolysis (7-9). This glycopeptide is also referred to as caseinomacropeptide or caseinoglycopeptide. GMP lacks aromatic amino acids (phenylalanine, tyrosine and tryptophan), and contains varying amounts of phosphate and sugars, including sialic acid, galactose and N-acetylgalactosamine (8, 10). Structure and composition of bovine GMP have been studied (9, 11-16). Bovine GMP has a number of biological activities (7, 17-19), and is thought to be a potential ingredient for functional foods and pharmaceuticals.

In contrast to bovine GMP, there is relatively limited information available concerning goat GMP. Alais and Jollès (20) and Jollès *et al.* (21) isolated GMP from caprine κ-casein and reported its chemical composition. Mercier *et al.* (13, 22) reported the amino acid sequence of κ-casein. There are, however, few reports of attempts to purify GMP from goat sweet whey, or comparative studies of when GMP is obtained from raw or pasteurized milk.

During the isolation of the GMP, a fraction referred to as GMP-depleted whey is also produced. There is limited information available concerning chemical composition of this GMP-depleted whey fraction. Such information may

be important for developing value-added products with this fraction as well. Due to the relatively high content of the amino acid threonine in GMP, it was suggested that the use of GMP-depleted whey fraction (reduced in threonine) may be useful as an ingredient in infants' formulas to prevent hyperthreoninemia (abnormally high threonine in blood), a well-known irregularity found in infants fed sweet whey-based formulas (23).

Production of GMP-depleted whey might be more practical if whey powder were used as a raw material. Currently, the use of bovine sweet whey powder in the dairy industry is convenient due to costs, storage and transportation issues and because of milk-production fluctuations.

Therefore, this chapter introduces the fundamentals of the studies presented in the subsequent chapters. The topics covered in these chapters include isolation and characterization of goat GMP, a comparison of this glycomacropeptide when it is isolated from raw and pasteurized goat's milk, and the chemical characterization of GMP-depleted whey obtained from goat's milk, and also from cow's sweet whey powder.

1.2 SIALIC ACID CONCENTRATION IN GOAT SWEET WHEY AND MILK

Since most sialic acid in bovine or goat sweet whey is found in GMP (24, 25), the concentration of sialic acid in sweet whey is an important indicator for estimating the amount of GMP present. Our recent study (24) showed that the concentration of sialic acid in sweet whey prepared from commercial goat's milk is 79 μg/ml. This is apparently lower than the sialic acid concentration (116 μg/ml) found previously in bovine sweet whey (25).

There is little published information available on the sialic acid content in goat sweet whey. It can, however, be estimated from the total sialic acid content in goat's milk, assuming that 99.9 % (26) of sialic acid is attached to protein (but not ganglyoside) and released into sweet whey, which account for 75% of milk (v/v, ref. 24). The sialic acid concentration in goat sweet whey shown above is comparable to the corresponding value (68 μg/ml) calculated from the data (5.1 mg sialic acid in 100 ml of mid-lactation goat's milk) reported by Morrisey (27)

The sialic acid concentration in milk changes during lactation. Puente *et al.* (26), in a study of goat's milk during the first two months lactation period, reported that the sialic acid concentration in milk was the highest on day 1 (1147 mg/kg), rapidly decreased to 558 mg/kg on day 2, and gradually decreased to 203 mg/kg after two months. The value after two months is, however, three-fold higher than that found in the mid-lactation goat's milk by Morrisey (27) (see above). Whether this difference is due to animal variation or other factors involved is unknown.

1.3 ISOLATION OF GMP

Bovine GMP has been isolated from sweet whey or whey protein concentrate by various techniques, including ultrafiltration, gel filtration chromatography, protein precipitation, ion-exchange chromatography and hydrophobic interaction chromatography (reviewed in ref. 28).

Highly purified GMP is difficult to prepare by a single-step procedure of either ultrafiltration or gel filtration chromatography. Anion-exchange chromatography may be the most practical method. Removal of small molecular weight materials from sweet whey by dialysis, is important to maximize the yield of GMP adsorbed to the anion-exchanger. Elimination of minor impurities from GMP fraction prepared by anion-exchange chromatography may be achieved by ethanol precipitation of proteins, hydrophobic interaction chromatography and cation-exchange chromatography (28).

In chapters 2 and 3, a procedure to purified GMP from non-dialyzable fraction of goat's milk sweet whey using a two-step technique is presented. This process includes anion-exchange chromatography on DEAE-Sephacel and hydrophobic interaction chromatography on phenyl-agarose. This method provided GMP fraction with its amino acid composition showing a trace of phenylalanine (24). In addition, the GMP-depleted whey, obtained during the ion-exchange chromatography, showed a significantly lower content of threonine, which might be suitable for its use as an ingredient in threonine-reduced infants'

formulas (see "hyperthreoninemia and gmp-depleted whey" below and chapters 3, 4 and 5).

1.4 CHEMICAL COMPOSITION AND STRUCTURE OF GMP

Published information on the carbohydrate and phosphorous analysis in goat GMP is limited to the report of Alais and Jollès (20). These authors isolated GMP from goat κ-casein, and reported the contents of sialic acid, galactose, galactosamine and phosphorous to be 3.0, 4.0, 2.8 and 0.72%, respectively, by dry weight. The content of sialic acid is comparable to that (2.5%) we found (24), but three times lower than that in bovine GMP reported by Kawakami *et al.* (29) (average 8.03% calculated from the author's results) or Nakano and Ozimek (28) (7.75%). From the phosphorous content of Alais and Jollès (20), it is estimated that the GMP contains approximately 1.7 residues of phosphate per peptide, which was calculated assuming that the molecular weight of goat GMP is 7.6 kDa (see below). This appears to be consistent with the previous reports showing that in caprine GMP Ser-151 and Ser-168 are phosphorylated (9, 10, 22).

From the concentrations of carbohydrates and phosphorous (see above), the proportion of peptide in goat GMP is estimated to be approximately 90%. Both amino acid sequence and composition of goat GMP (residues 106-171) differ from those of bovine GMP (residues (106-169). Amino acid sequences of caprine and bovine GMPs are given in Figure 1.1. There are 19 substitutions and two insertions (Val-132 and His-133) in the goat sequences compared to the bovine sequence (9, 13, 22). Differences in the primary structure of GMP among goats and other species, including humans, pigs, sheep and buffalo, have been discussed elsewhere (13,30).

Table 1.1 shows amino acid composition determined in GMP purified from either goat or bovine sweet whey in our laboratory, and theoretical amino acid composition of GMP for either species. Threonine and glutamic acid (and/or glutamine) are the most abundant amino acids in either GMP. The third abundant amino acids are serine and proline, respectively, in goat and bovine GMPs. Glycine and leucine, which are found in bovine GMP, do not occur in goat GMP,

and histidine, absent in bovine GMP, occurs in goat GMP. Traces (<1 residue/ peptide) of glycine and leucine detected in the goat GMP purified from sweet whey (Table 1.1) were probably derived from contaminating protein and/or peptide.

In a study of species differences in GMP structure, Plowman *et al.* (15) predicted that the residues 136-152 of bovine κ-casein are largely in the helical conformation, and that the occurrence of helical structure is in a low probability in goat GMP. Residues Glu-140, Ser-114, Ala-144 and Leu-146 found in the bovine sequence are replaced by Val, Gln, Asp and Pro, respectively in the goat sequence, suggesting a species difference in GMP conformation.

1.5 ISOELECTRIC POINT

By using a program, Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html), the isoelectric point (pI) of peptide can be computed. The pI of the 66-residue goat GMP peptide is 4.18. This is slightly higher than the pIs (4.04 and 4.14 for genetic variants A and B, respectively) of the 64-residue bovine GMP peptide. The pI of goat GMP with sialic acid (pK_a=2.6, ref. 31) and phosphate (pK_a=2.0) is expected to be lower than that of its peptide (see above). The precise pI value of goat or bovine GMP is unknown. A previous study indicated that the apparent pI of bovine GMP determined by chromatofocusing is <3.8 (32). However, this technique does not give accurate measurements for a pI below 4 (Pharmacia Fine Chemicals, 1980).

1.6 GEL ELECTROPHORESIS

We have recently studied goat GMP using sodium dodecyl sulfate (SDS)-gel electrophoresis in the presence and absence of 8 M urea (24). The GMP gave major and minor bands on urea-free gels with their average mobilities being faster than those of carbonic anhydrase (31.0 kDa) and lysozyme (14.4 kDa), respectively (Figure 1.2). However, when electrophoresed on gels containing 8 M urea (dissociative condition), the GMP demonstrated a major broad band with its average mobility slightly faster than that of lysozyme (Figure 1.3). These results

suggest that the majority of goat GMP forms dimmers on urea-free gels and dissociates into monomers on gels containing 8 M urea. Similar findings have been reported in bovine GMP (32, 33). This may be due in part to the acidic nature of GMP (see above) to which sufficient amounts of SDS cannot bind to dissociate the aggregated GMP. If so, the electrophoretic mobility of GMP is dependent on its size and charge, and thus an accurate estimation of an apparent molecular weight (M_r) using SDS-gel electrophoresis is difficult. The mechanism of dimerization of GMP is unknown. Previous studies of bovine GMP (34) also suggest that determination of its M_r using gel chromatography on Sephadex G-75 in the presence of 6 M guanidine hydrochloride (dissociative solvent) is difficult because there is no appropriate glycoprotein or glycopeptide standards available to calibrate the gel chromatographic column. Use of protein standards to calibrate the column results in an overestimation of the M_r of GMP (34). We therefore, suggest other methods, including mass spectrometry, sedimentation equilibrium and light scattering techniques, to determine the M_r of GMP. The M_r of goat GMP should be close to 7.6 kDa. This value can be obtained from summation of the theoretical molecular weight of the 66-residue peptide (6.824 kDa), and the molecular weights of sialic acid, galactose, N-acetylgalactosamine and phosphate estimated from the analytical data for goat GMP reported by Alais and Jollès (20) (see above).

Results from our laboratory also showed that goat GMP in SDS-gel is intensely stained with Coomassie blue in 50% methanol containing 12.5% (w/v) trichloroacetic acid (solution-A), but shows very weak staining in 45% methanol containing 10% acetic acid (solution-B), a commonly used preparation to stain protein (11). Similar results were demonstrated previously in bovine GMP (33). It appears that the acidic GMP with low pI (see above) can bind to anionic Coomassie blue in solution-A (pH=~1.6), but not in solution-B (pH=~3.5). Coomassie blue is, however, not specific to GMP. Immunological methods (e.g. immunoblotting) are superior to Coomassie blue staining for identification of GMP, if specific antibodies are available. Little information is available concerning antibodies raised against goat GMP.

1.7 HYPERTHREONINEMIA AND GMP-DEPLETED WHEY

Hyperthreoninemia is a well-known phenomenon characterized by abnormally high levels of threonine in blood. It has been reported that hyperthreoninemia is relatively common in infants fed with sweet whey-based formulas (35-40). Rigo *et al.*, (23) have suggested that high levels of threonine in blood should be avoided since it might cause problems during brain development. Reduction of threonine in diets has been suggested as effective to decrease hyperthreoninemia (42, 43). GMP is rich in threonine, and perhaps the most important component that causes hyperthreoninemia. Depletion of GMP from sweet whey would provide a product with a reduced threonine content, which might be used in infants' formulas to prevent hyperthreoninemia. Chapters 4 and 5 present the chemical characterization of GMP-depleted whey, and its comparison with human milk.

1.8 RESEARCH OBJECTIVES

Adequate isolation and characterization of potential functional foods and ingredients must be carried out before reaching the market. Cow glycomacropeptide has been extensively studied. There is, however, relatively limited information available concerning structure and composition of goat GMP. Little is known about the effect of pasteurization of milk on the structure and composition of GMP, as well as little information available regarding the GMP-depleted whey fraction. This study is comprised of four experiments. The first experiment was undertaken to isolate GMP from goat sweet whey by anion-exchange and hydrophobic interaction chromatography and to characterize it using chemical analyses, gel chromatography and electrophoresis. The second compared GMP isolated from raw and pasteurized goat's milk using chemical analysis and sodium dodecyl sulfate (SDS) gel electrophoresis. The third and fourth experiments were carried out to prepare GMP-depleted whey fraction prepared from goat's milk, as well as from bovine sweet whey powder, and to

determine whether they are suitable for infants' formulas by examining their chemical composition.

1.9 REFERENCES

1. Hilliam M. The market for functional foods. *Int. Dairy J.* **1998,** *8,*349-353.
2. Roberfroid, M.B. Prebiotics and symbiotics: concepts and nutritional properties. *Brit. J. Nutr.* **1998**, *80(Suppl 2),* S197–S202.
3. Horton, B. Commercial utilization de minor milk components in the health and food industries. *J. Dairy Sci.* **1995**, *78*, 2584-2589.
4. Jelen, P.; Lutz, S. *Functional foods, Biochemical y Processing aspects.* Lancaster, Basel: Technomic Publishing CO., Inc. 1998, 357-380.
5. Wit, J.N. Nutritional and functional characteristics de sueroproteins in food products. *J. Dairy Sci.* **1998**, *81*, 597-608.
6. Paquin, P. The key to added value: Fractionation and use de milk constituents. *Can. J. Anim. Sci.* **1998**, *78(Suppl),* 149-157.
7. Brody, E. Biological activities of bovine glycomacropeptide. *Br. J. Nutr.* **2000,** *84* (*Suppl. 1),* S39-S46.
8. Eigel, W.N.; Butler, J.E.; Ernstrom, C.A.; Farrel, H.M. Jr.; Harwalker, V.R.; Jenness, R.; Whitney, R.McL. Nomenclature of proteins of cow's milk: fifth revision. *J. Dairy Sci.* **1984**, *27,* 1599-1631.
9. Rasmussen, L.K.; Sorensen, E.K.; Petersen, T.E.; Nielsen, N.C.; Thomsen, J.K. Characterization of phosphate sites in native ovine, caprine and bovine casein micelles and their caseinomacropeptides: A solid-state phosphorus-31 nuclear magnetic resonance and sequence and mass spectrometric study. *J. Dairy Sci.* **1997,** *80*, 607-614.
10. Addeo, F.; Soulier, S.; Pelissier J.P.; Chobert, J.M.; Mercier, J.C.; Ribadeau-Dumas, B. Preparation and purification of goat κ-casein: analysis of the glycan and peptide components. *J. Dairy Res.* **1978**, *45,* 191-196.
11. Bollag, D.M.; Edelstein, S.J. *Protein Methods*; New York, 1991; pp 114-116.

12. Coolbear, K.P.; Elgar, D.F.; Ayers, J.S. Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. *Int. Dairy J.* **1996,** *6,* 1055-1068.
13. Mercier, J.C.; Chobert, J.M.; Addeo F. Comparative study of the amino acid sequences of the caseinomacropeptides from seven species. *Febs. Lett.* **1976,** *72,* 208-214.
14. Ono, T.; Yada, R.; Yutani, K.; Nakai, S. Comparison of conformations of κ-casein, para-κ-casein and glycomacropeptide. *Biochim. Biophys. Acta,* **1987**, 911, 318-325.
15. Plowman, J.E.; Creamer, L.K.; Liddell, M.J.; Cross, J.J. Solution conformation of a peptide corresponding to bovine k-casein B residues 130-153 by circular dichroism spectroscopy and 1H-nuclear magnetic resonance spectroscopy. *J. Dairy Res.*, **1997**, *64,* 377-397.
16. Vreeman, H.J.; Visser, S.; Slanger, C.J.; Van Riel, A.M. Characterization of bovine kappa-casein fractions and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. *Biochem. J.* **1986**, *240,* 87-97.
17. Abd El-Salam, M.H.; El-Shibiny, S.; Buchheim, W. Characteristics and potential uses of the casein macropeptide. *Int. Dairy J.* **1996,** *6,* 327-341.
18. Dziuba, J.; Minkiewicz, P. Influence of glycosylation on micelle stabilizing ability and biological properties of C-terminal fragment of cow's κ-casein. *Int. Dairy J.* **1996,** *6,* 1017-1044.
19. López-Fediño, R.; Ramos, M. Revisión: El caseinomacropéptido bovino. I. Características fisicoquímicas y actividad biológica (in Spanish). *Rev. Esp. Cienc. Tecnol. Aliment.* **1992,** *32,* 575-588.
20. Alais, C.; Jollès, P. Étude comparée des caséino-glycopeptides formés par action de la présure sur les caséines de vache, de brebis et de chèvre. II. Étude de la partie non-peptidique (in French). *Biochim. Biophys. Acta* **1961,** *51,* 315-322

21. Jollès, P.; Alais, C.; Jollès, J. Étude comparée des caséino-glycopeptides formés par action de la présure sur les caséines de vache, de brebis et de chèvre. I. Étude de la partie peptidique (in French). *Biochim. Biophys. Acta* **1961,** *51,* 309-314.

22. Mercier, J.C.; Addeo F.; Pelissier, J.P. Structure primaire du caseinomacropeptide de la caséine κ caprine (in French). *Biochimie* **1976**, *58,* 1303-1310.

