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

ERYCK R. SILVA-HERNANDEZ,* TAKUO NAKANO, AND LECH OZIMEK

Alberta Dairy Association Research Unit, Department of Agricultural, Food and Nutritional Science, 410 Agriculture/Forestry Center, University of Alberta, Edmonton, Alberta T6G 2P5, Canada

Glycomacropeptide (GMP) was purified from goat sweet whey by anion-exchange and hydrophobic interaction chromatography. Approximately 0.06% (w/v) of sweet whey was recovered as GMP. Amino acid analysis of the GMP preparation showed that the content of phenylalanine (an amino acid that does not occur in goat GMP) was negligible, indicating that the GMP was of high purity. The goat GMP contained 25 μg sialic acid per mg of dry weight. This was approximately 3-fold lower than the sialic acid concentration in bovine GMP reported in the literature. Gel electrophoretic results demonstrated that most of the goat GMP occurs as a dimer. The GMP was intensely stained with Coomassie blue in 50% methanol containing 12.5% (w/v) trichloroacetic acid, but showed very weak metachromasia with the same dye in 45% methanol containing 10% acetic acid, a preparation commonly used to stain protein.

KEYWORDS: Anion-exchange chromatography; hydrophobic interaction chromatography; glycomacropeptide; purification; goat sweet whey

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 has been 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.

MATERIALS AND METHODS

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 20,000*g* 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 cutoff. 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, ON. Diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-200, and blue dextran were from Pharmacia Biotech Inc., Baie d'Urfe, PQ. Molecular weight standards for gel electrophoresis were from Bio-Rad Laboratories Canada Ltd., Mississauga, ON.

Fractionation of Goat Sweet Whey. Samples of sweet whey were thawed and centrifuged at 20000*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 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 with 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, which have higher isoelectric points (pIs) or hydrophobicity than GMP, are expected to be desorbed from DEAE-Sephacel or adsorbed on phenyl-agarose under the experimental conditions. Thus, the major sialic acid peak

* To whom correspondence should be addressed. Telephone (780)492-0381. Fax (780)492-4265. E-mail eryck@ualberta.ca.

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eluting from the phenyl-agarose column should contain sialic acid from GMP but not from the sialylated sweet whey proteins.

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*).

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

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 gel 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.

RESULTS

After chymosin treatment, an 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. The nondialyzable fraction of sweet whey was then chromatographed on DEAE-Sephacel (**Figure 1**). On 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 1**) was then chromatographed on phenyl-agarose (**Figure 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** 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.

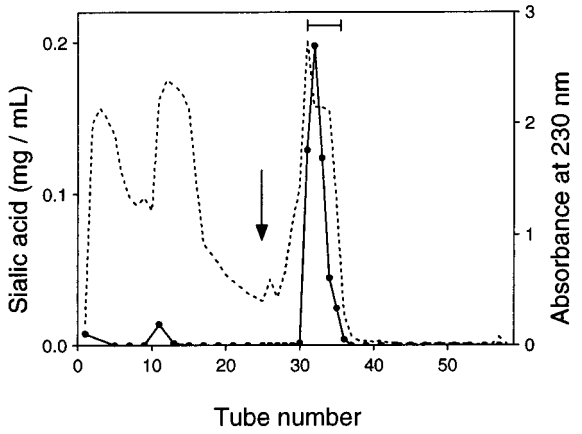

Figure 1. Anion-exchange chromatography of goat sweet whey (44 mL) on DEAE-Sephacel: (●—●) sialic acid; (- - -) protein/peptide. An arrow shows the position of application of 1 M NaCl. A horizontal bar denotes eluates pooled for further study. See Materials and Methods for other details.

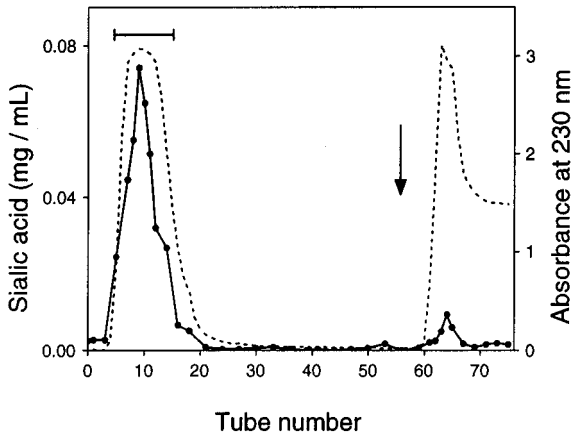

Figure 2. Hydrophobic interaction chromatography on phenyl-agarose of major sialic acid-containing fraction from DEAE-Sephacel (**Figure 1**). A sample containing approximately 46 mg of sialic acid in 5 mL of buffer-A was chromatographed: (●—●) sialic acid; (- - -) protein/peptide. An arrow shows the position of application of water. A horizontal bar denotes eluates pooled for further study. See Materials and Methods for other details.