23. Rigo, J.; Boehm, G.; Georgi, G.; Jelinek, J.; Nyambugabo, K.; Sawatzki, G.; Studzinski, F. An infant formula free of glycomacropeptide prevents hyperthreoninemia in formula-fed preterm infants. *J. Pediatr. Gastroenterol. Nutr.* **2001,** *32,* 127-130.

24. Silva-Hernandez, E.R.; Nakano, T.; Ozimek, L. Isolation and Analysis of κ-Casein Glycomacropeptide from Goat Sweet Whey. *J. Agric. Food Chem.* **2002,** *50,* 2034–2038.

25. Nakano, T.; Ozimek, L. Determination of sialic acid by the thiobarbituric acid reaction in sweet whey and its fractions. *J. Agric. Food Chem.* **1999,** *47,* 2613-2616.

26. Puente, R.; Garcia-Pardo, L.A.; Rueda, R.; Gil, A.; Hueso, P. Changes in ganglioside and sialic acid contents of goat milk during lactation. *J. Dairy Sci.* **1994,** *77,* 39-44.

27. Morrissey, P.A.. The N-acetyl neuraminic acid content of the milk of various species. *J. Dairy Res.* **1973,** *40,* 421-425.

28. Nakano, T.; Ikawa, N.; Silva-Hernandez, E.R.; Wendakoon, C.; Ozimek, L. Purification of glycomacropeptide from cheese whey. *Recent Res. Devel. Agricultural & Food Chem.,* **2000,** *4,* 177-191.

29. Kawakami, H.; Kawasaki, Y.; Dosako, S.; Tanimoto, M.; Nakajima, I. Determination of κ-casein glycomacropeptide by high performance liquid chromatography without trichloroacetic acid pretreatment. *Milchwissenschaft* **1992,** *47,* 688-693.

30. Jenness, R. Composition and characteristics of goat milk: Review 1968-1979. *J. Dairy Sci.* **1980,** *63,* 1605-1630.

31. Gottschalk, A. *The Chemistry and Biology of Sialic Acid and Related Substances*. Cambridge University Press. London. 1960; p 26.
32. Nakano, T.; Ozimek, L. Purification of glycomacropeptide from non-dialyzable fraction of sweet whey by hydrophobic interaction chromatography on phenyl-agarose. *Biotechnol. Lett.* **2000,** *22,* 413-416.
33. Nakano, T.; Ozimek, L. Purification of glycomacropetide from casinate hydrosylate by gel chromatography and treatment with acidic solution. *J. Food Sci.* **2000,** *65,* 588-590.
34. Nakano, T.; Ozimek, L. Gel chromatography of glycomacropeptide (GMP) from sweet whey on Sephacryl S-200 at different pH's and on Sephadex G-75 in 6M guanidine hydrochloride. *Milchwissenschaft* **1998,** *53,* 629-633.
35. Hanning, R.M.; Paes, B.; Atkinson, S.A. Protein metabolism and growth of term infants in response to a reduced-protein, 40:60 whey: casein formula with added tryptophan. *Am. J. Clin. Nutr.* **1992,** *56,*1004-1011.
36. Janas, L.M.; Picciano, M.F.; Hatch, T.F. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula. *Pediatrics* **1985,** *75,* 775-784.
37. Järvenpää, A.-L.; Rassin, D.K. Räihä, N.C.R.; Gauill, G.E. Milk protein quantity and quality in term infant: II. Effects on acidic and neutral amino acids. *Pediatrics* **1982,** *70,* 221-230.
38. Picone, T.A.; Benson, J.D.; Moro, G.; Minoli, I.; Fulconis, F.; Rassin, D.K.; Räihä, N.C.R. Growth, serum biochemistries, and amino acid of term infants fed formulas with amino acid and protein concentrations similar to human milk. *J. Pediatr. Gastroenterol. Nutr.* **1989,** *9,* 351-360.
39. Rassin, D.K.; Gauill, G.E.; Heinonen, K.; Räihä, N.C.R. Milk protein quantity and quality in low-birth-weight infants: II. Effects on selected aliphatic amino acids in plasma and urine. *Pediatrics* **1977,** *59,* 407-422.
40. Rigo, J.; Verloes, A.; Senterre, J. Plasma amino acid concentrations in term infants fed human milk, a whey-predominant formula, or a whey hydrosylate formula. *J Pedriatr* **1989,** *115,* 752-755.

41. Nakano, T.; Silva-Hernandez, E.R.; Ikawa, N.; Ozimek, L.; Goat κ-casein glycomacropetide. *Biotech. Progr.* **2002,** *18,* 409–412.
42. Rigo, J.; Salle, B.L.; Cavero, E.; Putet, G.; Senterre, J. Plasma amino acid and protein concentrations in term infants fed human milk or a whey protein hydrosylate formula during the first month of life. *Acta Paedriat,* **1994,** *83,* 127-131
43. Quero, J.; Carcia, P.; Vento, M.; Garcia-Sala, F.; Georgi, G.; Boehm, G.; Sawatzki, G. Reduction of hyperthreoninemia in term infants fed a whey predominant formula without glycomacropeptide. *J. Pediat. Gastr. Nutr.*, **1997,** *24,* 491.

Table 1.1. Amino acid compositions of goat and bovine GMPs.

Amino acid	Purified GMPs		Theoretical values[a]		
	Goat GMP[b]	Cow GMP[c]	Goat GMP	Cow GMP (V_A)	Cow GMP (V_B)
			Mol %		
Asx	11.4	7.7	10.6	7.8	6.3
Thr	14.3	16.3	16.7	18.8	17.2
Ser	10.7	8.9	12.1	9.4	9.4
Glx	17.9	16.7	13.6	15.6	15.6
Gly	0.8	2.6	0	1.6	1.6
Ala	12.3	8.5	13.6	7.8	9.4
Val	7.6	10.0	9.1	9.4	9.4
Met	ND	1.0	1.5	1.6	1.6
Ile	8.5	9.4	7.6	9.4	10.9
Leu	1.3	1.8	0	1.6	1.6
Tyr	0[d]	0	0	0	0
Phe	0.4	0	0	0	0
His	1.8	0.2	1.5	0	0
Lys	4.3	4.1	4.5	12.5	12.5
Arg	0	0	0	0	0
Pro	8.8	12.8	9.1	12.5	12.5

[a] Each value is based on the primary structure of goat GMP (13,22) or cow GMP (8).

[b] Silva-Hernandez *et al.* (24)

[c] Nakano *et al.* (41).

[d] A value of "zero" in purified GMP fraction indicates undetectable level of amino acid.

ND: Not determined.

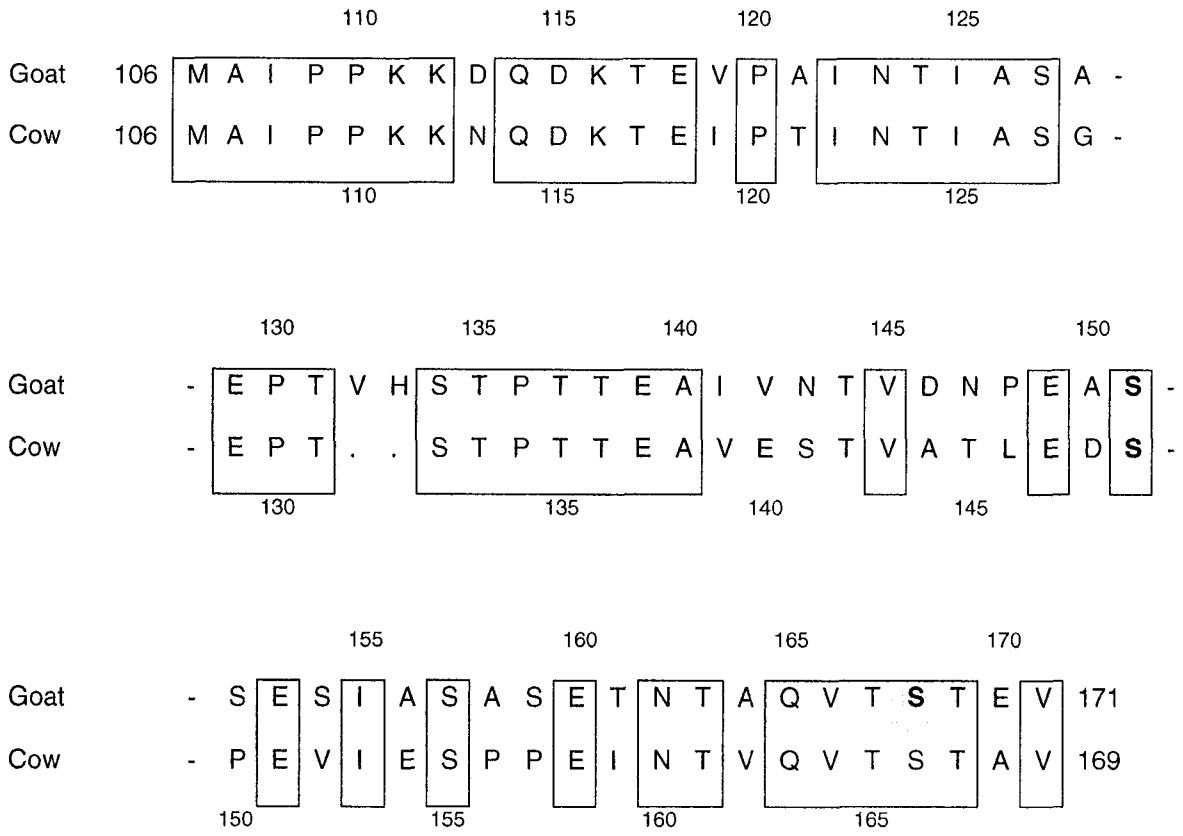

Figure 1.1. Amino acid sequences of bovine variant A and caprine GMPs. Boxes denote identical residues between the two species, and introduced gaps are designated by dots. Phosphorylated serines are in boldface type (adapted from ref. 9).

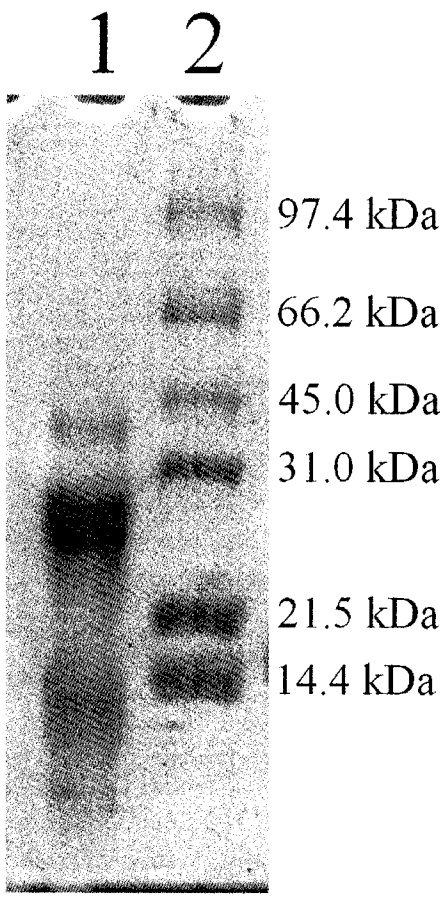

Figure 1.2. SDS-gel electrophoresis of goat GMP (24). Approximately 50µg of GMP purified from goat sweet whey was electrophoresed on a 12% polyacrylamide gel containing no urea. The gel was stained with Coomassie blue R250 as previously described (32). Lane 1; the purified GMP. Lane 2, Standard proteins, phospholylase b, serum albumin, ovalbmin, carbonic anhydrase, trypsin inihibitor and lysozyme in descending order of molecular weight.

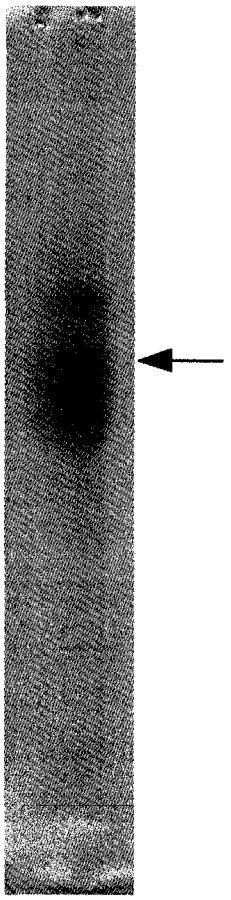

Figure 1.3. SDS-gel electrophoresis of goat GMP containing 8 M urea (24). Arrow shows the mobility of lysozyme (14.4 kDa). See Fig. 1.2 for details.

CHAPTER 2

Isolation and Analysis of κ-Casein Glycomacropeptide from Goat Sweet Whey[1]

2.1 INTRODUCTION

Glycomacropeptide (GMP) found in cheese whey (or sweet whey) is a C-terminal hydrophilic glycopeptide released from κ-casein by the action of chymosin during cheese making. Glycomacropeptide lacks aromatic amino acids (phenylalanine, tyrosine and tryptophan) (1, 2), and contains varying amounts of sugars including N-acetylneuraminic acid (sialic acid), galactose and N-acetylgalactosamine (1, 3). Glycomacropeptide has been isolated from bovine sweet whey and its chemical composition analyzed (4 - 6). Glycomacropeptide has a number of biological activities (7 - 10), and is thought to be a potential ingredient for functional foods and pharmaceuticals.

There is relatively limited information available concerning chemical and quantitative analysis of goat GMP. Jollès *et al.* (11) and Alais and Jollès (12) isolated GMP from caprine κ-casein and reported its chemical composition. Amino acid sequence in goat GMP differs from that of bovine GMP (2, 13, 14). The present study was, therefore, undertaken to isolate GMP from goat sweet whey by anion-exchange and hydrophobic interaction chromatography and to characterize it using chemical analyses, gel chromatography and electrophoresis.

---

[1] A version of this chapter has been published. Silva-Hernandez, E.R.; Nakano, T.; Ozimek, L. Isolation and Analysis of κ-Casein Glycomacropeptide from Goat Sweet Whey. *J. Agric. Food Chem.* **2002,** *50,* 2034–2038.

2.2 MATERIALS AND METHODS

2.2.1 Materials.

Samples of pasteurized homogenized milk were obtained from local milk processing plants in Alberta, Canada. Sweet whey was prepared by chymosin treatment (15) followed by centrifugation at 20,000g and 4 °C for 30 min to remove fat and protein precipitate (16). The goat sweet whey obtained was dialyzed using dialysis tubes with 6-8 kDa molecular weight cut-off. After dialysis, the sweet whey sample was stored at –25 °C for approximately one month before it was analyzed. Phenyl-agarose, sialic acid from sheep submaxillary glands, 2-thiobarbituric acid, galactose and galactosamine-HCl were obtained from Sigma Chemical Co., Mississauga, Ontario, Canada. Diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-200 and blue dextran were from Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada. Molecular weight standards for gel electrophoresis were from Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario, Canada.

2.2.2 Fractionation of Goat Sweet Whey.

Samples of sweet whey were thawed and centrifuged at 20,000g and 10 °C for 15 min. After centrifugation, the supernatant was adjusted to pH 3.0 with acetic acid and applied to a 1.5 × 5.5 cm column of DEAE-Sephacel equilibrated with water adjusted to pH 3.0. Materials adsorbed on the column were eluted by applying 1 M NaCl. Fractions (5 mL) were collected at a flow rate of 20 mL/h and monitored for sialic acid by the thiobarbituric acid reaction, and protein and/or peptide (protein/peptide) by measuring absorbance at 230 nm. Major sialic acid containing fractions from the DEAE-Sephacel column were pooled, dialyzed in water and freeze-dried. A portion of freeze-dried sample was dissolved in 0.01 M sodium phosphate buffer, pH 6.8 containing 5 M NaCl (buffer-A) and applied to a 1.5 × 5.5 cm column of phenyl-agarose equilibrated with buffer-A (17). The column was eluted first with 10-15 bed volumes of buffer-A, and then water to release materials adsorbed on the column. Fractions (2 mL) were collected at a flow rate of 20 mL/h and monitored for sialic acid, and protein/peptide contents

(see above). Fractions containing GMP sialic acid were pooled, dialyzed in water and freeze-dried to obtain the final preparation of GMP.

Sweet whey contains relatively small amounts of sialylated proteins including immunoglobulins and lactoferrin. These proteins having higher isoelectric points (pIs) or hydrophobicity than GMP are supposed to be desorbed from DEAE-Sephacel or adsorbed on phenyl-agarose under the experimental conditions. Thus, the major sialic acid peak eluting from the phenyl-agarose column is supposed to contain sialic acid from GMP but not from the sialylated sweet whey proteins.

2.2.3 Gel Chromatography.

The purified GMP was applied to a 1 × 109 cm column of Sephacryl S-200 equilibrated and eluted with 0.1 M sodium acetate, pH 7.0 containing 0.02% sodium azide. Fractions (1 mL) collected at a flow rate of 9 mL/h were monitored for sialic acid and protein/peptide contents (see above). Blue dextran and tritiated water were used to determine void volume (V_0) and total column volume (V_t), respectively. The partition coefficient (K_{av}) of sialic acid peak was calculated from the formula $K_{av} = (V_e - V_o) / (V_t - V_o)$, in which V_e represents the volume of the peak fraction. No attempt was made in this study to estimate the apparent molecular weight (M_r) of GMP because there were no appropriate glycoprotein or glycopeptide standards available to calibrate the column of Sephacryl S-200. Use of protein standards to calibrate a column results in an overestimation of the M_r of GMP (16).

2.2.4 Analytical Methods.

Sialic acid contents in goat sweet whey samples and chromatographic fractions were determined by the thiobarbituric acid reaction as previously described (16, 18). Amino acid analysis was performed by using a Beckman Model 6300 amino acid analyzer on samples hydrolyzed under nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol. The content of galactose was estimated by the anthron reaction (19) using galactose as a

standard, and that of galactosamine by the indole reaction (20) using galactosamine-HCl as a standard. The content of phosphorus was determined by using molybdate-vanadate reagent (21).

2.2.5 Gel Electrophoresis.

Electrophoresis in 0.1% (w/v) sodium dodecyl sulfate (SDS) was carried out on 12% polyacrylamide gels in Tris-borate buffer, pH 8.6 (22) using a mini-PROTEAN II cell (Bio-Rad, Laboratories) with 0.75 mm thick spacers and 10 well combs. Samples were dissolved in 0.04 M Tris-borate containing 2% (w/v) SDS, 6 M urea and 10% (v/v) 2-mercaptoethanol, and boiled for 5 min prior to application. Two gels per sample were prepared at the same time. One was stained with 0.5% (w/v) Coomassie blue R250 in 50% (v/v) methanol containing 12.5% (w/v) trichloroacetic acid (solution-A) and destained in 5% (w/v) trichloroacetic acid (5, 23). The other was stained with 0.1% (w/v) Coomassie blue R250 in 45% (v/v) methanol containing 10% glacial acetic acid (solution-B) and destained in 10% glacial acetic acid (24). Electrophoresis was also performed using the same 12% polyacrylamide gels (see above) but containing 8 M urea, and gels were stained as described above. The mobility of electrophoretic band was expressed using the R_f value, which is calculated as a ratio of the migration distance of electrophoretic band to that of marker dye, bromophenol blue.

2.3 RESULTS

After chymosin treatment, average 75% (v/v) of goat milk was recovered as sweet whey. The content of sialic acid determined in sweet whey averaged 79 μg/mL. Non-dialyzable fraction of sweet whey was then chromatographed on DEAE-Sephacel (Figure 2.1). Average 97% of recovered sialic acid (carbohydrate moiety of GMP) was adsorbed on the column and eluted as a single peak, while the remaining 3% of sialic acid did not bind to the column. This indicated that most GMP sialic acid was adsorbed on the anion-exchanger. The sialic acid peak fraction (tube numbers 31 to 35) from DEAE-Sephacel

(Figure 2.1) was then chromatographed on phenyl-agarose (Figure 2.2). Most (96%) of the recovered sialic acid was not adsorbed on the column and eluted as a single peak, while the remaining small proportion (4%) of sialic acid tightly bound to the column. The eluates from the major sialic acid peak were pooled as indicated in Figure 2.2 to obtain the final product, referred to as GMP fraction. The eluates containing small amounts of sialic acid adsorbed on the column were not analyzed further. The dry matter of GMP fraction accounted for 0.06% (w/v) of sweet whey. This corresponded to 0.8% of dry weight of sweet whey. The GMP fraction was further examined for its chemical composition and molecular size.

Table 2.1 shows amino acid profiles for major fractions obtained during purification of GMP and the theoretical composition of amino acid in goat GMP. The contents of glycine, leucine and phenylalanine, the amino acids absent in goat GMP, were relatively high in the GMP-containing fraction from DEAE-Sephacel, and rapidly decreased in the GMP fraction, a final product obtained by chromatography on phenyl-agarose. The GMP fraction contained traces (each <1 residue/peptide estimated from the data in Table 2.1) of glycine, leucine and phenylalanine, indicating that the GMP is of high purity. The contents of all amino acids present in the GMP fraction with the exception of threonine, and glutamine/glutamic acid were in general comparable to their theoretical contents in goat GMP. The content of threonine was lower, and that of glutamine/glutamic acid was higher compared to the theoretical content of each amino acid.

Sialic acid, galactose, galactosamine and phosphorus contents determined in the GMP fraction were 25.2, 31.9, 20.5 and 1.9 μg/mg dry weight, respectively. The sialic acid content was 25 times higher than that in sweet whey (1.0 μg/mg dry weight).

Gel chromatography of the GMP fraction on Sephacryl S-200 (Figure 2.3) gave a major peak (K_{av} = 0.46) of sialic acid accounting for approximately 84% of recovered sialic acid. The peak eluted later than dimeric β-lactoglobulin (36.6 kDa). The GMP fraction also demonstrated two minor sialic acid peaks (K_{av} = 0.29 and 0.64) both accounting for approximately 8% of recovered sialic acid.

The first peak eluted slightly earlier than bovine serum albumin (66.0 kDa), while the second peak eluted later than α-lactalbumin (14.2 kDa).

On gel electrophoresis (Figure 2.4), the GMP fraction gave major and minor broad bands stained with Coomassie blue in solution-A (Figure 2.4a, lane 1). The average R_f values were 0.55 and 0.78 for the major and minor bands, respectively. The major band had a mobility faster than that of carbonic anhydrase (31.0 kDa), while the minor band had its slowest moving portion showing the mobility similar to that of lysozyme (14.4 kDa). The major band was irregularly stained with Coomassie blue and showed presence of at least three components (R_f = 0.52, 0.56 and 0.59). The broadness and irregularly stained pattern of the band are probably due in part to variations in the content of sialic acid among GMPs. The GMP fraction also demonstrated two weakly stained minor bands (R_f = 0.42 and 0.89). The first one had a mobility slower than that of of carbonic anhydrase (31.0 kDa), and the second one a mobility much faster than that of lysozyme (14.4 kDa).

Electrophoresis of the major sialic acid peak fraction from Sephacryl S-200 (Figure 2.3) gave major and minor bands (electrophoretogram not shown). Both the staining intensity and R_f value for each band were similar to those observed in the corresponding band from the GMP fraction (Figure 2.4a, lane 1). This suggests that the majority of goat GMP contains at least two separate components, one that does not dissociate and the other that dissociates in the presence of SDS, but the latter is the minor component.

Electrophoresis was further carried out on gels containing 8 M urea (dissociative condition) (Figure 2.4b). The GMP fraction demonstrated a major broad band with its average mobility slightly faster than the mobility of lysozyme (14.4 kDa), indicating that the majority of goat GMP forms dimmers and dissociates into monomers in the presence of 8 M urea. In contrast to the above results, all bands containing goat GMP were very weakly stained with Coomassie blue in solution-B (eletrophoretogram not shown) and demonstrated slight pink coloration (metachromasia).

2.4 DISCUSSION

There is little information available on the sialic acid content in goat sweet whey. It can be estimated from the total sialic acid content in goat milk assuming that 99.9% (25) of sialic acid is attached to protein (but not ganglyoside) and released into sweet whey, which accounts for 75% (v/v, see Results) of milk. The sialic acid concentration found in goat sweet whey in this study is comparable to the corresponding value (68 μg/mL) calculated from the data (5.1 mg sialic acid in 100 mL of goat milk) reported by Morrissey (26). The sialic acid concentration in goat sweet whey is, however, 1.5 fold lower than that found in bovine sweet whey (18) reflecting the lower concentration of sialic acid in goat GMP (see below).

From the amino acid composition (Table 2.1), it is suggested that the goat GMP prepared in this study using the two step procedure with anion-exchange chromatography on DEAE-Sephacel and hydrophobic interaction chromatography on phenyl-agarose is of considerably high purity. However, the higher than theoretical content of glutamine/glutamic acid and the presence of amino acids not specific to goat GMP observed in the GMP fraction are probably due to the small amounts of contaminating protein/peptide present. The lower than theoretical content of threonine observed in the goat GMP fraction may be due to degradation during acid hydrolysis (27).

Amino acid composition and sequence of goat GMP (residues 105-171) differs from that of bovine GMP (residues 105-169). There are 19 substitutions and two insertions (Val-132 and His-133) in the goat sequence compared to the bovine sequence (2, 13, 14). These differences between the species are in part reflected by the present findings in the GMP fraction of traces of glycine and leucine (the amino acids which do not occur in goat but in bovine GMP), and of histidine occurring in goat (but not in bovine) GMP with its concentration close to the theoretical content (Table 2.1).

The concentrations of sialic acid, galactose and galactosamine in the GMP fraction (see above) are fairly comparable to those of sialic acid (30 μg/mg), galactose (40 μg/mg) and galactosamine (28 μg/mg) reported by Alais and Jollès

(12) in GMP prepared from caprine κ-casein. The sialic acid concentration in the GMP fraction is, however, approximately three times lower than that in bovine GMP reported by Kawakami *et al.* (ref. 4, average 80.3 μg/mg) or Nakano and Ozimek (ref. 17, 77.5 μg/mg). Assuming that the M_r of goat GMP is 7.5 kDa (see below for the estimation of M_r), the number of sialic acid residues per peptide calculated from its content in the GMP fraction (see above) is 0.6. This value is within the range (0.2 to 1.9 moles/mole) of sialic acid residue in goat κ-casein (and thus GMP) reported by Addeo *et al.* (3). Most (99 %) of sialic acid in goat κ-casein is found in its GMP position (28).

Sialic acid is an acidic sugar with pK_a value of 2.6 (Svennerholm, 1956, cited by ref. *29*). A higher concentration of sialic acid in GMP gives lower pI of this glycopeptide as demonstrated by previous studies, in which higher concentrations of sialic acid resulted in tighter binding of GMP to anion-exchanger (4, 6). The pI of 66-residue peptide in goat GMP computed by a Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html) is 4.18. This is slightly higher than pIs (4.04 and 4.14, respectively) for 64-residue peptide from bovine GMP variants A and B. Since the concentration of phosphate (pK_a = 2.0), which also contributes to the low pI of GMP, is similar between the bovine (17) and goat (see above) GMP, the lower concentration of sialic acid found in the latter (see above) suggests that the pI is higher in the goat GMP prepared in the present study. This may be related to the difference observed in the staining intensity with anionic Coomassie blue between the bovine (30) and goat (see Results) GMPs on SDS-polyacrylamide gels. The goat GMP showed very weak but significant metachromatic staining (an evidence of dye binding) in solution-B containing 45% methanol and 10% acetic acid, while the bovine GMP showed no detectable staining under the same condition (30). The precise pI value of goat GMP is unknown. A recent chromatofocusing study from our laboratory indicated the pI of bovine GMP to be below 3.8 (6). Occurrence of metachromasia in GMP has not been reported previously. Little is known about the factor involved in the formation of pink coloration in goat GMP.

The present results indicate that the majority of goat GMP forms dimmers. A similar situation occurs in bovine GMP, which is observed as an aggregate when examined by gel chromatography (16) or gel electrophoresis (30). The mechanism of aggregation is unknown. It is also not well understood why the aggregate of GMP does not dissociate in SDS-gels. Our speculation is that the affinity of SDS to anionic GMP is too weak to dissociate the dimeric GMP. If so, the electrophoretic mobility of GMP is dependent on its size and charge, and thus an accurate estimation of M_r using protein standards is difficult. The M_r of GMP can be determined by other methods including mass spectrometry, sedimentation equilibrium and light scattering techniques. Since its theoretical molecular weight of the 66-residue peptide is 6.824 kDa, the M_r of goat GMP calculated by including its concentrations of carbohydrates and phosphorous (see above) is approximately 7.5 kDa.

Most (~80% of total, ref. 18) of sialic acid from sweet whey is found in GMP. However, relatively small amounts of sialic acid are also found in sialylated sweet whey proteins including immunoglobulins and lactoferrin. In this experiment, we suggest that all sialic acid peaks eluting from Sephacryl S-200 (Figure 2.3) contained sialic acid from GMP only. Contamination with sialylated proteins unlikely occurs (see Methods). The three sialic acid peaks with K_{av} values 0.29, 0.46 and 0.64 appear to coincide with the electrophoretic bands with R_f values 0.42, 0.55 and 0.78, respectively. It is unknown whether the fourth band with R_f 0.89 contained GMP. More specific detection of GMP may be possible if antibodies raised against goat GMP are available.

Bovine GMP has been reported to have a number of biological activities (7 - 10). For example, bovine GMP was shown to bind cholera toxin to neutralize it (31). Oh *et al.* (32) reported that the cholera toxin-binding activity is rapidly reduced when the GMP was treated with N-acetylneuraminidase (sialidase), suggesting the importance of sialic acid for the binding activity of GMP. These authors, however, did not report the content of sialic acid in GMP used in their experiment. It is interesting to know whether the sialic acid concentration in the goat GMP prepared in this study is sufficient to neutralize cholera toxin.

Because of the absence of phenylalanine, GMP has been reported to be useful for the dietary treatment of phenylketonuria (PKU), a hereditary disorder of phenylalanine metabolism. The goat GMP prepared in this study with a negligible amount of phenylalanine (see above) can be used for partial replacement of amino acids in commercial diets for PKU patients.

Goat milk is an important source of nutrients in many parts of the world. World production of goat milk has been more rapidly increasing compared to that of cow milk during the past two decades (33). Chemical composition of goat milk protein has been relatively extensively studied (34). However, there are few reports of chemical and quantitative analysis of goat GMP. The present study provides previously unreported data on the purification and analysis of GMP from goat sweet whey, a by-product of cheese manufacturing. The information may contribute to the development of beneficial ways of utilization of goat cheese whey by producing value-added products. Such approach is of economical importance in the dairy industry especially in European countries and Mexico, where the majority (about 70% calculated from the data obtained from refs. *35* and *36*) of caprine milk is used for cheese production. Utilization of goat sweet whey also contributes to the prevention of pollution caused by disposal of this by-product, a long standing problem of dairy industry.

In conclusion, acidic hydrophilic GMP can be isolated from goat sweet whey by anion-exchange and hydrophobic interaction chromatography. The isolated GMP is of high purity sufficient for partial substitution of amino acids used for dietary treatment of PKU. Goat GMP differs from bovine GMP in amino acid composition, sialic acid concentration and staining intensity with Coomassie blue.

2.5 REFERENCES

1. Eigel, W.N.; Butler, J.E.; Ernstrom, C.A.; Farrel, H.M. Jr.; Harwalker, V.R.; Jenness, R.; Whitney, R.McL. Nomenclature of proteins of cow's milk: fifth revision. *J. Dairy Sci.* **1984**, *27,* 1599-1631.

2. Rasmussen, L.K.; Sorensen, E.K.; Petersen, T.E.; Nielsen, N.C.; Thomsen, J.K. Characterization of phosphate sites in native ovine, caprine and bovine casein micelles and their caseinomacropeptides: A solid-state phosphorus-31 nuclear magnetic resonance and sequence and mass spectrometric study. *J. Dairy Sci.* **1997,** *80,* 607-614.
3. Addeo, F.; Soulier, S.; Pelissier J.P.; Chobert, J.M.; Mercier, J.C.; Ribadeau-Dumas, B. Preparation and purification of goat κ-casein: analysis of the glycan and peptide components. *J. Dairy Res.* **1978**, *45,* 191-196.
4. Kawakami, H.; Kawasaki, Y.; Dosako, S.; Tanimoto, M.; Nakajima, I. Determination of κ-casein glycomacropeptide by high performance liquid chromatography without trichloroacetic acid pretreatment. *Milchwissenschaft* **1992,** *47,* 688-693.
5. Coolbear, K.P.; Elgar, D.F.; Ayers, J.S. Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. *Int. Dairy J.* **1996,** *6,* 1055-1068.
6. Nakano, T.; Ozimek, L. Purification of glycomacropetide from dialyzable and non-dialyzable sweet whey by anion-exchange chromatography at different pH values. *Biotechnol. Lett.* **2000,** *22,* 1081-1086.
7. Abd El-Salam, M.H.; El-Shibiny, S.; Buchheim, W. Characteristics and potential uses of the casein macropeptide. *Int. Dairy J.* **1996,** *6,* 327-341.
8. Brody, E. Biological activities of bovine glycomacropeptide. *Br. J. Nutr.* **2000,** *84* (*Suppl. 1),* S39-S46.
9. Dziuba, J.; Minkiewicz, P. Influence of glycosylation on micelle stabilizing ability and biological properties of C-terminal fragment of cow's κ-casein. *Int. Dairy J.* **1996,** *6,* 1017-1044.
10. López-Fediño, R.; Ramos, M. Revisión: El caseinomacropéptido bovino. I. Cracterísticas fisicoquímicas y actividad biológica (in Spanish). *Rev. Esp. Cienc. Tecnol. Aliment.* **1992,** *32,* 575-588.
11. Jollès, P.; Alais, C.; Jollès, J. Étude comparée des caséino-glycopeptides formés par action de la présure sur les caséines de vache, de brebis et de

chèvre. I. Étude de la partie peptidique (in French). *Biochim. Biophys. Acta* **1961,** *51,* 309-314.