Table 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 1**) of glycine, leucine, and phenylalanine, indicating that the GMP was 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 3**) gave a major peak (K_{av} = 0.46) of sialic acid accounting for approximately 84% of recovered sialic acid. The

Table 1. Amino Acid Compositions of GMP-Containing Fraction from DEAE-Sephacel (**Figure 1**, GMP-DEAE) and GMP Fraction (**Figure 2**) and the Theoretical Amino Acid Composition of Goat GMP

	mol %		
amino acid	GMP-DEAE	GMP fraction	goat GMP[a]
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.

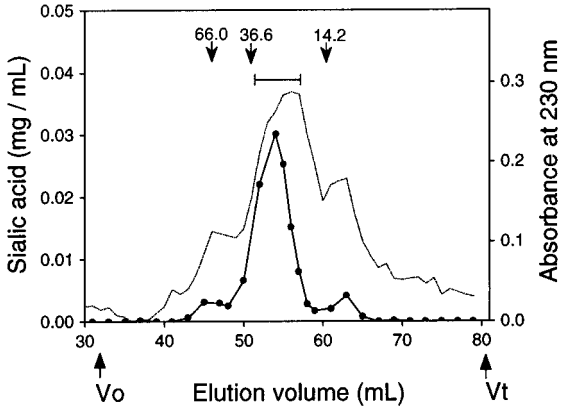

Figure 3. Gel chromatography of goat GMP on Sephacryl S-200. Approximately 9 mg of the GMP fraction from phenyl-agarose (**Figure 2**) was chromatographed: (●—●) sialic acid; (- - -) protein/peptide. Elution positions of protein standards, bovine serum albumin (66.0 kDa), dimeric β-lactoglobulin (36.6 kDa), and α-lactalbumin (14.2 kDa) are shown. A horizontal bar denotes eluates pooled for further study. V_0 and V_t show void and total column volumes, respectively. See Materials and Methods for other details.

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 4**), the GMP fraction gave major and minor broad bands stained with Coomassie blue in solution-A (**Figure 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 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

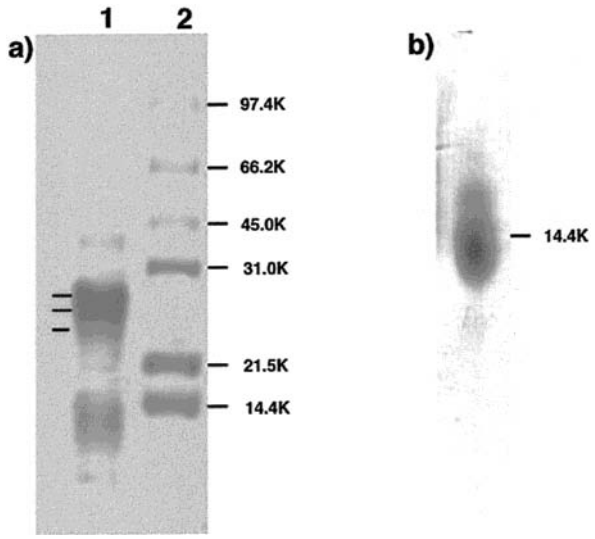

Figure 4. Gel electrophoresis of goat GMP. The GMP fraction from phenyl-agarose (**Figure 2**) (approximately 50 μg/well) was electrophoresed on SDS-gels containing no urea and SDS gels containing 8 M urea. (a) Gel containing no urea: Lane 1, the GMP fraction; Lane 2, molecular weight standards phospholylase *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). Bars indicate the three components in the major band. (b) Gel containing 8 M urea. See Materials and Methods for other details.

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 carbonic anhydrase (31.0 kDa), and the second one had a mobility much faster than that of lysozyme (14.4 kDa).

Electrophoresis of the major sialic acid peak fraction from Sephacryl S-200 (**Figure 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 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 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 dimers 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).

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 ganglioside) 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 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 do occur in bovine GMP), and of histidine occurring in goat (but not in bovine) GMP with its concentration close to the theoretical content (**Table 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 mol/mol) 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 the pIs (4.04 and 4.14, respectively) for 64-residue peptide from bovine GMP variants A and B. Because the concentration of phosphate (pK_a = 2.0), which also contributes to the low pI of GMP, is similar in 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, whereas 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 dimers. 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. As 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 phosphorus (see above) is approximately 7.5 kDa.

Most (~80% of total, ref *18*) of the 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 3**) contained sialic acid from GMP only. Contamination with sialylated proteins is unlikely to occur (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 would be of interest 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 increasing more rapidly than that of cow milk during the past two decades (*33*). The 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 byproduct of cheese manufacturing. The information may contribute to the development of beneficial ways to use goat cheese whey by producing value-added products. Such an 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 would also contribute to the prevention of pollution caused by disposal of this byproduct, a long-standing problem of the 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.

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