12. Alais, C.; Jollès, P. Étude comparée des caséino-glycopeptides formés par action de la présure sur les caséines de vache, de brebis et de chèvre. II. Étude de la partie non-peptidique (in French). *Biochim. Biophys. Acta* **1961,** *51,* 315-322
13. Mercier, J.C.; Chobert, J.M.; Addeo F. Comparative study of the amino acid sequences of the caseinomacropeptides from seven species. *Febs. Lett.* **1976,** *72,* 208-214.
14. Mercier, J.C.; Addeo F.; Pelissier, J.P. Structure primaire du caseinomacropeptide de la caséine κ caprine (in French). *Biochimie* **1976**, *58,* 1303-1310.
15. Chu, L.; MaCleod, A.; Ozimek, L. Isolation of glycomacropeptide from sodium caseinate hydrosylate solution by ultrafiltration. *Milchwissenschaft* **1996,** *51,* 303-306.
16. Nakano, T.; Ozimek, L. Gel chromatography of glycomacropeptide (GMP) from sweet whey on Sephacryl S-200 at different pH's and on Sephadex G-75 in 6M guanidine hydrochloride. *Milchwissenschaft* **1998,** *53,* 629-633.
17. Nakano, T.; Ozimek, L. Purification of glycomacropeptide from non-dialyzable fraction of sweet whey by hydrophobic interaction chromatography on phenyl-agarose. *Biotechnol. Lett.* **2000,** *22,* 413-416.
18. Nakano, T.; Ozimek, L. Determination of sialic acid by the thiobarbituric acid reaction in sweet whey and its fractions. *J. Agric. Food Chem.* **1999,** *47,* 2613-2616.
19. Trevelyan, W.; Harrison, J.S. Studies of yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. *Biochem. J.* **1952**, *50*, 298-303.
20. Dische, Z.; Borenfrend, E. A spectrophotometric method for the micro-determination of hexosamines. *J. Biol. Chem.* **1950,** *184*, 517-522.

21. AOAC, *Official Methods of Analysis of AOAC International.* 16th Ed. Association of Official Analytical Chemists; Gaithersburg, Maryland, 1998.
22. Mechanic, G.L. Collagen biochemistry. In: *Skeletal Research;* Simmons D.J., Kunin, A.S., Eds.; Academic Press: New York, 1979; pp 227-241.
23. Reid, J.R.; Coolbear, T.; Ayers, J.S.; Coolbear, K.P. The action of chymosin on κ-casein and its macropeptide: Effect of pH and analysis of products of secondary hydrolysis. *Int. Dairy J.* **1997,** *7,* 559-569.
24. Bollag, D.M.; Edelstein, S.J. *Protein Methods*; New York, 1991; pp 114-116.
25. Puente, R.; Garcia-Pardo, L.A.; Rueda, R.; Gil, A.; Hueso, P. Changes in ganglioside and sialic acid contents of goat milk during lactation. *J. Dairy Sci.* **1994,** *77,* 39-44.
26. Morrissey, P.A.. The N-acetyl neuraminic acid content of the milk of various species. *J. Dairy Res.* **1973,** *40,* 421-425.
27. Kawasaki, Y.; Kawakami, H.; Tanimoto, M.; Dosako, S.; Tomizawa, A.; Kotake, M.; Nakajima, I. pH-Dependent molecular weight changes of κ-casein glycomacropeptides and its preparation by ultrafiltration. *Milchwissenschaft* **1993,** *48,* 191-196.
28. Hofi, A.A.; Asker, A.A.; Hamzawi, L.F.; Abdel-Salam, M.H. Carbohydrate moiety of protein fractions from some mammals' milk. *Asian J. Dairy Res.* **1982,** *1,* 91-96.
29. Gottschalk, A. *The Chemistry and Biology of Sialic Acid and Related Substances.* Cambridge University Press. London. 1960; p 26.
30. Nakano, T.; Ozimek, L. Purification of glycomacropetide from casinate hydrosylate by gel chromatography and treatment with acidic solution. *J. Food Sci.* **2000,** *65,* 588-590.
31. Kawasaki, Y.; Isoda, H.; Tanimoto, M.; Dosako, S.; Idota, T.; Ahiko, K. Inhibition by lactoferrin and κ-casein glycomacrepeptide of binding of *Cholera* toxin to its receptor. *Biosci. Biotechnol. Biochem.* **1992,** *56,* 195-198.

32. Oh, S.; Worobo, R.W.; Kim, B.; Rheem, S.; Kim, S. Detection of the cholera toxin-binding activity of κ-casein macropeptide and optimization of its production by the response surface methodology. *Biosci. Biotechnol. Biochem.* **2000,** *64,* 516-522.

33. Morand-Fehr, P.; Boyazoglu, J. Present state and future outlook of the small ruminant sector. *Small Ruminant Res.* **1999,** *34,* 175-188.

34. Jenness, R. Composition and characteristics of goat milk: Review 1968-1979. *J. Dairy Sci.* **1980,** *63,* 1605-1630.

35. Haenlein, G.F.W. Status and prospects of the dairy goat industry in the U.S. *Proceedings, American Dairy Science Association Annual Meeting,* Minneapolis, MN. 1994.

36. Food and Agriculture Organization of the United Nations. Production 1997. *FAO yearbook.* 1998. pp 218-225.

Table 2.1. *Amino acid compositions of GMP-containing fraction from DEAE-Sephacel (Figure 2.1, GMP-DEAE) and GMP fraction (Figure 2.2) and the theoretical amino acid composition of goat GMP.*

Amino acid	GMP-DEAE	GMP fraction	Goat GMP[a]
		Mol%	
Asparagine and aspartic acid	10.4	11.4	10.6
Threonine	12.4	14.3	16.7
Serine	11.1	10.7	12.1
Glutamine and glutamic acid	18.6	17.9	13.6
Glycine	1.3	0.8	0
Alanine	10.3	12.3	13.6
Valine	7.5	7.6	9.1
Methionine	ND	ND	1.5
Isoleucine	7.9	8.5	7.6
Leucine	2.6	1.3	0
Tyrosine	0.3	0[b]	0
Phenylalanine	1.0	0.4	0
Histidine	2.0	1.8	1.5
Lysine	5.1	4.3	4.5
Arginine	0.7	0	0
Proline	8.8	8.8	9.1
Cysteine	ND	ND	0
Tryptophan	ND	ND	0

[a] Values are based on the primary structure of goat GMP (13, 14).

[b] A value of "zero" in the GMP fraction indicates undetectable level of amino acid.

ND: Not determined.

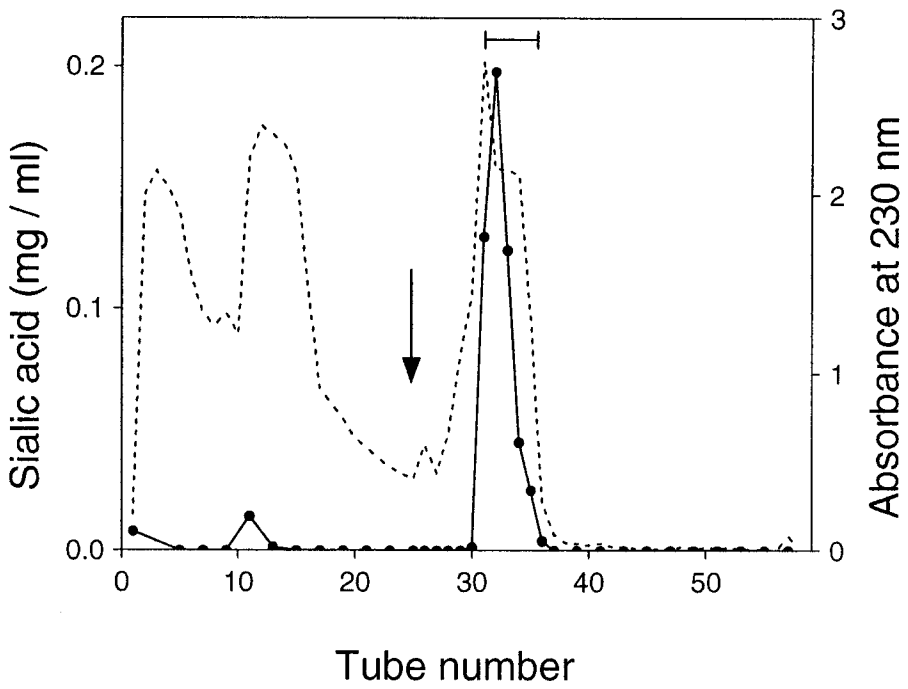

Figure 2.1. Anion-exchange chromatography of goat sweet whey (44 ml) on DEAE-Sephacel: (•—•) sialic acid; (----) protein/peptide. An arrow shows the position of application of 1 M NaCl. A horizontal bar denotes eluates pooled for further study. See Methods for other details.

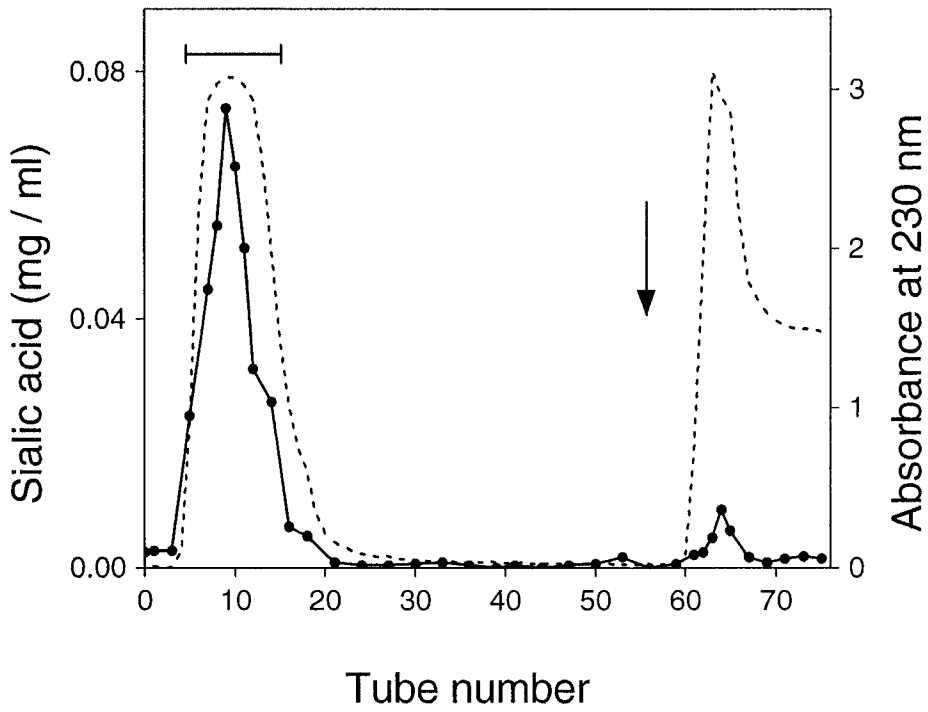

Figure 2.2. Hydrophobic interaction chromatography on phenyl-agarose of major sialic acid containing fraction from DEAE-Sephacel (Figure 2.1): A sample containing approximately 46 mg of sialic acid in 5 ml of buffer-A was chromatographed. (•—•) sialic acid; (----) protein/peptide. An arrow shows the position of application of water. A horizontal bar denotes eluates pooled for further study. See Methods for other details.

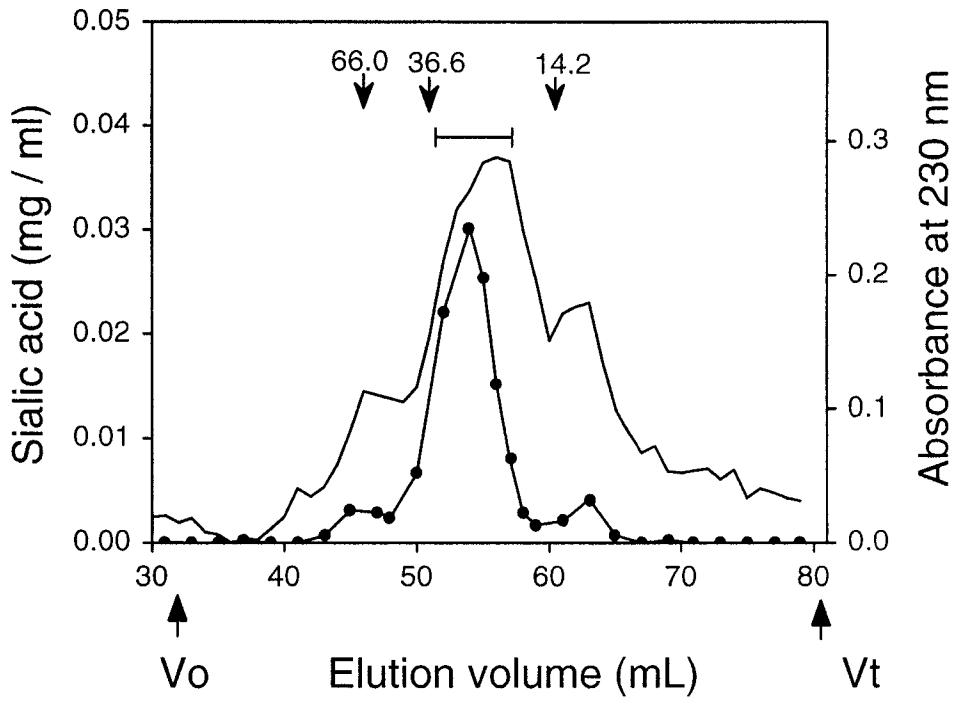

Figure 2.3. Gel chromatography of goat GMP on Sephacryl S-200: Approximately 9 mg of the GMP fraction from phenyl-agarose (Figure 2.2) was chromatographed. (•—•) sialic acid; (----) protein/peptide. Elution positions of protein standards, bovine serum albumin (66.0 kDa), dimeric β-lactoglobulin (36.6 kDa) and α-lactalbumin (14.2 kDa) are shown. A horizontal bar denotes eluates pooled for further study. Vo and Vt show void and total column volumes, respectively. See Methods for other details.

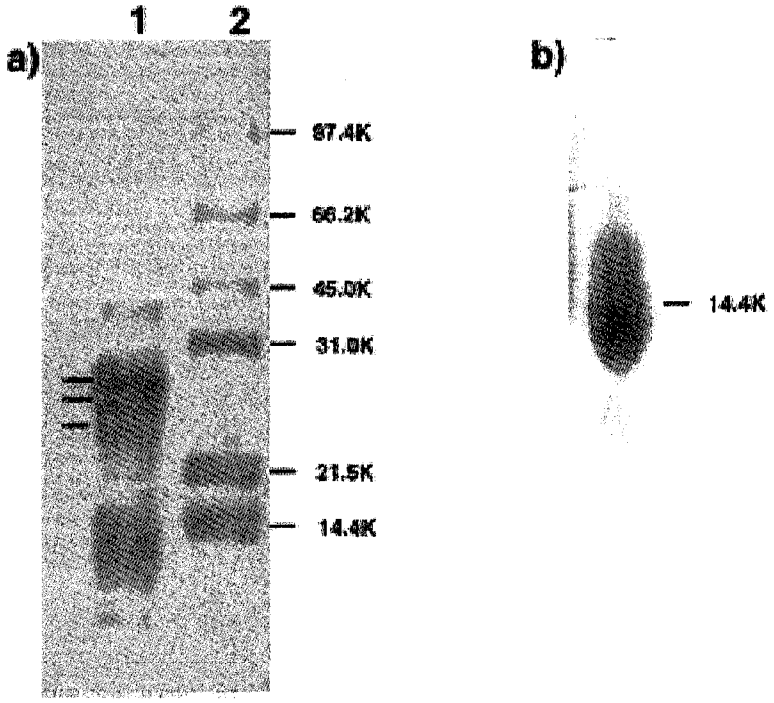

Figure 2.4. Gel electrophoresis of goat GMP: The GMP fraction from phenyl-agarose (Figure 2) (approximately 50 μg/well) was electrophoresed on SDS-gels containing no urea and those containing 8 M urea. a) Gel containing no urea: Lane 1; the GMP fraction. Lane 2; M_r standards, phospholylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inihibitor (21.5 kDa) and lysozyme (14.4 kDa). The bars indicate the three components in the major band. b) Gel containing 8 M urea: See Methods for other details.

CHAPTER 3

Comparison of Glycomacropeptide Isolated from Raw and Pasteurized Goat Milk[1]

3.1 INTRODUCTION

A proteinase, chymosin added to goat milk for cheese making, catalyzes cleavage between Phe105 and Met106 in κ-casein. This results in generation of two polypeptides, a hydrophobic N-terminal para-κ-casein (residues 1-105), and a hydrophilic phosphorylated and glycosylated C-terminal polypeptide (residues 106-171). The latter is known as caseinomacropeptide or glycomacropeptide (GMP), which is recovered in whey (more commonly called sweet whey), a by-product containing β-lactoglobulin and α-lactalbumin as major proteins.

We have recently isolated GMP from goat sweet whey and characterized it using chemical analysis, gel chromatography and gel electrophoresis (1). This sweet whey was, however, prepared from pasteurized milk (but not raw milk), which led us to question if pasteurization or heat treatment of milk affects the structure and composition of GMP. There has been no published information available in this regard. The present study was, therefore, undertaken to investigate such a possibility. GMP fractions prepared from goat milk with and without heat treatment were examined using chemical analysis and gel electrophoresis, and results obtained were compared. A sweet whey fraction obtained after isolating GMP (GMP-depleted whey fraction) was also analyzed. Methods of heat treatment included high temperature short time (HTST), and low temperature long time (LTLT) pasteurizations. Since goat cheese is made from raw as well as pas-

[1] A version of this chapter has been published. Silva-Hernández, E.R.; Nakano, T.; Verdalet-Guzmán, I.; Ozimek, L. Comparison of glycomacropeptide isolated from raw and pasteurized goat milk. *Milchwissenschaft.* **2004,** *1-2,* 27-31.

teurized milk, the present approach may be of importance to researchers studying goat cheese technology.

3.2 MATERIALS AND METHODS

3.2.1 Materials.

Samples of fresh raw goat milk were obtained from a local supplier (Natricia Dairy, Alberta, Canada). A portion of each sample was subjected to HTST pasteurization (72 °C for 15 s) and another portion to LTLT pasteurization (63.5 °C for 30 min), while the remaining portion of sample was used as raw milk without pasteurization. Sweet whey was prepared from raw and pasteurized milk by chymosin treatment (2) followed by centrifugation at 20,000 g and 4 °C for 30 min to remove fat and protein precipitate (3). The goat sweet whey obtained was dialyzed using dialysis tubes with 6-8 kDa molecular weight cutoff to obtain a non-dialyzable fraction of sweet whey. This was stored at –20 °C until used for isolation of GMP. Diethylaminoethyl (DEAE)-Sephacel was obtained from Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada. Phenyl-agarose, sialic acid from sheep submaxillary glands, 2-thiobarbituric acid, galactose and galactosamine-HCl were from Sigma Chemical Co., Mississauga, Ontario, Canada. Molecular weight standards for gel electrophoresis were from Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario, Canada.

3.2.2 Purification of GMP.

Samples of non-dialyzable fraction of sweet whey (see above) were thawed and centrifuged at 20,000 g and 10 °C for 15 min. After centrifugation, the supernatant was adjusted to pH 3.0 with acetic acid and applied to a 1.5 × 5.5 cm column of DEAE-Sephacel equilibrated with water adjusted to pH 3.0. Materials adsorbed on the column were eluted by applying 1 M NaCl. Fractions (5 ml) were collected at a flow rate of 20 ml/h and monitored for sialic acid content by the thiobarbituric acid reaction, and protein and/or peptide (protein/peptide) content by measuring absorbance at 230 and 280 nm. Major sialic acid containing fractions

from the DEAE-Sephacel column were pooled, and applied to a 1.5 × 5.5 cm column of phenyl-agarose equilibrated with 0.01 M sodium phosphate buffer, pH 6.8 containing 5 M NaCl (buffer-A) (4). The column was eluted first with 10–15 bed volumes of buffer-A, and then water to release materials adsorbed on the column. Fractions (2 ml) were collected at a flow rate of 20 ml/h and monitored for sialic acid, and protein/peptide contents (see above). Fractions containing GMP sialic acid were pooled, dialyzed in water and freeze-dried to obtain the final preparation of GMP.

Sweet whey contains relatively small amounts of sialylated proteins including immunoglobulins and lactoferrin. These proteins having higher isoelectric points or hydrophobicity than GMP are supposed to be desorbed from DEAE-Sephacel or adsorbed on phenyl-agarose under the experimental conditions. Thus, the major sialic acid peak eluting from the phenyl-agarose column is supposed to contain sialic acid from GMP but not from the sialylated sweet whey proteins.

3.2.3 Analytical methods.

Sialic acid contents in goat sweet whey samples and chromatographic fractions were determined by the thiobarbituric acid reaction as previously described (3, 5). Amino acid analysis was performed by using a Beckman Model 6300 amino acid analyzer on samples hydrolyzed under nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol. The content of galactose was estimated by the anthron reaction (6) using galactose as a standard, and that of galactosamine by the indole reaction (7) using galactosamine-HCl as a standard. The content of phosphorus was determined by using a molybdate-vanadate reagent (8). Calcium contents were determined by using a Perkin Elmer Model 400 atomic absorption spectrophotometer (8). Protein contents were estimated by the method of LOWRY *et al.* (9) using bovine serum albumin as a standard.

3.2.4 Gel electrophoresis.

Electrophoresis in 0.1% (w/v) sodium dodecyl sulfate (SDS) was carried out on 12% polyacrylamide gels in Tris-borate buffer, pH 8.6 (10) using a mini-PROTEAN II cell (Bio-Rad, Laboratories) with 0.75 mm thick spacers and 10 well combs. Samples were dissolved in 0.04 M Tris-borate containing 2% (w/v) SDS, 6 M urea, and 10% (v/v) 2-mercaptoethanol, and boiled for 5 min prior to application. Gels were stained with 0.5% (w/v) Coomassie blue R250 in 50% (v/v) methanol containing 12.5% (w/v) trichloroacetic acid, and destained in 5% (w/v) trichloroacetic acid (11, 12). Destained gels were scanned using a Bio-Rad Model GS-670 imaging densitometer. Coomassie blue R250 in 45% (v/v) methanol containing 10% glacial acetic acid, a commonly used preparation to stain protein (13), was not suitable for staining GMP. It stained bovine and goat GMP very poorly giving weak metachromasia with the latter (1). The mobility of electrophoretic band was expressed using an R_f value, which is calculated as a ratio of the migration distance of electrophoretic band to that of marker dye, bromophenol blue.

3.3 RESULTS AND DISCUSSION

Quantitative data obtained for preparation and chromatographic fractionation of sweet whey were similar among raw and pasteurized samples of goat milk. The recovery of sweet whey averaged 76.3% (v/v) of milk, and that of dry matter of non-dialyzable product 0.56% (w/v) of sweet whey (Table 3.1). The average concentration of sialic acid determined in sweet whey was 79 µg/ml, which corresponded to 1.5 µg/mg dry weight. The non-dialyzable product of sweet whey was then fractionated by column chromatography on DEAE-Sephacel (Figure 3.1). An average 90% of recovered sialic acid was adsorbed on the column and eluted as a single peak, while the remaining 10% of sialic acid did not bind to the column. This indicated that most GMP sialic acid (and thus GMP) was adsorbed on the DEAE-Sephacel anion-exchanger. The sialic acid peak fraction (tube no. 41 to 48, Figure 3.1) was further chromatographed on phenyl-agarose

(Figure 3.2), while the material desorbed from the anion exchanger (tube no. 12 to 15, Figure 3.1), referred to as GMP-depleted whey fraction, was subjected to chemical analyses (see below).

On phenyl-agarose chromatography, most (92%) of the recovered sialic acid was desorbed from the column and eluted as a single peak, while the remaining small portion (8%) of sialic acid tightly bound to the column (Figure 3.2). The eluates from the major sialic acid peak were pooled as indicated in Figure 3.2 to obtain a GMP fraction. The minor sialic acid peak fraction (Figure 3.2) was not analyzed further. The dry matter of the GMP fraction accounted for an average 20.7% of dry weight of non-dialyzable fraction of sweet whey. This corresponded to 0.16% (w/v) of sweet whey, which is close to the yield of bovine GMP (0.12–0.15%) reported elsewhere (14).

Carbohydrate and mineral concentrations determined in the GMP fractions are shown in Table 3.2. There was apparently no treatment effect on the concentrations of sialic acid, galactose, galactosamine and phosphorous, averaging 21.3, 34.8, 39.2 and 3.7 μg/mg dry weight, respectively. The calcium concentration was, however, higher in the raw than in the pasteurized sample, suggesting loss of calcium ions attached to phosphorylated serine in κ-casein during heating of milk. This may be expected, but not much information is available concerning heat susceptibility of calcium in GMP.

Amino acid compositions (Table 3.3) were similar among the GMP fractions from the raw and pasteurized milk both of which contained traces (each < 1 residue/peptide estimated from the data in Table 3.3) of glycine, leucine, tyrosine, phenylalanine and arginine (amino acids not found in goat GMP). The contents of threonine and valine were lower and those of serine and glutamine/glutamic acid were higher than the theoretical contents (Table 3.3). However, the contents of remaining amino acids present in the GMP fractions were in general comparable to their theoretical contents. These results indicate that the purity of each preparation of GMP is considerably high. The high contents of serine and glutamine/glutamic acid and amino acids not specific to GMP (see above) ap-

pear to contribute to the small amount of impurity present. The reason for the low contents of threonine and valine is unknown.

SDS gel electrophoresis patterns for the purified GMP samples are given in Figure 3.3a. A densitometric scan of each electrophoretogram is also shown in Figure 3.3b. All samples produced an irregularly stained broad band with its mobility faster than that of carbonic anhydrase (31.0 kDa) but slower than that of trypsin inhibitor (21.5 kDa). The broad band appears to reflect variability in the contents of carbohydrates including sialic acid present in GMP. The mobility of the band suggests that goat GMPs having molecular weight of approximately 7.5 kDa (1) form aggregates on SDS gels. Each band had at least 3 components, but its profile varied among the samples with different treatments. The sample from raw milk (Figures. 3.3a-lane 2 and 3.3b-lane 2) displayed components with Rf values of 0.65, 0.71 and 0.76, which were inversely related to their staining intensity and broadness. This was apparently different from the electrophoretic patterns of samples prepared from pasteurized milk, in which the third component (Rf = 0.76) detected in the GMP prepared from raw milk (see above) was not clearly seen (Figures 3.3a-lanes 3 and 4, and 3.3b-lanes 3 and 4). Instead the samples from pasteurized milk had a component with faster mobility (Rf = 0.78) as the third component with the staining intensity being less in the sample with HTST treatment (Figures 3.3a-lane 3 and 3.3b-lane 3) than that with LTLT treatment (Figures 3.3a-lane 4 and 3.3b-lane 4). The staining intensity and broadness for the first component (Rf = 0.65) as well as the second component (Rf = 0.71) tended to be the greatest in the sample from raw milk and greater in the sample with HTST treatment compared to that with LTLT treatment. These results suggest that the structure of goat GMP is affected by the heat treatment of milk with a greater extent by the LTLT than the HTST treatment. Little information is available concerning the mechanism of heat induced changes detected by SDS gel electrophoresis. Only the sample with LTLT treatment (Figures 3.3a-lane 4 and 3.3b-lane 4) demonstrated a band (Rf = 0.59) with its mobility similar to that of carbonic anhydrase (31.0 kDa). This band was not characterized further.

Gel electrophoresis results obtained in this study are consistent with the previous report from this laboratory demonstrating occurrence of a single broad band of aggregated GMP purified from bovine sweet whey (17). The present findings, however, do not confirm the previous report showing occurrence of monomeric GMP as a minor component of goat GMP detected by SDS gel electrophoresis (1). No satisfactory explanation is available concerning the formation of monomeric GMP.

Analysis of GMP-depleted whey fraction showed that the concentrations of protein, sialic acid, phosphorous and calcium (Table 3.4) and amino acid compositions (Table 3.5) were relatively constant among the preparations from raw and pasteurized milk samples. The GMP-depleted whey fraction had high contents of glutamine/glutamic acid, leucine, asparagine/aspartic acid and lysine. Since GMP contains relatively large amounts of threonine and sialic acid, the GMP-depleted fraction generated after removal of GMP from sweet whey is expected to contain less threonine and sialic acid than sweet whey. The sialic acid concentration was approximately 6-fold lower in the GMP-depleted whey fraction (average 2.0 μg/mg, Table 3.4) compared to sweet whey (11.5 μg/mg, E.R. Silva-Hernández, T. Nakano and L. Ozimek, unpublished data), and the threonine content (5.8 mol%, Table 3.5) was 69% of that found in sweet whey (8.4 mol%, E.R. Silva-Hernández, T. Nakano and L. Ozimek, unpublished data). Most sialic acid is present in non-GMP materials (*e.g.* immunoglobulins) in the GMP-depleted whey fraction, while GMP sialic acid is a component of oligosaccharide attached to threonine. The higher contents of phenylalanine (3.1 mol%) and tyrosine (2.7 mol%) observed in the GMP-depleted whey fraction (Table 3.5) than in goat sweet whey (2.4 and 1.8 mol% for phenylalanine and tyrosine, respectively (E.R Silva-Hernández, T. Nakano and L. Ozimek, unpublished data obtained from dialyzed samples) are also not surprising because goat GMP does not contain these aromatic amino acids.

The GMP-depleted whey fraction that contains less threonine than does sweet whey (see above) may be useful as an ingredient for infant formulas. It has been reported that the threonine rich GMP is the major factor causing hyper-

threoninemia, a well known phenomenon in preterm infants fed sweet whey based formulas, and that feeding acid whey based formula free of GMP prevents hyperthreoninemia (18). The GMP-depleted whey fraction may be as effective as acid whey to prevent the abnormality.

The whole experiment including preparation and analysis of GMP and GMP-depleted whey fraction was repeated with goat milk samples obtained from the same supplier (see Materials) but different from those used in the first experiment. Results obtained were in general consistent with those obtained in the first experiment.

In conclusion, the present study provides previously unreported information on GMP prepared from goat milk with and without pasteurization. Results showed that GMP from non-pasteurized milk contained more calcium than that from pasteurized milk, and that pasteurization generates GMP aggregates with increased mobility determined by SDS gel electrophoresis. We have also shown that the GMP-depleted whey fraction obtained after purification of GMP is a product with low threonine content useful as an ingredient for infant formula.

3.4 REFERENCES

1. Silva-Hernandez, E.R.; Nakano, T.; Ozimek, L. Isolation and Analysis of κ-Casein Glycomacropeptide from Goat Sweet Whey. *J. Agric. Food Chem.* **2002,** *50,* 2034–2038.
2. Chu, L.; MaCleod, A.; Ozimek, L. Isolation of glycomacropeptide from sodium caseinate hydrosylate solution by ultrafiltration. *Milchwissenschaft* **1996,** *51,* 303-306.
3. Nakano, T.; Ozimek, L. Gel chromatography of glycomacropeptide (GMP) from sweet whey on Sephacryl S-200 at different pH's and on Sephadex G-75 in 6M guanidine hydrochloride. *Milchwissenschaft* **1998,** *53,* 629-633.

4. Nakano, T.; Ozimek, L. Purification of glycomacropeptide from non-dialyzable fraction of sweet whey by hydrophobic interaction chromatography on phenyl-agarose. *Biotechnol. Lett.* **2000,** *22,* 413-416.
5. Nakano, T.; Ozimek, L. Determination of sialic acid by the thiobarbituric acid reaction in sweet whey and its fractions. *J. Agric. Food Chem.* **1999,** *47,* 2613-2616.
6. Trevelyan, W.; Harrison, J.S. Studies of yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. *Biochem. J.* **1952**, *50*, 298-303.
7. Dische, Z.; Borenfrend, E. A spectrophotometric method for the micro-determination of hexosamines. *J. Biol. Chem.* **1950,** *184*, 517-522.
8. AOAC, *Official Methods of Analysis of AOAC International.* 16th Ed. Association of Official Analytical Chemists; Gaithersburg, Maryland, 1998.
9. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **1951**, *195,* 265–275.
10. Mechanic, G.L. Collagen biochemistry. In: *Skeletal Research;* Simmons D.J., Kunin, A.S., Eds.; Academic Press: New York, 1979; pp 227-241.
11. Coolbear, K.P.; Elgar, D.F.; Ayers, J.S. Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. *Int. Dairy J.* **1996,** *6,* 1055-1068.
12. Reid, J.R.; Coolbear, T.; Ayers, J.S.; Coolbear, K.P. The action of chymosin on κ-casein and its macropeptide: Effect of pH and analysis of products of secondary hydrolysis. *Int. Dairy J.* **1997,** *7,* 559-569.
13. Bollag, D.M.; Edelstein, S.J. *Protein Methods*; New York, 1991; pp 114-116.
14. Abd El-Salam, M.H.; El-Shibiny, S.; Buchheim, W. Characteristics and potential uses of the casein macropeptide. *Int. Dairy J.* **1996,** *6,* 327-341.
15. Mercier, J.C.; Addeo F.; Pelissier, J.P. Structure primaire du caseinomacropeptide de la caséine κ caprine (in French). *Biochimie* **1976**, *58,* 1303-1310.

16. Mercier, J.C.; Chobert, J.M.; Addeo F. Comparative study of the amino acid sequences of the caseinomacropeptides from seven species. *Febs. Lett.* **1976,** *72*, 208-214.

17. Nakano, T.; Silva-Hernandez, E.R.; Ikawa, N.; Ozimek, L.; Goat κ-casein glycomacropetide. *Biotech. Progr.* **2002,** *18,* 409–412.

18. Rigo, J.; Boehm, G.; Georgi, G.; Jelinek, J.; Nyambugabo, K.; Sawatzki, G.; Studzinski, F. An infant formula free of glycomacropeptide prevents hyperthreoninemia in formula-fed preterm infants. *J. Pediatr. Gastroenterol. Nutr.* **2001,** *32,* 127-130.

Table 3.1. Recoveries of goat sweet whey and GMP from raw milk and HTST and LTLT pasteurized milk.

	Raw	HTST	LTLT
Sweet whey (ml/100 ml of milk)	75	77	77
Non-dialyzable fraction of sweet whey (g/100 ml of sweet whey)	0.60	0.52	0.57
GMP (g/100 ml of sweet whey)	0.14	0.16	0.19
GMP (g/100 g of dried sweet whey)	19	19	24
GMP (g/liter of milk)	1.05	1.23	1.46

Table 3.2. Analysis of goat GMP isolated from raw milk, HTST and LTLT pasteurized milk (μg/mg dry weight)

	Raw	HTST	LTLT
Sialic acid	23.0	19.8	21.2
Galactose	32.7	37.0	34.7
Galactosamine	41.3	32.4	44.4
Phosphorus	3.8	3.4	3.8
Calcium	15.7	12.7	11.8

Table 3.3. Amino acid compositions of goat GMP isolated from raw milk and HTST and LTLT pasteurized milk and theoretical amino acid composition of goat GMP (Mol %)

	Raw	HTST	LTLT	Goat GMP*
Asparagine and aspartic acid	9.9	10.9	10.9	10.6
Threonine	14.1	14.8	14.7	16.7
Serine	13.7	14.4	14.1	12.1
Glutamine and glutamic acid	15.9	15.9	16.7	13.6
Glycine	0.6	0.7	0.6	0
Alanine	12.3	11.7	11.6	13.6
Valine	6.3	6.5	6.2	9.1
Methionine	ND	ND	ND	1.5
Isoleucine	7.7	7.1	7.3	7.6
Leucine	1.6	1.5	1.8	0
Tyrosine	0.3	0.2	0.2	0
Phenylalanine	0.6	0.6	0.7	0
Histidine	1.6	1.5	1.7	1.5
Lysine	5.6	5.0	4.6	4.5
Arginine	0.7	0.7	0.6	0
Proline	9.2	8.5	8.4	9.1
Cysteine	ND	ND	ND	0
Tryptophan	ND	ND	ND	0

*Values are based on the primary structure of goat GMP (15, 16). ND: Not determined

Table 3.4. Analysis of the GMP-depleted goat whey fractions obtained from raw milk and HTST and LTLT pasteurized milk*

	Raw	HTST	LTLT
Protein	79.3	86.7	78.6
Water	8.2	6.0	7.1
Ash	9.9	7.3	8.6
Sialic acid	1.8	2.1	2.0
Phosphorus	0.4	0.2	0.3
Calcium	1.7	2.0	2.1

*Contents of protein, water and ash: g/100 g of dry weight, of sialic acid, phosphorus and calcium: μg/mg dry weight.

Table 3.5. Amino acid compositions of the GMP-depleted goat whey fractions obtained from raw milk and HTST and LTLT pasteurized milk (Mol%)

	Raw	HTST	LTLT
Asparagine and aspartic acid	10.6	11.4	11.0
Threonine	6.0	5.7	5.7
Serine	5.6	5.6	5.8
Glutamine and glutamic acid	15.4	16.5	16.6
Glycine	3.8	3.6	3.5
Alanine	9.0	8.5	8.4
Valine	6.4	6.0	6.1
Methionine	ND	ND	ND
Isoleucine	5.3	5.2	5.1
Leucine	11.3	12.6	12.6
Tyrosine	2.7	2.8	2.7
Phenylalanine	3.3	3.0	3.0
Histidine	2.1	1.9	2.0
Lysine	10.6	10.3	10.1
Arginine	1.8	1.8	1.8
Proline	6.0	5.2	5.6
Cysteine	ND	ND	ND
Tryptophan	ND	ND	ND

ND: Not determined

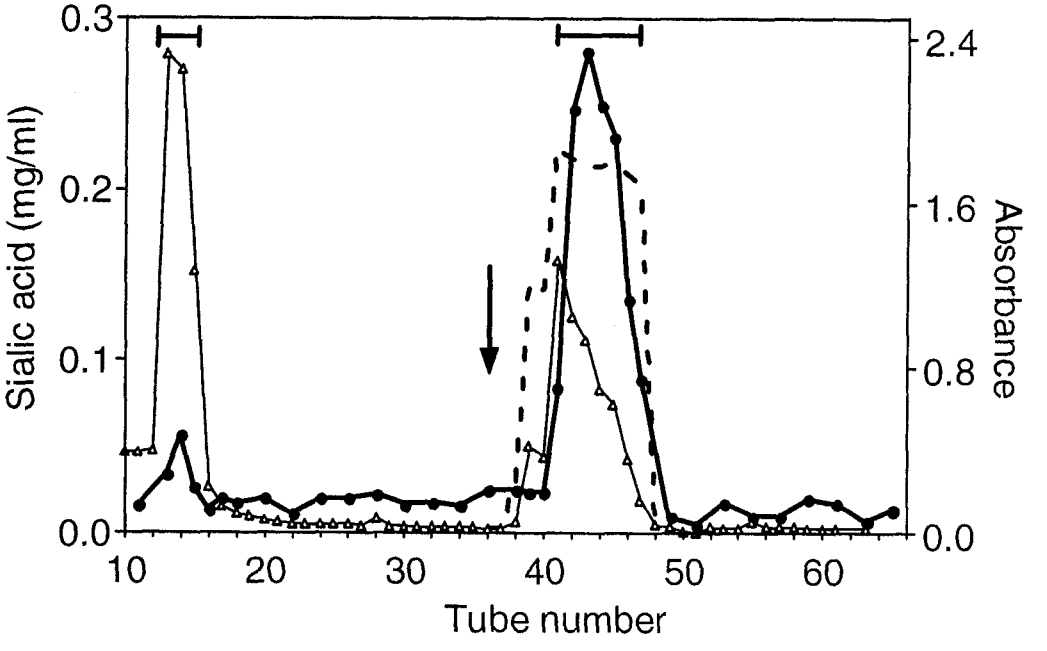

Figure 3.1. Anion-exchange chromatography of goat sweet whey (44 ml) on DEAE-Sephacel: (●—●) sialic acid; (----) absorbance at 230 nm, and (△---△) absorbance at 280 nm. Absorbance at 230 nm was read in tubes 36-65 only. An arrow shows the position of application of 1 M NaCl. Bars denote eluates pooled for further study.

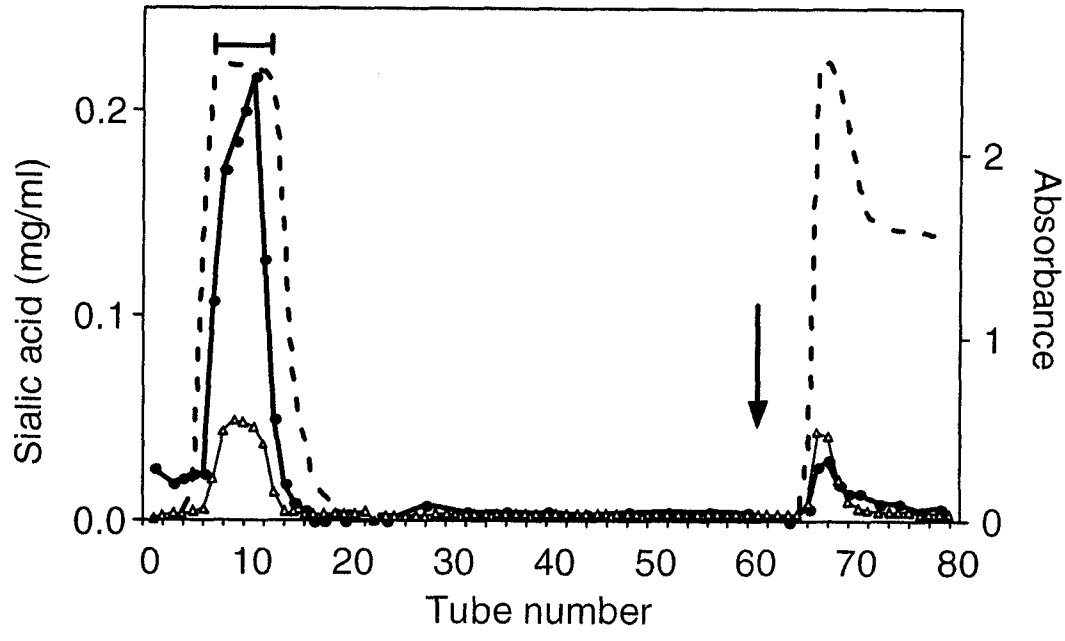

Figure 3.2. Hydrophobic interaction chromatography on phenyl-agarose of major sialic acid containing fraction from DEAE-Sephacel (Fig. 3.1): (●—●) sialic acid; (----) absorbance at 230 nm, and (△---△) absorbance at 280 nm. An arrow shows the position of application of water. A bar denotes eluates pooled for further study.

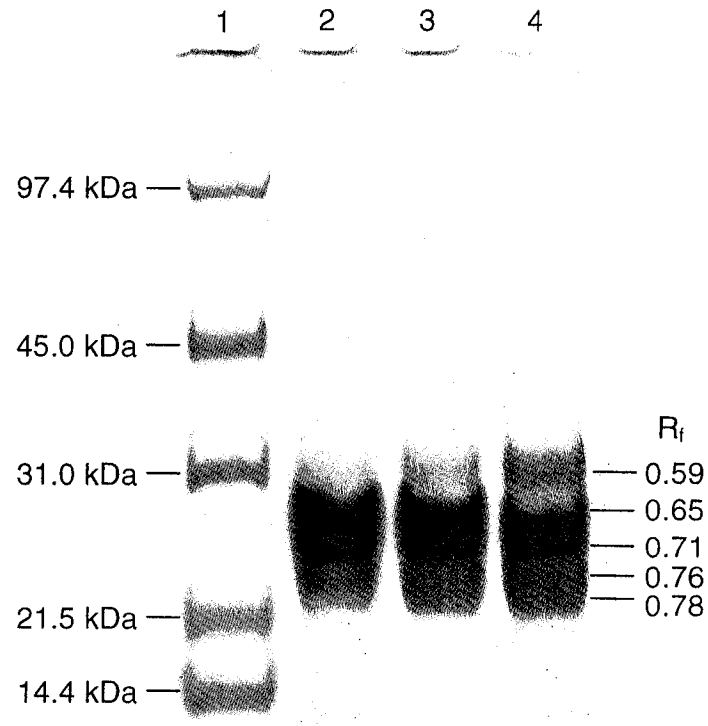

Figure 3.3a. Gel electrophoresis of goat GMP: (a) GMP fractions from phenyl-agarose (Fig. 3.2) (~50 µg/well) were electrophoresed on SDS-gel. Lane 1; Molecular weight standards, phospholylase b (97.4 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Lanes 2–4: GMP isolated from raw milk, and HTST and LTLT pasteurized goat milk, resp. Bars indicate components in major band.

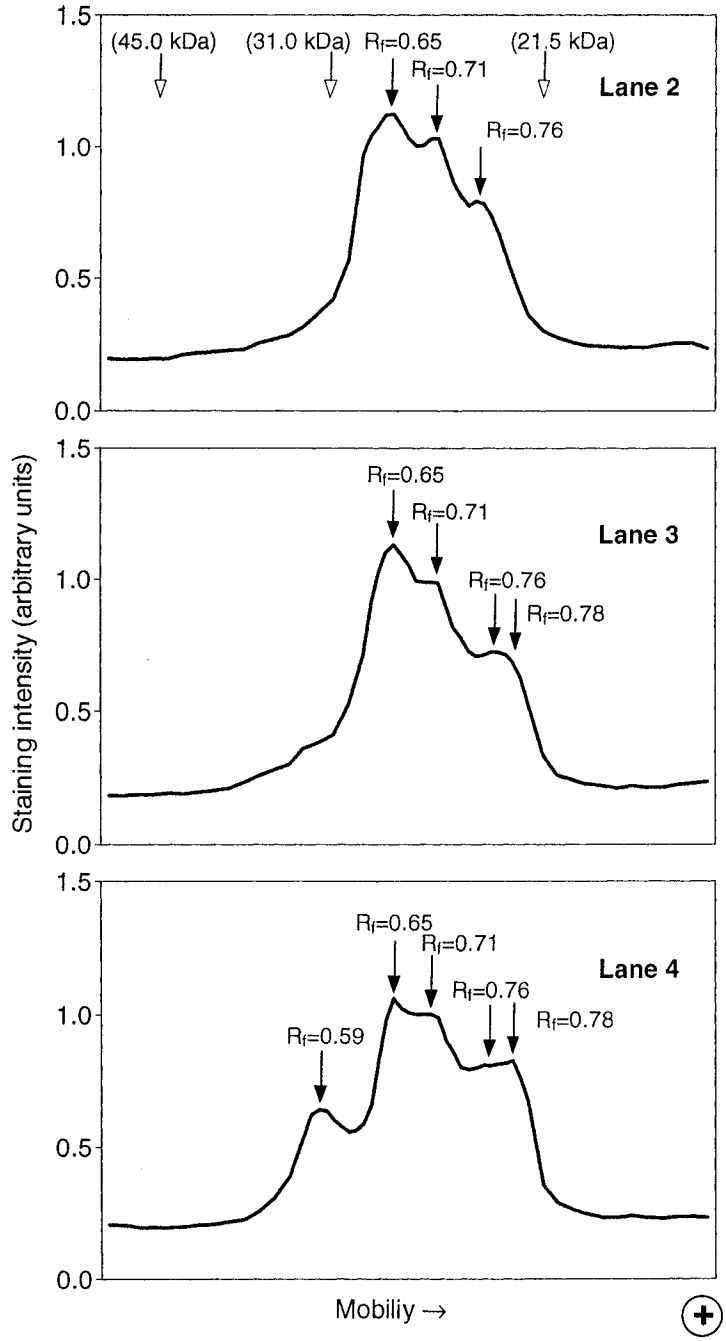

Figure 3.3b. Gel electrophoresis of goat (GMP). Densitometric scans of electrophoretograms (lanes 2–4 in Fig. 3.3a).

CHAPTER 4

Chemical Composition of Glycomacropeptide-Depleted Goat Sweet Whey and its Potential Use in Infant Formulas[1]

4.1 INTRODUCTION

Production of novel high value-added nutraceuticals from milk has been the subject of increasing interest in the pharmaceutical and food industries, and thus much attention has been given to fractionating milk proteins and peptides into individual components to take advantage of their biological and/or functional properties. We have recently isolated a glycomacropeptide from goat sweet whey, and reported that the GMP preparation with traces of aromatic amino acids (e.g. phenylalanine) may be suitable for the diet of patients suffering from phenylketonuria, a hereditary disorder of phenylalanine metabolism (1). There is limited information available concerning chemical composition of the fraction (referred to as GMP-depleted whey fraction) obtained after separation of GMP. Such information may be important for developing value-added products with this fraction.

It has been reported that infants fed with bovine sweet whey-based formulas suffer from hyperthreoninemia (2-7), which is a condition manifested as abnormally high concentrations of serum threonine. GMP, a threonine rich sialylated peptide found in sweet whey is suggested as a compound contributing to the cause of this abnormality. If so, use of GMP-depleted whey fraction as an ingredient of infant formula may help prevent hyperthreoninemia, which may affect brain development (8). The present study was, therefore, undertaken to prepare

---

[1] A version of this chapter has been submmited to be considered for publication. Silva-Hernandez, E.R.; Nakano, T.; Ozimek, L. Chemical Composition of Glycomacropeptide-Depleted Goat Sweet Whey and its Potential Use in Infant Formulas. *Brit. J. Nutr.* **2004**.

GMP-depleted whey fraction from goat milk and to determine whether it is suitable for infant formulas by examining its chemical composition.

4.2 MATERIALS AND METHODS

4.2.1 Materials.

Samples of pasteurized goat milk were obtained from local milk processing plants in Alberta and British Columbia, Canada. Sweet whey was prepared by chymosin treatment (9) of milk, followed by centrifugation at 20,000g and 4 °C for 30 min to remove fat and protein precipitate (10).

All samples of sweet whey were dialyzed using dialysis tubes with 6-8 kDa molecular weight cut-off. A portion of dialyzed sweet whey was freeze-dried and stored at 4 °C until used for chemical analysis. The remaining portion of dialyzed whey was stored at -25 °C for approximately one week before it was fractionated by anion-exchange chromatography. Diethylaminoethyl (DEAE)-Sephacel was obtained from Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada. Sialic acid from sheep submaxillary glands, and 2-thiobarbituric acid were from Sigma-Aldrich, Canada Ltd., Oakville, Ontario, Canada.

4.2.2 Preparation of GMP-depleted whey fraction.

Frozen samples of dialyzed sweet whey (see above) were thawed and centrifuged at 20,000g and 10 °C for 15 min. After centrifugation, each supernatant obtained was adjusted to pH 3.0 with acetic acid and applied to a 1.5 $\times$ 5.5 cm column of DEAE-Sephacel equilibrated with water adjusted to pH 3.0. Fractions (2 ml) were collected at a flow rate of 20 ml/h and monitored for protein content by measuring absorbance at 230 nm, and for the sialic acid content determined by the thiobarbituric acid reaction (10). Fractions containing protein desorbed from the anion-exchanger were pooled, dialyzed in water, and freeze-dried to obtain a GMP-depleted whey fraction. GMP was adsorbed on the anion-exchanger and eluted with 1M NaCl (1).

4.2.3 Analytical methods.

Amino acid analysis was performed by using a Beckman Model 6300 amino acid analyzer on samples hydrolyzed under nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol. The content of phosphorus was determined by using a molybdate-vanadate reagent (11), and that of calcium by using a Perkin Elmer Model 400 atomic absorption spectrophotometer (11). Protein contents were estimated by the method of Lowry *et al.* (12) using bovine serum albumin as a standard.

4.2.4 Homology of amino acid composition.

Amino acid profiles obtained from goat sweet whey and GMP-depleted goat whey fraction were compared to those from human milk by calculating the index ($S\Delta Q$) value proposed by Marchalonis and Weltman (13) for the difference of amino acid composition. The index value was calculated from the formula:

$$S\Delta Q = \sum_j (X_{i,j} - X_{k,j})^2$$

where i and k identify the products being compared and X_j is the content, in mol/100 mol, of the amino acid of type j. Pairs of homologous amino acid profiles generally have $S\Delta Q$ below 100.

4.3 RESULTS AND DISCUSSION

Concentrations of protein, ash and calcium in the sweet whey (Table 4.1) are fairly comparable to those (93.7 and 6.0 g/100g and 4.1 μg/mg, respectively) calculated from the corresponding values reported by Pintado *et al.* (14). The GMP-depleted whey fraction obtained after DEAE-Sephacel chromatography accounted for 71% of total protein recovered. The protein concentration of GMP-depleted fraction was similar to that of sweet whey averaging 89.6 g/100g dry weight. Phosphorous and calcium concentrations were, however, approximately three times lower in GMP-depleted whey fraction.

Amino acid analysis (Table 4.2) showed that the GMP-depleted whey fraction has high contents of glutamine/glutamic acid, leucine, asparagine/aspartic

acid and lysine. The contents of threonine and serine were lower and those of leucine and lysine were higher in the GMP-depleted whey fraction compared to sweet whey. The contents of remaining amino acids in the GMP-depleted whey fraction were similar to those in sweet whey. Comparison of amino acid composition between the human milk and GMP-depleted whey fraction indicated that the contents of glutamine/glutamic acid, alanine, leucine and lysine are higher and the content of proline is lower in the latter (Table 4.2). The contents of remaining amino acids including threonine were similar between the two products.

The $S\Delta Q$ values calculated between the GMP-depleted whey fraction and human milk A or B were 57 and 61, respectively. The corresponding values (47 and 54) between the goat sweet whey and human milk A or B were slightly lower. Marchalonis and Weltman (13) reported that pairs of homologous amino acids profiles generally have $S\Delta Q$ values below 100. Therefore, the index values obtained in this study indicate that there is high homology of amino acid composition between human milk and goat GMP-depleted whey fraction. The homology is slightly higher in goat sweet whey (with lower $S\Delta Q$ value) compared to its GMP-depleted whey fraction.

Bovine sweet whey has been used as an ingredient for various food products including baby formulas. However, Rigo *et al.* (8) reported that feeding infants with sweet whey-based formulas with high threonine content causes hyperthreoninemia. The present results suggest that GMP-depleted whey fraction with its threonine content lower than that found in sweet whey but similar to that in human milk can be used as an ingredient suitable for baby formulas. The GMP-depleted whey fraction with high protein content (Table 4.1) could also be used to replace sweet whey to be added to various processed food products including bakery products and beverages.

4.4 REFERENCES

1. Silva-Hernandez, E.R.; Nakano, T.; Ozimek, L. Isolation and Analysis of κ-Casein Glycomacropeptide from Goat Sweet Whey. *J. Agric. Food Chem.* **2002,** *50,* 2034–2038.
2. Hanning, R.M.; Paes, B.; Atkinson, S.A. Protein metabolism and growth of term infants in response to a reduced-protein, 40:60 whey: casein formula with added tryptophan. *Am. J. Clin. Nutr.* **1992,** *56,*1004-1011.
3. Janas, L.M.; Picciano, M.F.; Hatch, T.F. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula. *Pediatrics* **1985,** *75,* 775-784.
4. Järvenpää, A.-L.; Rassin, D.K. Räihä, N.C.R.; Gauill, G.E. Milk protein quantity and quality in term infant: II. Effects on acidic and neutral amino acids. *Pediatrics* **1982,** *70,* 221-230.
5. Picone, T.A.; Benson, J.D.; Moro, G.; Minoli, I.; Fulconis, F.; Rassin, D.K.; Räihä, N.C.R. Growth, serum biochemistries, and amino acid of term infants fed formulas with amino acid and protein concentrations similar to human milk. *J. Pediatr. Gastroenterol. Nutr.* **1989,** *9,* 351-360.
6. Rassin, D.K.; Gauill, G.E.; Heinonen, K.; Räihä, N.C.R. Milk protein quantity and quality in low-birth-weight infants: II. Effects on selected aliphatic amino acids in plasma and urine. *Pediatrics* **1977,** *59,* 407-422.
7. Rigo, J.; Verloes, A.; Senterre, J. Plasma amino acid concentrations in term infants fed human milk, a whey-predominant formula, or a whey hydrosylate formula. *J Pedriatr* **1989,** *115,* 752-755.
8. Rigo, J.; Boehm, G.; Georgi, G.; Jelinek, J.; Nyambugabo, K.; Sawatzki, G.; Studzinski, F. An infant formula free of glycomacropeptide prevents hyperthreoninemia in formula-fed preterm infants. *J. Pediatr. Gastroenterol. Nutr.* **2001,** *32,* 127-130.
9. Chu, L.; MaCleod, A.; Ozimek, L. Isolation of glycomacropeptide from sodium caseinate hydrosylate solution by ultrafiltration. *Milchwissenschaft* **1996,** *51,* 303-306.

10. Nakano, T.; Ozimek, L. Gel chromatography of glycomacropeptide (GMP) from sweet whey on Sephacryl S-200 at different pH's and on Sephadex G-75 in 6M guanidine hydrochloride. *Milchwissenschaft* **1998,** *53,* 629-633.
11. AOAC, *Official Methods of Analysis of AOAC International.* 16th Ed. Association of Official Analytical Chemists; Gaithersburg, Maryland, 1998.
12. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **1951**, 195, 265–275.
13. Marchalonis, J.J.; Weltman, J.K. Relatedness among proteins: a new method of estimation and its application to immunoglobulins. *Comp. Biochem. Physiol.* **1971**, *38B,* 609–625.
14. Pintado, M.E.; Lopes da Silva, J.A.; Malcata, F.X. Comparative characterization of whey protein concentrates from ovine, caprine and bovine breeds. *Lebensm. Wiss. Technol.* **1999,** *32,* 231-237.
15. Darragh, A.J.; Moughan, P.J. The amino acid composition of human milk corrected for amino acid digestibility. *Brit. J. Nutr.* **1998,** *80,* 25-34.
16. Rigo, J.; Salle, B.L.; Cavero, E.; Putet, G.; Senterre, J. Plasma amino acid and protein concentrations in term infants fed human milk or a whey protein hydrosylate formula during the first month of life. Acta Pediatr **1994**, 83, 127-131.

Table 4.1. Analysis of goat sweet whey and GMP-depleted whey fraction*.

Component	Sweet whey	GMP-depleted whey fraction
Protein	91.4 ± 3.1	87.7 ± 5.1
Ash	4.0 ± 1.1	1.8 ± 0.3
Phosphorous	3.3 ± 0.2	1.1 ± 0.1
Calcium	7.7 ± 0.6	2.8 ± 1.2

* Contents of protein and ash are expressed as g/100 g of dry weight, and those of phosphorus and calcium as μg/mg dry weight.

Table 4.2. Amino acid compositions of goat sweet whey and GMP-depleted whey fraction, and human milk. Contents are expressed as moles of amino acid per 100 moles of total amino acids.

Amino acid	Goat sweet whey	GMP-depleted whey fraction	Human milk	
			A	B
Asparagine and aspartic acid	10.6	11.3	9.7	9.3
Threonine	7.9	5.8	5.8	5.1
Serine	9.3	6.9	5.7	6.3
Glutamine and glutamic acid	17.8	17.2	16.2	15.0
Glycine	2.8	3.6	4.1	4.7
Alanine	8.1	8.2	5.2	5.6
Cysteine	ND	ND	3.2	1.6
Valine	5.5	5.2	6.2	5.7
Methionine	ND	ND	1.3	1.1
Isoleucine	5.0	4.2	5.5	5.1
Leucine	9.2	12.2	10.1	9.7
Tyrosine	1.9	2.6	2.9	3.6
Phenylalanine	2.5	3.1	3.2	3.7
Histidine	1.8	1.7	2.2	2.9
Lysine	8.5	10.3	6.1	6.4
Tryptophan	ND	ND	---	1.5
Arginine	1.5	1.8	2.5	2.9
Proline	7.8	6.1	10.2	10.0

A: Calculated from Darragh & Moughan (15)

B: Calculated from Rigo *et al.* (16)

ND: Not determined

CHAPTER 5

Analysis of Glycomacropeptide-Depleted Sweet Whey Fraction[1]

5.1 INTRODUCTION

Sweet whey, a by-product of cheese manufacturing, can be separated into glycomacropeptide (GMP)-rich and GMP-depleted fractions by ion-exchange chromatography (1-3). We have analyzed a GMP-rich fraction, accounting for approximately 18% of dry weight of dialyzed sweet whey, and confirmed that GMP is a sialylated peptide with high threonine content (3). However, not much information is available concerning chemical composition of GMP-depleted whey fraction.

It has been reported that infants fed with sweet whey-based formulas frequently suffer from hyperthreoninemia (4-9), which is a condition manifested as abnormally high serum threonine levels. GMP (a threonine rich peptide) present in sweet whey is suggested as a compound contributing to the cause of this abnormality. Replacement of sweet whey by GMP-depleted whey fraction in infant formulas may help prevent occurrence of hyperthreoninemia. The present study was, therefore, undertaken to analyze chemical composition of GMP-depleted whey fraction prepared from sweet whey powder, and to determine whether the GMP-depleted fraction is suitable as an ingredient for infant formulas.

---

[1] A version of this chapter has been submitted to be considered for publication. Silva-Hernandez, E.R.; Nakano, T.; Ozimek, L. Analysis of Glycomacropeptide-depleted Sweet whey fraction. *Int. J. Food Sci. Technol.*, **2004.**

5.2 MATERIALS AND METHODS

5.2.1 Materials.

Samples of bovine sweet whey powder were obtained from Dairyland Canada, Vancouver, British Columbia, Canada, and Alpha Milk Co., Red Deer, Alberta, Canada. Six grams of each sample were mixed with 100 ml of water, and the mixture was dialyzed using dialysis tubes with 6-8 kDa molecular weight cut-off. A portion of dialyzed sweet whey was freeze-dried and stored at 4 °C until used for chemical analysis. The remaining portion of dialyzed whey was stored at -25 °C for approximately one week before it was fractionated by anion-exchange chromatography. Diethylaminoethyl (DEAE)-Sephacel was obtained from Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada. Sialic acid from sheep submaxillary glands, and 2-thiobarbituric acid were obtained from Sigma-Aldrich, Canada Ltd., Oakville, Ontario, Canada.

5.2.2 Preparation of GMP-depleted whey fraction.

Frozen samples of dialyzed sweet whey (see above) were thawed and centrifuged at 20,000g and 10 °C for 15 min. After centrifugation, each supernatant obtained was adjusted to pH 3.0 with acetic acid and applied to a 1.5 × 5.5 cm column of DEAE-Sephacel equilibrated with water adjusted to pH 3.0. Fractions (2 ml) were collected at a flow rate of 20 ml/h and monitored for protein content by measuring absorbance at 230 nm, and for the sialic acid content determined by the thiobarbituric acid reaction (10). Fractions containing protein desorbed from the anion-exchanger were pooled, dialyzed in water, and freeze-dried to obtain a GMP-depleted whey fraction. GMP was adsorbed on the anion-exchanger and eluted with 1M NaCl (11).

5.2.3 Analytical methods.

Amino acid analysis was performed by using a Beckman Model 6300 amino acid analyzer on samples hydrolyzed under nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol. The content of phosphorus was determined by using a molybdate-vanadate reagent (12), and that of calcium

by using a Perkin Elmer Model 400 atomic absorption spectrophotometer (12). Protein contents were estimated by the method of Lowry *et al.* (13) using bovine serum albumin as a standard.

5.2.4 Homology of amino acid composition.

Amino acid profiles obtained from sweet whey and GMP-depleted whey fraction were compared to those from human milk by calculating the index ($S\Delta Q$) value proposed by Marchalonis and Weltman (14) for the difference of amino acid composition. The index value was calculated from the formula:

$$S\Delta Q = \sum_j (X_{i,j} - X_{k,j})^2$$

where i and k identify the products being compared and X_j is the content, in mol/100 mol, of the amino acid of type j. Pairs of homologous amino acid profiles generally have $S\Delta Q$ below 100.

5.3 RESULTS AND DISCUSSION

Analysis of sweet whey (Table 5.1) showed that concentrations of protein, ash, and phosphorous in the sweet whey are fairly comparable to those (95.8 and 4.2 g/100g and 4.4 μg/mg, respectively) calculated from the corresponding values of WPC reported by Sienkiewicz & Riedel (15). Although the calcium content in sweet whey found in this study was higher than that in whey protein concentrate (3.3 μg/mg) calculated from Sienkiewicz & Riedel (15), it was comparable to the corresponding value reported for whey protein concentrate (7.6 μg/mg) calculated from Pintado *et al.* (16).

Figure 5.1 shows a representative DEAE-Sephacel chromatogram. The GMP-depleted whey fraction accounted for 69% of total protein recovered.

The protein concentration of GMP-depleted fraction was similar to that of sweet whey averaging 97.7 g/100g dry weight. Phosphorous and calcium concentrations were, however, approximately twice lower in GMP-depleted whey fraction.

Amino acid analysis (Table 5.2) showed that the GMP-depleted whey fraction has high contents of glutamine/glutamic acid, leucine and asparagine/aspartic acid. The contents of threonine, serine and proline were lower and those of leucine and asparagine/aspartic acid were higher in the GMP-depleted whey fraction compared to sweet whey. The contents of remaining amino acids in the GMP-depleted whey fraction were similar to those in sweet whey. Comparison of amino acid composition between the human milk and GMP-depleted whey fraction indicated that the contents of leucine, alanine, lysine, asparagine/aspartic acid, glutamine/glutamic acid and serine are higher and the content of proline is lower in the latter (Table 5.2). The contents of remaining amino acids including threonine were similar between the two products.

Homology values ($S\Delta Q$) calculated between the GMP-depleted whey fraction and human milk A (Ref. 17) or B (Ref. 18) were 55 and 62, respectively. The corresponding values (41 and 50) between the sweet whey and human milk A or B were slightly lower. Marchalonis and Weltman (14) indicated that pairs of homologous amino acids profiles of proteins usually have $S\Delta Q$ values below 100. Therefore, the homology index values obtained in this study show that there is high homology of amino acid composition between human milk and GMP-depleted whey fraction. The homology is slightly higher in sweet whey (with lower $S\Delta Q$ value) compared to its GMP-depleted whey fraction.

Sweet whey powder has been used as an ingredient for numerous food products including infant formulas. However, it has been reported that feeding infants with sweet whey-based formulas causes hyperthreoninemia (19). The present findings suggest that GMP-depleted whey fraction with its threonine content lower than that found in sweet whey but similar to that in human milk can be used as an ingredient suitable for infant formulas. The GMP-depleted whey fraction with high protein content (Table 5.1) could also be used to replace sweet whey used as an ingredient in various food products.

5.4 REFERENCES

1. Ayres, J.S.; Coolbear, K.P.; Elgar, D.F. (1998) Process for isolating glycomacropeptide form dairy products with a phenylalanine impurity of 0.5% w/w. PCT/WO 98/1407/.
2. Etzel, M.R. (1999) Production of κ-casein macropeptide. PCT/WO 99/18808.
3. Nakano, T.; Ozimek, L. Determination of sialic acid by the thiobarbituric acid reaction in sweet whey and its fractions. *J. Agric. Food Chem.* **1999,** *47,* 2613-2616.
4. Hanning, R.M.; Paes, B.; Atkinson, S.A. Protein metabolism and growth of term infants in response to a reduced-protein, 40:60 whey: casein formula with added tryptophan. *Am. J. Clin. Nutr.* **1992,** *56,*1004-1011.
5. Janas, L.M.; Picciano, M.F.; Hatch, T.F. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula. *Pediatrics* **1985,** *75,* 775-784.
6. Järvenpää, A.-L.; Rassin, D.K. Räihä, N.C.R.; Gauill, G.E. Milk protein quantity and quality in term infant: II. Effects on acidic and neutral amino acids. *Pediatrics* **1982,** *70,* 221-230.
7. Picone, T.A.; Benson, J.D.; Moro, G.; Minoli, I.; Fulconis, F.; Rassin, D.K.; Räihä, N.C.R. Growth, serum biochemistries, and amino acid of term infants fed formulas with amino acid and protein concentrations similar to human milk. *J. Pediatr. Gastroenterol. Nutr.* **1989,** *9,* 351-360.
8. Rassin, D.K.; Gauill, G.E.; Heinonen, K.; Räihä, N.C.R. Milk protein quantity and quality in low-birth-weight infants: II. Effects on selected aliphatic amino acids in plasma and urine. *Pediatrics* **1977,** *59,* 407-422.
9. Rigo, J.; Verloes, A.; Senterre, J. Plasma amino acid concentrations in term infants fed human milk, a whey-predominant formula, or a whey hydrosylate formula. *J Pedriatr* **1989,** *115,* 752-755.
10. Nakano, T.; Ozimek, L. Gel chromatography of glycomacropeptide (GMP) from sweet whey on Sephacryl S-200 at different pH's and on Sephadex

G-75 in 6M guanidine hydrochloride. *Milchwissenschaft* **1998,** *53,* 629-633.

11. Silva-Hernandez, E.R.; Nakano, T.; Ozimek, L. Isolation and Analysis of κ-Casein Glycomacropeptide from Goat Sweet Whey. *J. Agric. Food Chem.* **2002,** *50,* 2034–2038.
12. AOAC, *Official Methods of Analysis of AOAC International.* 16th Ed. Association of Official Analytical Chemists; Gaithersburg, Maryland, 1998.
13. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **1951**, 195, 265–275.
14. Marchalonis, J.J.; Weltman, J.K. Relatedness among proteins: a new method of estimation and its application to immunoglobulins. *Comp. Biochem. Physiol.* **1971**, *38B,* 609–625.
15. Sienkiewicz, T.; Riedel, C.-L. *Whey and whey utilization, 2nd ed.* P. 116. Verlag Th. Mann. Gelsenkirchen-Buer, Germany, 1990.
16. Pintado, M.E.; Lopes da Silva, J.A.; Malcata, F.X. Comparative characterization of whey protein concentrates from ovine, caprine and bovine breeds. *Lebensm. Wiss. Technol.* **1999,** *32,* 231-237.
17. Darragh, A.J.; Moughan, P.J. The amino acid composition of human milk corrected for amino acid digestibility. *Brit. J. Nutr.* **1998,** *80,* 25-34.
18. Rigo, J.; Salle, B.L.; Cavero, E.; Putet, G.; Senterre, J. Plasma amino acid and protein concentrations in term infants fed human milk or a whey protein hydrosylate formula during the first month of life. Acta Pediatr **1994**, 83, 127-131.
19. Rigo, J.; Boehm, G.; Georgi, G.; Jelinek, J.; Nyambugabo, K.; Sawatzki, G.; Studzinski, F. An infant formula free of glycomacropeptide prevents hyperthreoninemia in formula-fed preterm infants. *J. Pediatr. Gastroenterol. Nutr.* **2001,** *32,* 127-130.

Table 5.1. Analysis of sweet whey and GMP-depleted whey fraction*.

Component	Sweet whey	GMP-depleted whey fraction
Protein	97.0 ± 0.7	98.4 ± 4.3
Ash	2.4 ± 0.1	1.1 ± 0.5
Phosphorous	2.7 ± 0.3	1.2 ± 0.1
Calcium	8.5± 0.5	3.9 ± 0.4

* Contents of protein and ash are expressed as g/100 g of dry weight, and those of phosphorus and calcium as μg/mg dry weight.

Table 5.2. Amino acid compositions of sweet whey and GMP-depleted whey fraction, and human milk. Contents are expressed as moles of amino acid per 100 moles of total amino acids.

Amino acid	Sweet whey	GMP-depleted whey fraction	Human milk	
			A	B
Asparagine and aspartic acid	11.2	12.3	9.7	9.3
Threonine	8.4	6.0	5.8	5.1
Serine	8.9	7.5	5.7	6.3
Glutamine and glutamic acid	17.3	17.6	16.2	15.0
Glycine	3.2	3.3	4.1	4.7
Alanine	7.5	7.6	5.2	5.6
Cysteine	ND	ND	3.2	1.6
Valine	5.5	5.0	6.2	5.7
Methionine	ND	ND	1.3	1.1
Isoleucine	5.5	4.8	5.5	5.1
Leucine	10.4	12.9	10.1	9.7
Tyrosine	2.2	2.7	2.9	3.6
Phenylalanine	2.4	3.0	3.2	3.7
Histidine	1.4	1.7	2.2	2.9
Lysine	7.4	8.3	6.1	6.4
Tryptophan	ND	ND	---	1.5
Arginine	1.7	1.8	2.5	2.9
Proline	7.1	5.7	10.2	10.0

A: Calculated from Darragh & Moughan (1998)

B: Calculated from Rigo *et al.* (1994)

ND: Not determined

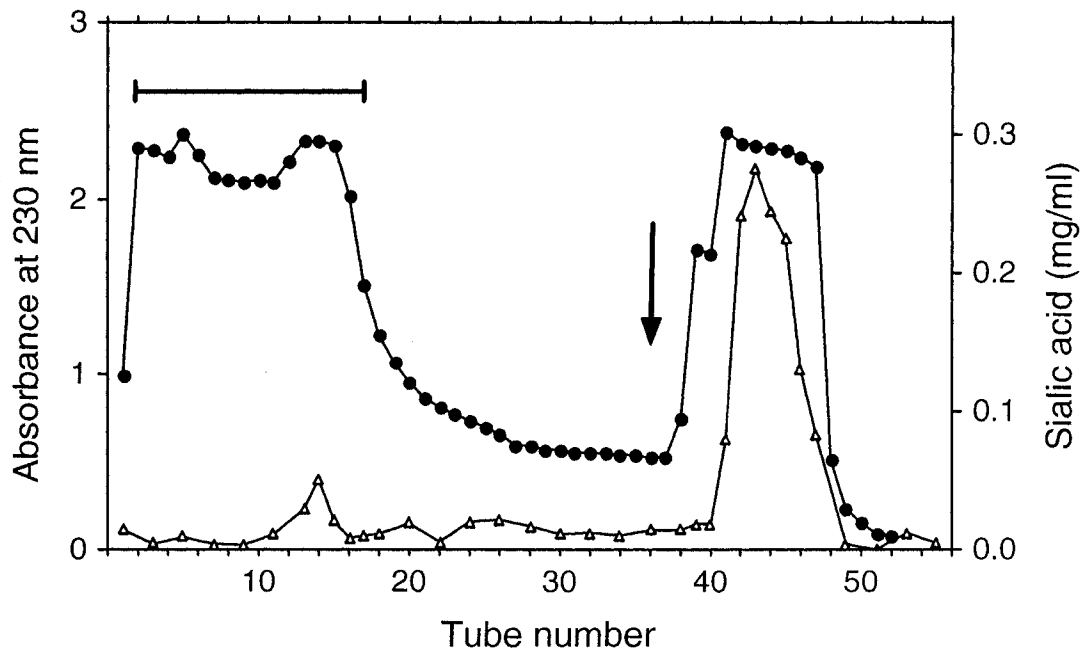

Figure 5.1. Anion-exchange chromatography of sweet whey powder (1.2g in 20 ml) on DEAE-Sephacel: (●—●) absorbance at 230 nm, and (△—△) Sialic acid. An arrow shows the position of application of 1 M NaCl to release the GMP adsorbed o0n the column. A bar denotes eluates pooled for further study. Eluates containing GMP adsorbed on the column were not analyzed further.

CHAPTER 6

Final Remarks[1]

6.1 INTRODUCTION

Research in functional foods has increased during the last fifteen years with the intention of increasing life expectancy and improving human health conditions. Probiotics, prebiotics, bioactive peptides or proteins, dietetic fibers and fatty acids, as well as the addition of fitochemical compounds in dairy products have become important areas of research in functional foods (1-3). The increase in life expectancy, the belief that it is possible to influence one's own health and the awareness of the importance of prevention of disease are the main factors that influence the demand for these kinds of products. Milk contains diverse constituents with physiological functionality (4), which might change the traditional point of view that we have about drugs. The topic of functional foods in general, and specifically the area related to milk and dairy products, has still not been completely explored.

6.2 SUMMARY OF RESEARCH FINDINGS

The goat glycomacropeptide (GMP) purified in our laboratory with negligible amount of phenylalanine appears to be useful for the dietary treatment of phenylketonuria, a hereditary disorder of phenylalanine metabolism (5). Gel electrophoresis of GMP demonstrated that pasteurization generates GMP aggregates with increased mobility on SDS gels and that the low-temperature, long-time compared to high-temperature, short-time pasteurization is more effective in inducing this change.

The GMP-depleted whey fraction, obtained from goat sweet whey as well as from bovine sweet whey powder, analyzed in our laboratory showed a high

[1] Part of of this chapter has been published. Silva-Hernandez, E.R.; Nakano, T.; Ikawa, N.; Ozimek, L. Goat kappa-casein Glyco-macropeptide In: *Food Science and Product Technology. Research* Signpost. India. 2002, Pp. 104-110.

homology in its amino acid composition, when compared to human milk. The GMP-depleted whey fraction from either goat or bovine sources has a threonine content similar to that of human milk (6, 7), making it suitable for use in infants' formulas that might prevent hyperthreoninemia (8). This condition, observed as abnormally high serum threonine levels, has frequently been reported in infants fed with whey-based formulas (9-14).

6.3 RECOMMENDATIONS FOR FUTURE RESEARCH

Although results presented in this document appear as a strong evidence of the potential of the caprine glycomacropeptide and the GMP-depleted whey, more research work should be performed with more samples to confirm the findings expressed here. Experiments in which individual samples are considered, as well as control of the seasonal variables, stage of lactation, and breed of the animals should be included in subsequent analyses. In addition, finding economical ways to separate GMP from sweet whey is required when considering the industrial production of this product.

Development of practical techniques to analyze contents of phenylalanine in GMP and threonine in GMP-depleted whey should be considered as well. Currently, estimations of phenylalanine and theronine are usually determined through acid hydrolysis of proteins and peptides, followed by the use of an amino acid analyzer such as the Beckman Model 6300. However, this technique is expensive and time consuming. Therefore, it might be interesting, for example, to see if it is possible to estimate the content of threonine in GMP-depleted whey through indirect methods. For instance, the thiobarbituric acid reaction used for the determination of sialic acid in this report might help in the estimation of theronine, since it seems that the amount of sialic acid in GMP-depleted whey is correlated to its threonine content.

Use of different sources of GMP and GMP-depleted whey may be an important consideration as well. Cow's milk is the most important milk in the world, and research related to the isolation of bioactive compounds from this kind of milk is also the most important in the dairy industry nowadays. However, the

use of other sources of milk would open new areas of research. This document presented the isolation and characterization of goat's GMP and GMP-depleted whey, but preparation and characterization of these products from milk obtained from other species, such as sheep and buffalo, might be important to be studied as well.

Numerous biological activities have been reported as associated with bovine GMP (15-18). It would be interesting to know whether goat GMP, whose composition and structure differ from those of bovine GMP, has the same biological activities as bovine GMP.

Goat's milk is an important source of nutrients in many parts of the world. World production of goat's milk has been increasing more rapidly than cow's milk during the past two decades (19). Chemical composition of goat-milk protein has been relatively extensively studied (20). However, there is still limited information available concerning goat sweet whey components, including GMP and GMP-depleted whey. Research into the potential uses of goat GMP and GMP-depleted whey in the pharmaceutical, cosmetic and functional food sectors may be important. This is especially the case in European countries and Mexico where the majority of caprine milk is used for cheese production. Utilization of goat sweet whey also helps prevent pollution caused by disposal of this byproduct, a long-standing problem for the dairy industry.

Milk and dairy products as sources of functional foods and ingredients are currently a reality. Many people around the world are already consuming them. However, many aspects in this field still remain to be explained before reaching firm conclusions. In addition, more studies in humans are needed, along with consideration of milk sources other than cow's milk. Exploring milk from other species, such as goats, sheep and buffalo, might be worthwhile.

6.2 REFERENCES

1. Hilliam M. The market for functional foods. *Int. Dairy J.* **1998,** *8,*349-353.
2. Roberfroid, M.B. Prebiotics and symbiotics: concepts and nutritional properties. *Brit. J. Nutr.* **1998**, *80(Suppl 2),* S197–S202.
3. Horton, B. Commercial utilization de minor milk components in the health and food industries. *J. Dairy Sci.* **1995**, *78*, 2584-2589.
4. Jelen, P.; Lutz, S. *Functional foods, Biochemical y Processing aspects.* Lancaster, Basel: Technomic Publishing CO., Inc. 1998, 357-380.
5. Yule, K.S. *The metabolic and molecular bases of inherited disease*; Mcgraw-Hill. New York, 2000; pp. 623-649.
6. Darragh, A.J.; Moughan, P.J. The amino acid composition of human milk corrected for amino acid digestibility. *Brit. J. Nutr.* **1998,** *80,* 25-34.
7. Rigo, J.; Salle, B.L.; Cavero, E.; Putet, G.; Senterre, J. Plasma amino acid and protein concentrations in term infants fed human milk or a whey protein hydrosylate formula during the first month of life. Acta Pediatr **1994**, 83, 127-131.
8. Rigo, J.; Boehm, G.; Georgi, G.; Jelinek, J.; Nyambugabo, K.; Sawatzki, G.; Studzinski, F. An infant formula free of glycomacropeptide prevents hyperthreoninemia in formula-fed preterm infants. *J. Pediatr. Gastroenterol. Nutr.* **2001,** *32,* 127-130.
9. Hanning, R.M.; Paes, B.; Atkinson, S.A. Protein metabolism and growth of term infants in response to a reduced-protein, 40:60 whey: casein formula with added tryptophan. *Am. J. Clin. Nutr.* **1992,** *56,*1004-1011.
10. Janas, L.M.; Picciano, M.F.; Hatch, T.F. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula. *Pediatrics* **1985,** *75,* 775-784.
11. Järvenpää, A.-L.; Rassin, D.K. Räihä, N.C.R.; Gauill, G.E. Milk protein quantity and quality in term infant: II. Effects on acidic and neutral amino acids. *Pediatrics* **1982,** *70,* 221-230.

12. Picone, T.A.; Benson, J.D.; Moro, G.; Minoli, I.; Fulconis, F.; Rassin, D.K.; Räihä, N.C.R. Growth, serum biochemistries, and amino acid of term infants fed formulas with amino acid and protein concentrations similar to human milk. *J. Pediatr. Gastroenterol. Nutr.* **1989,** *9,* 351-360.
13. Rassin, D.K.; Gauill, G.E.; Heinonen, K.; Räihä, N.C.R. Milk protein quantity and quality in low-birth-weight infants: II. Effects on selected aliphatic amino acids in plasma and urine. *Pediatrics* **1977,** *59,* 407-422.
14. Rigo, J.; Verloes, A.; Senterre, J. Plasma amino acid concentrations in term infants fed human milk, a whey-predominant formula, or a whey hydrosylate formula. *J Pedriatr* **1989,** *115,* 752-755.
15. Brody, E. Biological activities of bovine glycomacropeptide. *Br. J. Nutr.* **2000,** *84 (Suppl. 1),* S39-S46.
16. Abd El-Salam, M.H.; El-Shibiny, S.; Buchheim, W. Characteristics and potential uses of the casein macropeptide. *Int. Dairy J.* **1996,** *6,* 327-341.
17. Dziuba, J.; Minkiewicz, P. Influence of glycosylation on micelle stabilizing ability and biological properties of C-terminal fragment of cow's κ-casein. *Int. Dairy J.* **1996,** *6,* 1017-1044.
18. López-Fediño, R.; Ramos, M. Revisión: El caseinomacropéptido bovino. I. Características fisicoquímicas y actividad biológica (in Spanish). *Rev. Esp. Cienc. Tecnol. Aliment.* **1992,** *32,* 575-588.
19. Monrand-Fehr, P.; Boyazoglu, Present state and future outlook of the small ruminant sector. *J. Small Ruminant Res.,* **1999,** *34,* 175-188.
20. Jenness, R. Composition and characteristics of goat milk: Review 1968-1979. *J. Dairy Sci.* **1980,** *63,* 1605-1630.